Multiple context discrimination in adult rats: sex variability and dynamics of time-dependent generalization of an aversive memory

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ABSTRACT

Memory generalization can be defined as the transference of a conditioned fear response to novel contexts. It can be attained rapidly, by manipulating distinct hormonal and neurotransmitter systems, but, more importantly, in a time-dependent manner the interval between training and test sessions affects the quality of retrieved memory, due to a rearrangement in the recruitment of brain regions, as is the case in Systems Consolidation. However, less is known about how female and male subjects retrieve contextual memories over time. In this work, we aimed to investigate how rats of both sexes discriminate among multiple contexts gradually differentiated in similarity to the conditioning one, two days after training. In the pilot study, despite not obtaining, at first, a stepwise series of freezing responses, we found that – strangely - males displayed their higher levels of freezing to the least similar context, while females systematically displayed lower freezing levels compared to males as if they were better discriminators when tested in exact same contexts. With a second set of contexts, sorted according to other criteria, only males, at the 2 days interval, attained a staircase-like response to the successive contexts, while females displayed a completely different freezing pattern; in remote tests, 28 and 45 days later, freezings grouped into two levels, suggesting a partial time-dependent generalization for some of the contexts. Females and males, in discriminating between multiple, dissimilar aversive contexts, seem to deal with threat signals in distinct ways, through different sensory modalities, a clear demonstration of behavioural dimorphism.

Keywords: context discrimination, memory generalization, Systems Consolidation, sexual dimorphism, Contextual Fear Conditioning task, adult rats

INTRODUCTION

The ability to store and retrieve information is a highly adaptive, evolutionarily conserved trait in animals, and in many situations the ability to predict the safety or threat of future events overcomes the capacity to retain more precise memories of past events, which is understood as an adaptive advantage (Asok et al., 2019; Suddendorf, 2006). This is especially true of fear memories in which the generalization of threat cues allows animals to increase their survival through the transference of learned behaviours to these, new and potentially dangerous environments / situations based on similar cues, so that learning each specific case is not necessary in order to react accordingly (Bergstrom, 2020; Young, 2012).

Mammalian brains seem to have evolved to alternate between discrimination and generalization in the face of novel situations, aiming to maximize the possibility of survival. Yet, we still do not fully understand the exact “boundary conditions” that trigger one or other outcome, especially when choosing between slightly distinct contexts, and to which extent their recognition relies upon one or more different (or a combination of) sensory modalities. Furthermore, it is also difficult to define which contextual elements (floor textures, lighting, size, chamber shape, odour, etc.) actually support discriminatory scalability, with measurable statistically significant increments — that, for example, organize the data in a “descending staircase-like” fashion.

In rats, the retrieval of an aversive contextual memory depends, initially, on the hippocampus, but, with time, becomes more reliant on neocortical structures, a process known as *Systems Consolidation* (Dudai, 1996; Haubrich et al., 2016). Evidence suggests this process develops in two dimensions - a gradual neuroanatomical reorganization, in which different brain structures support memory retrieval as time passes, and a progressive loss of the ability to discriminate between contexts, in which animals display fear in contexts not previously paired with the aversive stimulus (Cullen et al., 2015; De Oliveira Alvares et al., 2012; Quillfeldt, 2019; Rudy, 2009; Wiltgen et al., 2010). This contextual memory generalization tends to increase as the interval between conditioning and test sessions increases (Wiltgen & Silva, 2007).

Overgeneralization - an exaggerated fear response to neutral cues or contexts - on the other hand, can have maladaptive consequences, such as restricting important resource-seeking behaviours or resulting in a mental disorder, in the case of humans. Therefore, to maximize the possibility of survival and generation of offspring, there must be an optimal balance between threat avoidance and perception of safety. This form of fear discrimination can also be understood as *learned fear inhibition* in the presence of safety signals whereas generalization may arise from impaired safety signalling (Day & Stevenson, 2020).

In humans, excessive fear generalization is a hallmark of anxiety and trauma-related disorders such as posttraumatic stress disorder (PTSD) and generalized anxiety disorder (GAD), with women being at disproportionately higher risk of developing these conditions (Besnard & Sahay, 2016; Jasnow et al., 2017; Keiser et al., 2017; Lissek & Grillon, 2012; McLean et al., 2011), suggesting a role of sex steroids in this process. While different behavioural phenotypes are nothing new, only recently has there been an increase in the awareness of the importance of symmetrically including subjects of both sexes in preclinical and clinical studies to avoid missing important differences (e.g., NIH Policy on Sex as a Biological Variable, 2016).

Despite the fact that sex hormones are known to interfere with spatial, social and fear memory formation (Taxier et al., 2020), discrimination / generalization studies have produced mixed results, with some showing females generalizing before males (J. Lynch et al., 2013; J. F. Lynch et al., 2014), while others indicate just the opposite (Crestani et al., 2022; Foilb et al., 2018), or even finding no difference between sexes (Clark et al., 2019; Keeley et al., 2015; Reppucci et al., 2013). This highlights the relevance of knowing the experimental parameters and contextual configurations in order to explain contradictory findings, i.e., knowing the extent to which they may rely on sex differences, sensory modality and how this allows a discriminatory scalability. In other words, knowing how individual cues present in a neutral, supposedly safe context can trigger the aversive memory, either partially or completely, and to which extent cues signalling safety can effectively induce learned fear inhibition (Asok et al., 2019), or straightforward time-dependent generalization.

In this work, we trained young adult female and male rats in the contextual fear conditioning task and tested them 2, 28 or 45 days later, either on the conditioning context (A) or in any of three novel / neutral contexts, graded according to different degrees of similarity to the training context. To our knowledge, no previous study has

compared fear responses between adult female and male rats in *multiple neutral contexts* in recent or remote test sessions.

EXPERIMENTAL PROCEDURES

Subjects

Naïve male and female Wistar rats weighing 270–340g and aged 70–75 days at the beginning of experimental procedures, were used. All animals were provided by our local breeding colony (CREAL/UFRGS) and housed in plastic cages up to four individuals per cage. Males and females were housed with same-sex littermates at opposite ends of the same facility room, which was kept under a 12h light/dark cycle, controlled temperature of 21 ± 2°C and approximately 65% humidity. Experimental procedures were conducted during the light phase. Regular chow and water were available *ad libitum* for all animals. To prevent additional or differential stress, estrus cycle was not verified. All experiments were conducted in accordance to local animal care legislation and guidelines (Brazilian Federal Law 11,794/2008) and approved by the Committee on Ethics in the Use of Experimental Animals of the Federal University of Rio Grande do Sul (CEUA – UFRGS, Project # 40,945).

Conditioning apparatus and novel contexts

All seven distinct contexts used in this study are depicted in **Figure 1** below. It is important to note that due to relevance, the presentation of results was not chronological, i.e., the experiment with 4 contexts and 3 time intervals was presented first – with contexts having letters assigned accordingly, A, B, C and D (**Figure 1.A**). The second experiment shown was, indeed, the pilot study, in which contexts E, E', F and G (**Figure 1.B**) were first tested, but only for recent memory (2 day training-test interval).

The Conditioning Context A was the same for both experiments, and consisted of an internally illuminated Plexiglass box (L x W x H: 22 x 22 x 33 cm) with three black and one light brown wall. The floor was a grid of parallel 0.1cm calibre stainless steel bars spaced 1 cm apart. Rats were observed through a webcam mounted on the opaque upper lid. The conditioning chamber A was cleaned with 70% ethanol solution before placing each animal.

Context B was a not-so-slightly modified version of context A, in which the metal grid floor was covered with a smooth brown vinyl mat and a semi-circular rubber wall placed inside the conditioning chamber. Both contexts A and B were cleaned with 70% ethanol before each session.

Context C consisted of a right triangular chamber (sides x H: 34, 28, 48 x 19 cm) with smooth black plywood floor and walls. Differently from A and B, Context C was cleaned with quaternary ammonium disinfectant prior to placing each animal.

Context D was a larger rectangular box (L x W x H: 50 x 25 x 29 cm) was placed in a different area of the conditioning room and contained three opaque beige walls, one transparent frontal wall and a semi-translucent lid. A desk lamp was placed beside the frontal wall. The floor consisted of a grid of parallel 0.1 cm calibre stainless copper bars spaced 1 cm apart. The test chamber was cleaned with 70% isopropyl alcohol.

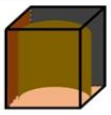
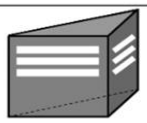
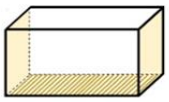
In the pilot study, context E was, in our view, the least similar to Context A, and consisted of polyvinyl chloride (PVC) cylindric container (diameter: 25 cm; height: 22 cm) with vertical black and white stripes on its internal walls and a heavy transparent glass lid. The floor consisted of a smooth black plywood surface covered with wood shavings, which was replaced before placing each animal. The test chamber was cleaned with 70% isopropyl alcohol. A slightly modified version E', identical to E but without wood shavings, was also assayed.

Context F consisted of a scalene triangular chamber, smaller than Context C (sides x H: 35, 14.5, 23 x 6.5 cm), with brown plywood walls, white tempered glass floor and no lid. Context F was cleaned with quaternary ammonium disinfectant cleaning solution before each animal.

Context G was a modified, half-sized version of Context D (L x W x H: 25 x 25 x 29 cm), with dimensions similar to the Conditioning chamber A, and was cleaned with 70% isopropyl alcohol.

Rats were kept in their home cages and habituated to the conditioning room under a constant fan background noise for at least 30 min before the start of each session. Contexts C, E, E' and F were located in separate rooms from that of the conditioning chamber.

A

Main Experiment				
				
Context	**A (conditioning)**	**B (modified conditioning)**	**C (neutral)**	**D (neutral)**
Chamber shape	square cuboid	semicircle	triangular	rectangular cuboid
Size	22 x 22 x 33 (LxWxH cm)	22 x 22 x 33 (LxWxH cm)	34 ; 28 ; 48 x 19 (S1;S2; S3 x H cm)	50 x 25 x 29 (LxWxH cm)
Floor texture	grid of parallel 0.1 cm caliber stainless steel bars spaced 1 cm apart	smooth brown vinyl	smooth black plywood	grid of parallel 0.1 cm caliber stainless copper bars spaced 1 cm apart
Wall texture and color	three black and one pastel brown	same as context A with an added brown rubber forming a semicircle	smooth black plywood with white markings	three opaque beige and one transparent
Illumination	webcam and chamber wall lights	webcam light	ceiling lamp	desk lamp near the transparent wall
Odor (cleaning agent)	70% ethanol	70% ethanol	ammonium quartenary disinfectant	70% isopropyl alcohol
Room used	conditioning room	conditioning room	novel room	conditioning room

B

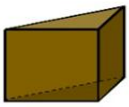
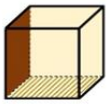
Pilot Study				
				
Context	**A (conditioning)**	**E (neutral)**	**F (neutral)**	**G (neutral)**
Chamber shape	square cuboid	circular	triangular	square cuboid
Size	22 x 22 x 33 (LxWxH cm)	25 x 22 (D x H)	35 ; 14.5 ; 23 x 6.5 (S1;S2; S3 x H cm)	25 x 25 x 29 (LxWxH cm)
Floor texture	grid of parallel 0.1 cm caliber stainless steel bars spaced 1 cm apart	smooth black plywood with wood shavings	white tempered glass	parallel 0.1 cm caliber stainless copper bars spaced 1 cm apart
Wall texture and color	three black and one pastel brown	vertical black and white stipes	brown plywood	three opaque beige and one transparent
Illumination	webcam and chamber wall lights	ceiling lamp	ceiling lamp	desk lamp near the transparent wall
Odor (cleaning agent)	70% ethanol	70% isopropyl alcohol	ammonium quartenary disinfectant	70% isopropyl alcohol
Room used	conditioning room	novel room	novel room	conditioning room

Figure 1. Schematic illustration depicting the specific contexts used in this study. (A) Main experiment (2, 28 and 45 day training-test intervals); (B) Pilot experiment (2 days only). In our pilot groups we also included E', a modified version of context E, without wood shavings in the floor. For more details, please, see text.

Behavioural procedures

Contextual fear conditioning (CFC)

Rats were habituated to the conditioning context (A) for 3 min before receiving two 2-s continuous 0.5 mA footshocks separated by a 30-s interval. Thirty seconds after the second footshock rats were removed from the chamber and returned to their homecage. Males and females were not trained in the same experimental session.

Test sessions

For each training-test interval, conditioned animals were divided into four different groups, each one tested in a different test chamber: either A, B, C or D - for the main experiment (**Figures 2-4**), or A, E/E', F or G – for the pilot study (**Figure 5**). In both experiments, test sessions lasted 5 min during which freezing responses were manually scored by an experienced observer using a stopwatch. Defensive freezing behaviour, defined as the absence of all muscle movements except those related to breathing (Blanchard & Blanchard, 1969), was used as an indicator of memory retention and expressed as a percentage of total session time. The number of attempted escapes (jumps) were also quantified as a secondary indicator of flight behaviour. To prevent reactivation of fear memory, which could influence the results, each rat group was tested in one context only. Males and females were never tested in the same experimental session.

Statistical Analyses

After assessing data distribution for **normality** (Shapiro-Wilk test) and **homoscedasticity** (Levene's test), we employed, for parametric data, the following tests to find statistical differences between groups (considering $P < 0.05$): for two independent groups, the unpaired two-tailed Student's t-test was used, and for K groups, we employed independent one or two-way ANOVAs, followed by Tukey Multiple Comparisons *post* hoc test in order to sort the significant differences found. In order to support *reproducibility*, for every statistically significant ($P < 0.05$) effect, **Effect Sizes** were calculated, either using Hedge's g (effect: small = 0.20, medium = 0.50, large = 0.80), or omega-squared (ω^2), for the ANOVAs (effect: small = 0.02, medium = 0.06, large = 0.14). Outliers beyond two standard deviations above or below the mean were excluded from analyses.

In order to estimate the degree of discrimination between contexts, individual and average **Z-scores** for every neutral context were calculated and plotted: Z-scores inform where each individual score is located inside the normal curve in terms of standard deviations, compared to the average percentual freezing score for same sex control groups with the same training-test interval. In **Figures 2.D, 3.D, 4.D** and **5.D**, each point represents the behavioural response of one individual animal.

RESULTS

MAIN EXPERIMENT

Discrimination between multiple contexts over time: Subjects of both sexes were fear conditioned to context A, and 2, 28 or 45 days later, submitted to retrieval tests in this or three other neutral contexts, sorted according to multidimensional criteria (chamber size and odour).

1. Tested 2 days after training – Independent two-way ANOVA of rats tested in one of four distinct contexts (See **Figure 1.A**) revealed significant effects in the interaction between Context x Sex ($F_{(3, 77)} = 9.182$; $P < 0.0001$, with effect size $\omega^2 = 0.22$), Context ($F_{(3, 77)} = 63.92$; $P < 0.0001$, $\omega^2 = 0.699$) and Sex ($F_{(1, 77)} = 82.17$; $P < 0.0001$, $\omega^2 = 0.49$) - **Figure 2.B**: according to the ω^2, all detected effects were large and robust. Number of subjects per context (A, B, C and D respectively): females, 10, 10, 10, 11, males, 11, 11, 11, 11.

In females, Tukey Multiple Comparisons *post* hoc test revealed different freezing levels in contexts A and B ($P = 0.002$, $g = 2.256$), but not between contexts C and D ($P = 0.67$); in males, there are significant differences between A and C ($P < 0.0001$, $g = 3.132$) and A and D ($P < 0.0001$, $g = 3.923$), B and C ($P = 0.0001$, $g = 2.483$) and C and D ($P = 0.002$, $g = 1.331$), but not between A and B ($P = 0.572$) – See **Figure 2.C**.

In order to estimate the degree of discrimination, we plotted the individual and average Z-scores for every neutral context (B, C or D): as seen in **Figure 2.D**, all data points have lower values compared to A, and they differ among them, except for context C, that seems to be equally well discriminated by both sexes. Importantly, the salient “staircase effect” in the degree of discrimination of the males was a sorting pattern not followed by the females in these contexts.

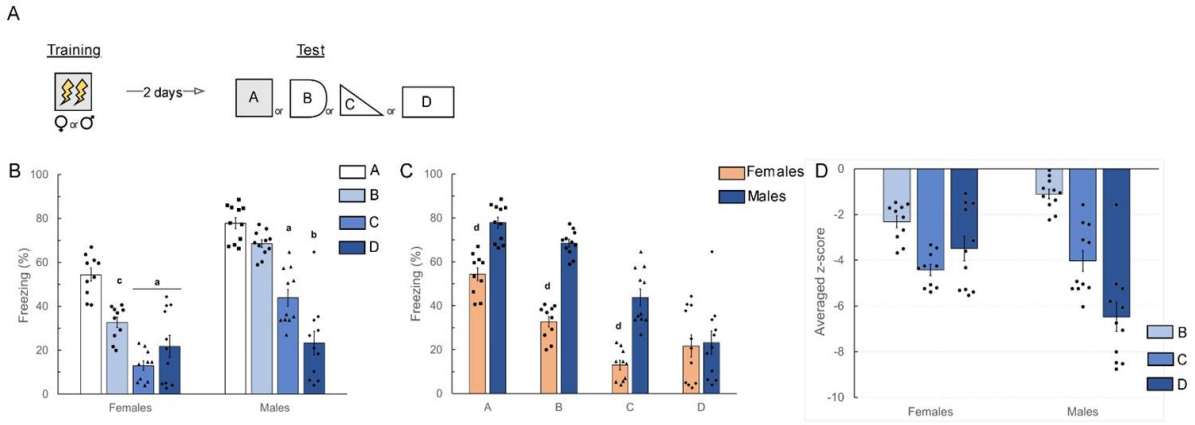


Figure 2. Context discrimination of female and male rats 2 days after training in a fear conditioning task. (A) Experimental protocol: all subjects were trained in Context A and subsequently tested either in A, B, C or D **2 days** later; (B) Histogram showing mean±SEM of the % freezing time in each context grouped by sex with individual dots overlayed representing individual animals; (C) Same data (and statistical analysis) grouped by context. B & C: data analyzed by independent two-way ANOVA followed by Tukey post hoc test. **a** (different from A / B), $P < 0.0001$, **b** (differs from same sex groups tested in all other contexts), $P < 0.002$, and **d** (females differ from males in this specific context E), $P < 0.0005$; (D) Individual (and average) Z-scores for contexts B, C and D (standard deviations compared to A).

2. Tested 28 days after training - Independent two-way ANOVA of rats tested in one of four distinct contexts (See **Figure 1.A**) revealed no significant effect in the interaction between Context x Sex ($F_{(3,81)} = 0.132$; $P=0.94$), while Context ($F_{(3,81)} = 43.81$; $P < 0.0001$, $\omega^2 = 0.62$) and Sex ($F_{(1,81)} = 8.65$; $P < 0.0043$, $\omega^2 = 0.09$) differ significantly - **Figure 3.B**: according to the ω^2, effect size of Sex was moderate to large only, while for Context it was large and robust. Number of subjects per context (A, B, C and D respectively): females, 11, 11, 12, 11, and males, 11, 11, 11, 11.

For both females and males, Tukey Multiple Comparisons *post* hoc test revealed similar freezing levels in all contexts, with no differences observed between contexts A and B ($P > 0.99$) and between C and D ($P > 0.99$) (**Figure 3.C**).

Similarly to what we did in the main experiment above, the degree of discrimination was estimated by plotting the individual and the average Z-scores for every other context (B, C or D) compared to A (**Figure 3.D**). The main effect is the dramatic response to context B – now indistinguishable from A – in both females and males, while discrimination of contexts C and D converged to a similar degree, but females appear to better discriminate these contexts (from A) than males.

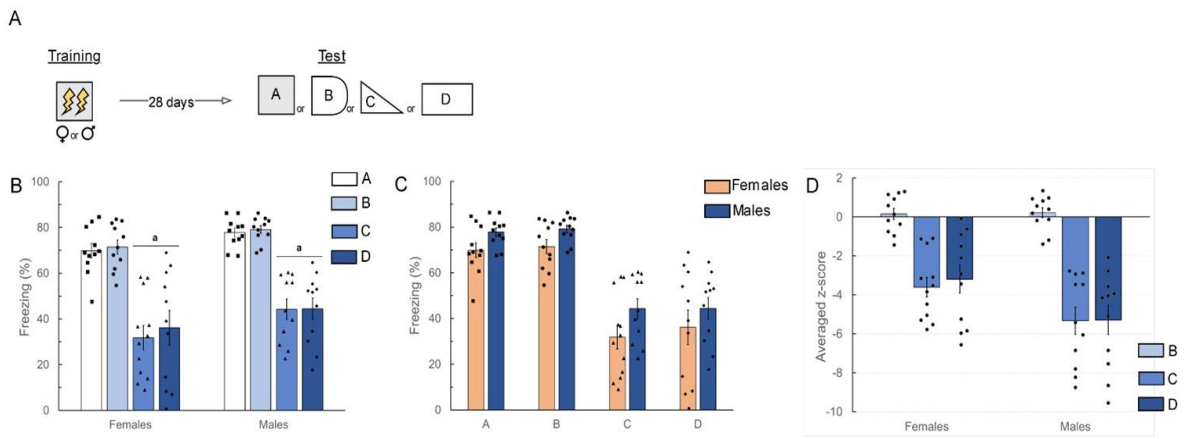


Figure 3. Context discrimination of female and male rats 28 days after training in a fear conditioning task. (A) Experimental protocol: all subjects were trained in Context A and subsequently tested either in A, B, C or D **28 days** later; (B) Histogram showing mean±SEM of the % freezing time in each context grouped by sex with individual dots overlayed representing individual animals; (C) Same data (and statistical analysis) grouped by context. B & C: data analyzed by independent two-way ANOVA followed by Tukey post hoc test. **a** (different from A / B), $P < 0.0001$; (D) Individual (and average) Z-scores for contexts B, C and D (standard deviations compared to A).

3. Tested 45 days after training (**Figure 4**) - Independent two-way ANOVA of rats tested in one of four distinct contexts (See **Figure 1.A**) revealed no significant effect in the interaction between Context x Sex ($F_{(3,70)} = 0.6543$; $P = 0.583$), while Context ($F_{(3,70)} = 15.52$; $P < 0.0001$, $\omega^2 = 0.36$) and of Sex ($F_{(1,70)} = 18.27$; $P < 0.0001$, $\omega^2 = 0.18$) differ significantly - **Figure 4.B**: according to the ω^2, all detected effects were large and robust. Number of subjects per context (A, B, C and D respectively): females, 9, 9, 9, 9, and males, 9, 11, 11, 11.

Tukey Multiple Comparisons *post* hoc test revealed that females maintained a profile similar to that of 28 days, with no significant differences between A and B ($P > 0.99$) or C and D ($P = 0.99$), while males keep discriminating between contexts A and C ($P = 0.029$, $g = 1.348$), but not between A and D ($P = 0.07$). Also, when compared in the same context, there were no differences between males and females ($P > 0.05$ for all comparisons) - **Figure 4.C**).

We also estimated the degree of discrimination with the Z-scores for every other context (B, C or D) compared to A (**Figure 4.D**). The same convergence of responses (% freezing level) between contexts B and A verified at 28 days (**Figure 3.D**), was observed here. Discrimination of contexts C and D, however, is much less evident now, suggesting that, at 45 days, all neutral contexts tend to evoke a fear response similar to A.

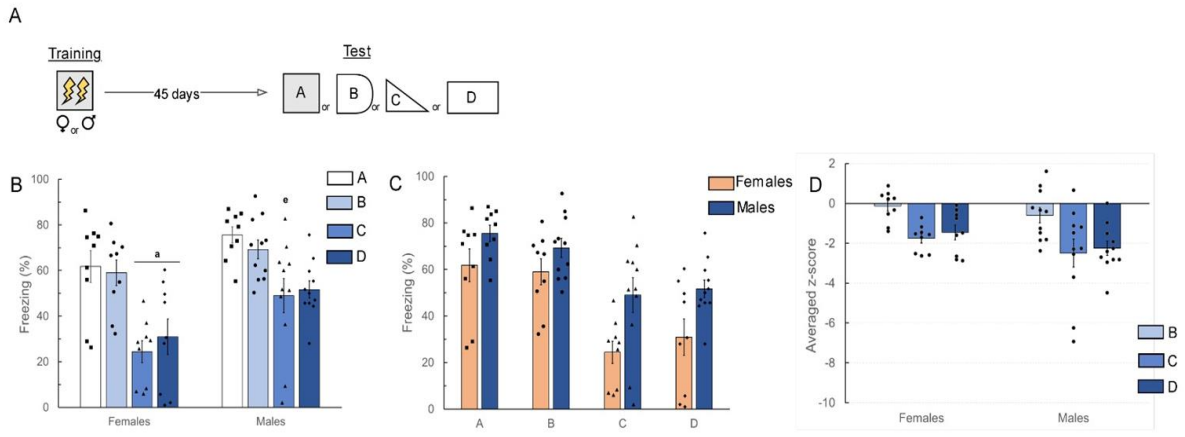


Figure 4. Context discrimination of female and male rats 45 days after training in a fear conditioning task. (A) Experimental protocol: all subjects were trained in Context A and subsequently tested either in A, B, C or D **45 days** later; (B) Histogram showing mean±SEM of the % freezing time in each context grouped by sex with individual dots overlayed representing individual animals; (C) Same data (and statistical analysis) grouped by context. B & C: data analyzed by independent two-way ANOVA followed by Tukey post hoc test. **a** (different from A / B), $P < 0.015$, and **e** (different from A), $P = 0.03$; (D) Individual (and average) Z-scores for contexts B, C and D (standard deviations compared to A).

1.4. Comparing the performance of each sex over time in the conditioning context A

We also compared the performance over these time intervals of females and males – separately – in context A,: independent two-way ANOVA of female rats tested in context A in three different training-test intervals revealed only the effect of Sex ($F_{(1,55)}$ = 25.13; P < 0.0001, ω^2 = 0.28), with no significant effect either in the interaction Sex x Time ($F_{(2,55)}$ = 2.389; P = 0.1012), or in Time ($F_{(2,55)}$ = 2.403; P = 0.0999). According to the ω^2, the detected effect was large and robust.

In females, Tukey post hoc multiple comparisons revealed a negligible difference in freezing levels for context A between 2 and 28 days (P = 0.0422, g =1.557), and no significant differences between 28 and 45 days (P = 0.6499). In males, there were no significant differences between 2 and 28 days (P > 0.999) and between 28 and 45 days (P = 0.9983). Comparing sexes, females differed from males only at 2 days (P = 0.0004, g = 2.70), while no significant differences were found at 28 (P = 0.6029), and 45 days (P = 0.1418). Number of subjects per sex at each time point: females (2, 28 and 45d) = 10, 11, 9, and males = 11, 11, 9.

For comparisons of context A performances for each sex and timepoint, please, see **Figures 2.B, 3.B** and **4.B**, above.

PILOT EXPERIMENT

Subjects of both sexes were fear conditioned to context A, and, 2 days later, retrieval tests were performed in this or in an alternative set of neutral contexts, sorted according to different criteria (chamber shape and floor texture).

Independent two-way ANOVA of female and male rats tested in one of a new set of four contexts (see **Figure 1.B**) plus a version of E without wood shavings on the floor (Context E') revealed significant effects of Context x Sex Interaction ($F_{(4, 86)} = 3.059$; $P = 0.0208$, $\omega^2 = 0.08$) , Context ($F_{(4, 86)} = 16.44$; $P < 0.0001$; $\omega^2 = 0.39$) and Sex ($F_{(1, 86)} = 30.62$; $P < 0.0001$, $\omega^2 = 0.24$) – **Figure 5.B**: According to the ω^2, all detected effects were large and robust. Number of subjects per context (A, E, E', F and G) respectively: females, 8, 15, 10, 9, 9, and males, 9, 10, 7, 10 and 9.

In females (**Figure 5.B**), Tukey *post* hoc test revealed significant differences between groups tested in the conditioning context A and all novel contexts ($P < 0.0001$) with large effect sizes (respectively, A x E, $g = 1.896$; A x F, $g = 4.55$; A x G, $g = 3.848$; A x E', $g = 2.069$), though freezing levels did not differ significantly between the novel contexts E, E', F and G ($P > 0.90$).

For males (also **Figure 5.B**), Tukey *post* hoc test showed groups differed significantly between Context A and novel contexts F ($P < 0.0001$, $g= 2.49$) and G ($P < 0.0001$, $g = 2.962$), but not between A and E ($P = 0.98$), A and E' ($P = 0.57$) or E and E' ($P =0.90$). Also, context E differed from F ($P = 0.0011$, $g = 1.727$) and G ($P = 0.0021$, $g = 1.872$). Male and female groups did not differ in freezing levels in the conditioning context A ($P >0.99$).

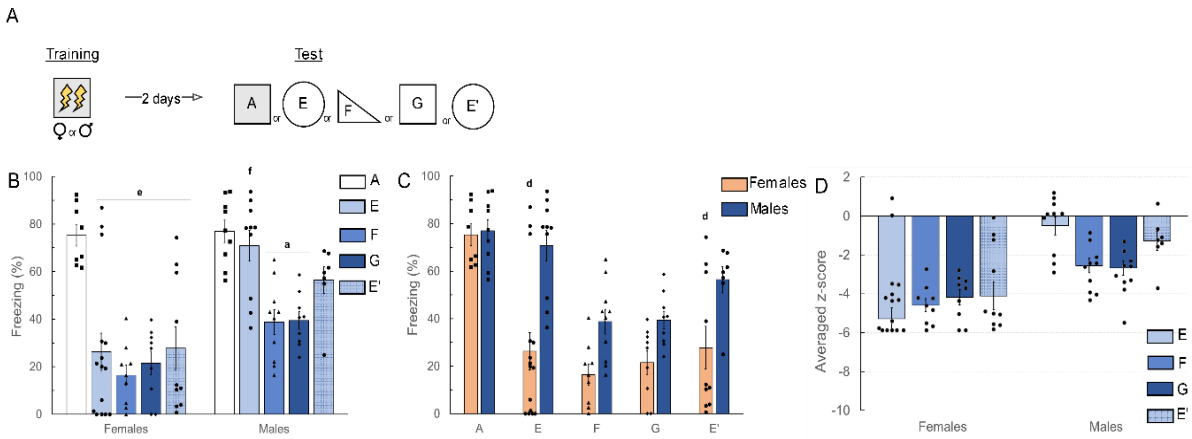


Figure 5. Contextual discrimination of female and male rats trained in a fear conditioning task and tested 2 days later.

(A) Experimental protocol: all subjects were trained in Context A and subsequently tested either in A, E, F, G or E' **2 days** after training (E' being a slight modification of context E); (B) Histogram showing mean±SEM of the % freezing time in each context grouped by sex, with individual dots overlayed representing individual animals; (C) Same data (and statistical analysis) grouped by context. B & C: Data analyzed by independent two-way ANOVA followed by Tukey post hoc test: **a** (different from A / B), $P < 0.0055$, **f** (different from F / G), $P < 0.032$, and **d** (females differ from males tested in the same context), $P < 0.0001$; (D) Individual (and the average) Z-scores for contexts E, F, G and E' (standard deviations, all compared to A).

DISCUSSION

Our main objective was to detect any differential generalization – though the increase in fear expression over time - in multiple novel contexts, and further verify whether this was expressed continuously or in discrete steps. In order to do this, it was necessary to be able to behaviourally quantify *degrees of similarity* between contexts, and obtain – if possible - a progressive, statistically significant reduction of freezing levels across novel contexts in relation to that of the conditioning apparatus. Since we did not obtain the desired 'staircase effect' in the pilot study (tested 2 days after training), we decided to explore a different set of novel contexts, sorted according to different criteria (see **Figure 1**), and having succeeded, but only for males. We then investigated how discrimination evolved over time for both sexes, testing recent memory (2 days after training), as well as remote memory (at 28 or 45 days). This more complete study is our main experiment, assaying contexts A, B, C and D, while the pilot study was shown after, mostly to emphasize some interesting findings.

In murine fear conditioning models, the most frequent indicator of poor context discrimination is the expression of higher freezing levels in neutral contexts, and when they do not differ from the conditioning context anymore, we interpret this as a case of "full generalization". In this work we tried to explore in some depth the time-dependent nature of generalization, whether it comes in progressive degrees, which, however, may not be the case for any neutral context. If this is true, we could talk about "partial vs. total generalization". In the main experiment, time intervals were chosen to be consistent with previous studies from our lab and others (De Oliveira Alvares et al., 2012; Haubrich et al., 2016; Huckleberry et al., 2016).

Instead of just varying, say, one sensory criterion, as is usually done (Shepard, 1987; Young, 2012), we tried a *multidimensional approach*, considering that multiple sensory modalities must be recruited to discriminate between contexts. As seen in **Figure 1**, the seven chosen criteria were (1) chamber shape, (2) size, (3) floor texture, (4) wall texture/colour, (5) illumination, (6) odour and (7) the room used. According to their sensory nature, five are mostly visual (1, 2, 4, 5 and 7), two tactile (2 again and 3) and two, olfactory (6 and 7). Of course, this is not an exhaustive list of possible sensorial criteria to cover all contextual elements of each experimental chamber, and besides, it would be exceedingly complex to examine all possible combinations of sensory modalities anyway. For the sake of practicality, they were ~~we~~ pooled and based on the more salient modalities in rat sensory perception, and combined these criteria two by

two, namely, tactile or olfactory features, each coupled with a visual cue (see **Figure 1** for more details): therefore, in this work we sorted contexts based on chamber size and odour (main experiment) or chamber shape and floor texture (pilot study), as depicted in **Figure 6.A**, below.

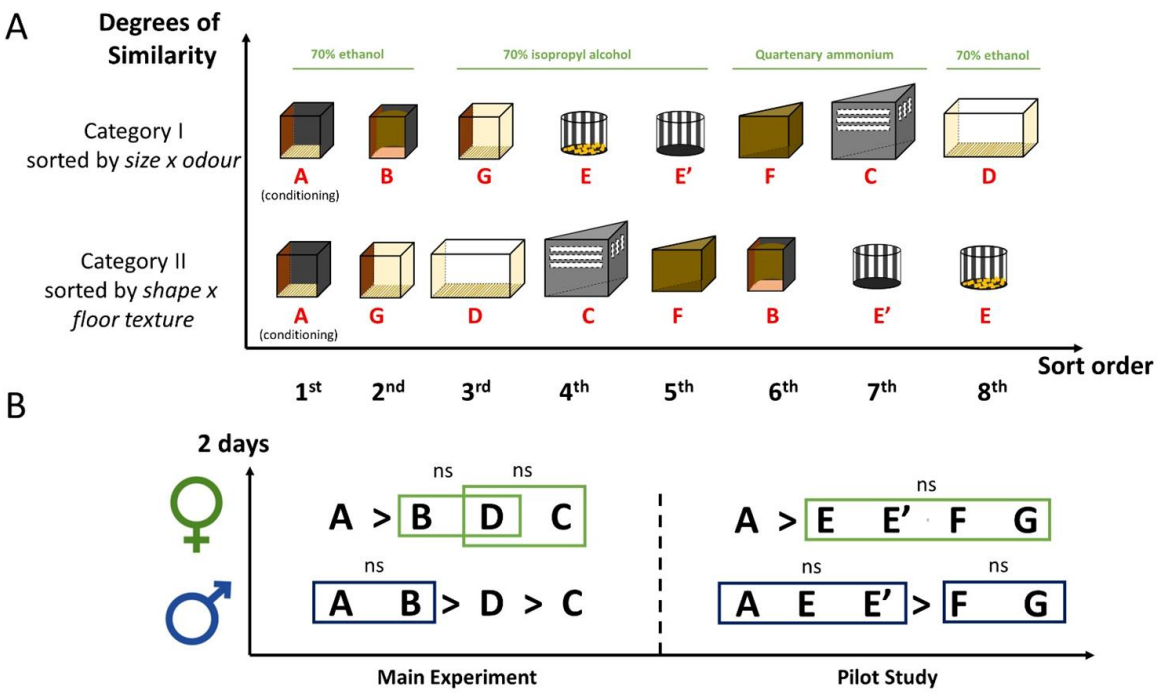


Figure 6. (A) Two possible classifications of our contexts in terms of *degrees of similarity* to A - the conditioning context in both experiments – according to two combined criteria, one mostly tactile/visual (chamber size or shape), and the other, either tactile (floor texture) or olfactory (cleaning solution) cues. (B) Graphic summary of the behavioural response of females and males in each context for recent memory for both experiments. All subjects were trained in Context A (3 min habituation plus two 0.5 mA / 2 sec footshocks within 30 sec intervals) and, 2 days later, tested either in A, B, C or D (Main Experiment), or in A, E, F, G or E' - a slightly different version of E (Pilot experiment). ">" or "<", statistically significant difference, "ns", non-significant difference.

SORTING OF MULTIPLE CONTEXTS: RECENT MEMORY VS. SEX

In our pilot study, we tried to explore contextual arrangements sorted according to intuitive criteria based on shape and floor texture (E, F, G and E' – see **Figure 1** and **Figure 6.A** for more details), looking to obtain a graduated response across

successive contexts (e.g., A > B > C > D), that, unfortunately, was unsuccessful. Surprisingly, *only females* were able to discriminate the least similar context E (and its modified version E') from A. Since context E had wood chips spread on the floor, we suspected this could have acted as a reminder of their homecage, thus signalling safety in a new environment, or acting as a distractor: we, therefore, tested these animals in a cleaner variant of E, E', but even without the wood chips, there was no increase in the freezing response to this context. Males, however, displayed the opposite discrimination pattern, with equal freezing levels to E / E' and the conditioning context A (**Figure 5.B**).

This higher fear response seen in males could be due to neophobia (Greggor et al., 2015) or of novelty-induced fear (Maren, 2014) facing a very dissimilar context. Somehow, this constitutes a behavioural sexual dimorphism. Indeed, all females were able to discriminate equally well among all the "neutral" contexts - there was no significant difference among their freezing performances, all freezings being significantly lower than the response to the conditioning context A.

In order to estimate the degree of discrimination, we plotted the individual (and average) Z-scores for every neutral context (B, C or D in the main experiment, E, F, G and E' in experiment 2): all data points have lower values compared to A, but only in the recent memory test (2 days) there are differences among them (**Figures 2.D, 3.D and 4.D**). These findings are summarized in **Figure 6.B**.

In order to avoid the possible interference of neophobia in recent memory and verify which contextual features might endure, as expected, a time-dependent generalization, in our main experiment we compared male and female fear responses at different training-test intervals, employing a different set of neutral contexts, now mainly based on chamber size and odour (see **Figures 1** and **6.A** for more details). With these contexts, males expressed a "staircase-like effect" in their degrees of discrimination – see **Figure 2.B**. Females, however, displayed a completely different pattern of fear expression, evincing another important difference between the sexes.

Employing this protocol, high levels of freezing were obtained in the conditioning context A in all training-test intervals, indicating *good retention* of the aversive experience (**Figures 2 to 5**). Two days after training, males were definitely not able to distinguish context B from A, while females were, since displaying a (statistically significant) lower freezing level in B compared to A (**Figure 2.B**). This is consistent

with other studies comparing conditioned fear between sexes (Colon et al., 2018; Morena et al., 2021; Trott et al., 2022).

Of relevance, context B is a modified version of the original conditioning chamber A, which may be the main reason for the high expression of fear by males, since we suspect this to be due to males recognizing olfactory cues, the presence of the scent of the original context (plus the use of the same cleaning agent) must have “transferred” enough of the aversive information from context A to B, notwithstanding the other modified parameters (inner chamber shape and floor texture), at least for young adult male rats. This is consistent with the well-known fact that olfactory threat signals are very prominent in rodent life (Canteras et al., 2008; Grella et al., 2020; Takahashi et al., 2005), but it came as a surprise that females seemed not be valuing scent the same way males do: apparently, females did not transfer the same contextual predictors of threat to the new context. This behavioural sexual dimorphism might be attributed to some parameter preference, with males attributing more salience to olfactory cues when assessing threat levels than females, that were “valuing” other aspects of the environment, such as chamber geometry and floor texture, but at this point we did not pursue this line of investigation.

This poses the question: are males generalizing the response to context B 2 days after training, while females keep discriminating them from A? Is this a representative case of "generalization" by itself? Since generalization is defined exactly by this “transference of conditioned responding to stimuli that perceptually differ from the original one” (Bergstrom, 2020), could this be considered a case of “rapid generalization”? Perhaps, but at this recent timepoint it would differ from a *bona fide* time-dependent loss of contextual precision due to neuroanatomical reorganizational processes. At 28 days, however, B becomes indistinguishable from A for both males and females (**Figure 3.B**), continuing like this until 45 days (**Figure 4.B**).

Also noticeable in the recent memory test is that females express *a lower maximum freezing level* compared to males in contexts A, B and C, but not in D, the least similar context (**Figure 2.C**), in which performance was equal between sexes. This is consistent with other studies comparing conditioned fear between males and females (Colon et al., 2018; Morena et al., 2021; Trott et al., 2022), and point to a possible superior discriminative capacity of young adult females relative to males. However, this pattern was not reproduced in our pilot experiment – in which freezing levels in A are equal for both sexes – thus there is not much to be said about this.

At remote memory tests, 28 and 45 days after training, both sexes discriminate similarly, distinguishing contexts A and B (grouped in the same level) from C and D (also grouped) at similar levels (**Figures 3.C** and **4.C**). The raise in fear response in D and C may thus be considered a legitimate case of partial *time-dependent memory generalization*.

TIME-DEPENDENT PROGRESSIVE GENERALIZATION: REMOTE MEMORY

At 28 days, discrimination of contexts C and D converged to a similar, but still significatively lower level than the response to A and B (**Figures 3.B and 4.B**), a 'grouping effect' between contexts C and D (besides A and B), which effectively "broke" the multilevel pattern observed in the recent memory test, and, in particular, dismantled the 'staircase-like effect' exhibited by males at 2 days (**Figure 2.B**) All contexts besides the conditioning one, displayed an increase in freezing levels in these remote tests, which is consistent with a partial *time-dependent contextual generalization* process (Haubrich et al., 2016).

This relates to an important open question of *whether context generalization increases continually or discontinuously with time*, but to shed more light on this it would be necessary to examine much older remote memories than those explored in the present experimental design, and even that might be ultimately inconclusive, since forgetting would also be expected to increase and counterweigh the variable being measured (% freezing). For now, we can only support the idea of *generalization* taking place in incremental steps, of different sizes depending on sex, and not necessarily asymptotically reaching the conditioning context plateau.

Our experiments, employing two different ways to classify multiple contexts allowed the demonstration of a wide variety of behavioural responses, with different instances of sexual dimorphisms. These young adult subjects exhibited a diversity of sex- and time-dependent, contextual fear responses based on criteria we simply did not anticipate, besides showing different forms of time-dependent transitions from precise to generalized fear memory. The epistemological irony here is that this just proves the obvious fact that rodents do not necessarily agree with our poor, reductionistic classification criteria. This might explain the great difficulty in finding the *right* neutral contexts when planning experimental setups to investigate memory generalization (Bouton et al., 2021; Giachero et al., 2024; Rosas & Alonso, 1997): specific 'animal

preferences', including sexual differences, should be added to a much-needed list of *"Systems Consolidation Boundary Conditions"* (SCBC), since they can inadvertedly interfere with such experiments.

MEMORY GENERALIZATION OR WHAT?

As Shepard (1987) explains, "generalization is a cognitive act, not merely a failure of sensory discrimination" and amounts to inferring if an object or context belongs or not to what may be considered "a consequential region", i.e., a safety region in an individual's psychological space. The kind of fear generalization we try to evince here is specifically the time-dependent loss of trace precision, or simply the transference, with time, of a conditioned memory to novel contexts (Bergstrom, 2020; Young, 2012), but since our quantifiable variable is the fear response itself, different processes could explain this change. To this point, our data allows us to discard at least two confounding factors, forgetting and fear incubation.

While it is well established that high emotional arousal such as fear is important to consolidate long-lasting memories, less emotionally charged memories tend to decay easier with time. In a similar vein, we could ask which types of contextual safety-indicating cues can resist gradual decay, or, on the other hand, become true threat signals derived from prior experiences. The first theory proposes that *forgetting* of stimulus attributes is the main cause of generalization (Riccio et al., 1992), but there might be other explanations, such as *associative novelty-induced fear* (Kumaran & Maguire, 2006; Maren, 2014), *neophobia* (Greggor et al., 2015), or even – among the factors raised to explain avoidance generalization in PTSD animal models - *non-associative novelty-induced fear,* a kind of sensitization (Pamplona et al., 2011). With the exception of forgetting, none of these additional possibilities were examined here.

The specific contribution of forgetting in the retrieval of fear memories in novel contexts can, sometimes, be difficult to separate from a true generalization process. However, in our experiment, freezing performance remained high for both sexes even at 45 days after training – over 60% of the session time - meaning that the amount of forgetting taking place in the period covered in our experimental setup might be considered negligible.

Another important confounding factor is fear incubation - when fear responses increase or 'incubate' over time depending on certain experimental parameters (Pickens et al., 2009; Poulos et al., 2016). While the effect of fear incubation in novel contexts remains an open question, some studies suggest the high levels of fear may be due, at least in part, to this phenomenon, in association with retrieval error and the flattening of the generalization curve (Poulos et al., 2016; Rosas & Alonso, 1997).

It is not fully clear where the dividing line rests between Fear Generalization (FG) and Fear Incubation (FI): literature is scarce in experimental evidence that effectively distinguish them. One procedural way to approach this problem, however, would be to consider FI a time-dependent fear increase only when taking place *in the conditioning context*, while the increase *in neutral context(s)*, could be considered true instances of FG (Poulos et al., 2016; Wiltgen & Silva, 2007). In this sense, our findings seem be pointing in different directions for females and males (see **Figures 7.A** and **7.B**, below). Of course, it is still possible that both processes interact synergically, but the present data does not us allow to ascertain this hypothesis. For instance, 'generalization' could be, to some extent, the 'transference' of FI from the conditioning to the neutral context(s), which, as a result, will be indistinguishable from pure generalization by mere increase in expressed fear. However, this increase must first be present in the conditioning context – something verified only in our females. Another aspect to consider is that ours is a quite mild experimental protocol in terms of aversiveness, compared to the most reliable known protocols that induce FI, which usually involve extensive training combined with multiple high-intensity CS (Pickens et al., 2013): in this sense, FI would not be actually expected here.

On the other hand, it could have been the case that a possible *ceiling effect* (see Quillfeldt, 2018) – such as the one displayed by our male subjects, with much higher levels of freezing at all training-test intervals than females (**Figure 7.B**) – rendered us unable to observe fear incubation in those groups (**Figure 7.B**), while females tested in the conditioning context A and in B, showed a substantial increase in % freezing from day 2 to day 28, and remained stable up to 45 days (**Figure 7.A**), which perhaps suggests females exhibit some degree of fear incubation. Actually, in females the increase in fear in B was even steeper than in A. Would that mean that there are sex differences in the way incubated fear is generalized to neutral contexts? This is an interesting possibility, that, however, was outside the scope of the present study.

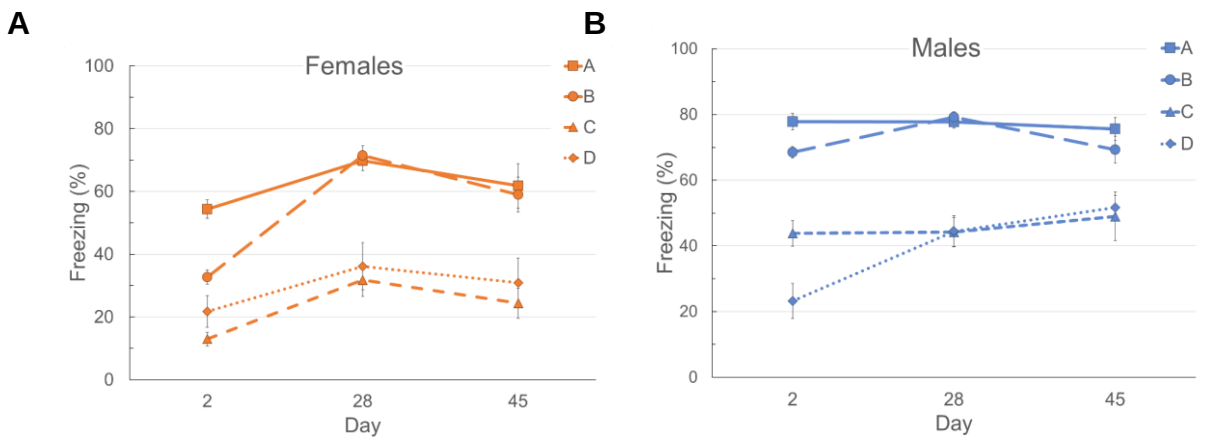


Figure 7. The temporal dynamics of females and males. (A) Line graphs representing freezing responses of females tested at 2, 28 and 45 days in contexts A, B, C and D. (B) *idem* for males tested at the same timepoints in the same contexts.

A CLOSER LOOK INTO THE TEMPORAL DYNAMICS OF FREEZING RESPONSES

In the CFC task, metal grid floors act as threat predictors, becoming more salient with time and facilitating fear generalization to neutral contexts (Huckleberry et al., 2016). Therefore, we used a novel context (D) with a grid floor very similar to the one in the conditioning context, but found that while males tested at 2 days displayed significantly less fear in context D (average 23% of session time spent freezing), males tested 45 days after training showed a clear time-dependent generalization for this context (average 52% of session time - **Figure 7.B**). Conversely, females, who displayed similar freezing levels to males in context D at 2 days (average 22% of session time spent freezing), showed less fear than their male counterparts when tested at 45 days (average 31% of session time), even though freezing levels between males and females in the conditioning context did not differ significantly at this remote timepoint, indicating both sexes learned the task equally well (**Figure 7.A**). Taken together, these results suggest that while this salient aversive contextual cue became progressively generalized to a novel context, other background features signalling safety for males might have become less salient and counteracted the fear response, while the female group on average still perceived the context in its entirety as relatively safe.

There are pitfalls in comparing fear responses between males and females only based in observable behavioural differences. The CFC task is an inescapable, classical conditioning, and even in auditory (tone) fear conditioning, subjects cannot escape the

aversive context and tend to express a passive response, such as freezing in response to threat, rather than engaging in more active escape mechanisms such as flight or fight. However, it is known that, compared to males, females are more prone to *darting*, a defensive behaviour in the presence of auditory cues: thus, freezing by itself might not always be the most reliable index of fear memory retention (Gruene et al., 2015). Furthermore, females can display heightened shock reactivity (Olivera-Pasilio & Dabrowska, 2023) or distinct startle responses compared to males, although results investigating these differences have been mixed, highlighting the need to include other variables in these comparisons (Graham et al., 2009; Russo & Parsons, 2021; Zhao et al., 2018). In our conditioned contextual fear experiments, neither males nor females were observed to engage in darting, and since most of our female subjects displayed relatively high levels of freezing in the conditioning context, we did not consider the possible interference of this defensive behaviour in our results. Interestingly, though infrequent, males in general displayed more *flight behaviou*r compared to females, depending on the context: females in A (zero of 10 subjects), B (zero of 10), C (zero of 10) and D (zero of 11); males in A (1 jumper among 11 subjects), B (zero of 11), C (1 jumper among 11 subjects) and D (zero of 11).

TO WHICH EXTENT IS DATA DISPERSION HORMONE-INDUCED?

One likely factor that may increase the dispersion of data as seen in our females in the neutral contexts may be due to the fact that they can have heightened sensitivity to stress hormones compared to males (Bangasser & Wicks, 2017; Daviu et al., 2014). Stress hormones are important modulators of memory generalization, and can increase or accelerate the natural time-course of memory generalization (Dos Santos Corrêa et al., 2019; Pedraza et al., 2016). We did not quantify corticosterone levels in our groups, and since we had no intention to submit males to pre-training handling, we opted also not to handle females to verify estrus cycle phase so as not to cause additional stress. Previous unpublished results of our lab compared adult females who had undergone several days of previous handling displayed significantly less fear when tested in the conditioning context 2 days after training, but did not differ in anxiety behaviour in the Elevated Plus-Maze compared to nonhandled females, suggesting that frequent handling increased resilience to fear in these females while having no effect upon anxiety (Lotz and Guerra, unpublished results from 2018) (**Figure 8**).

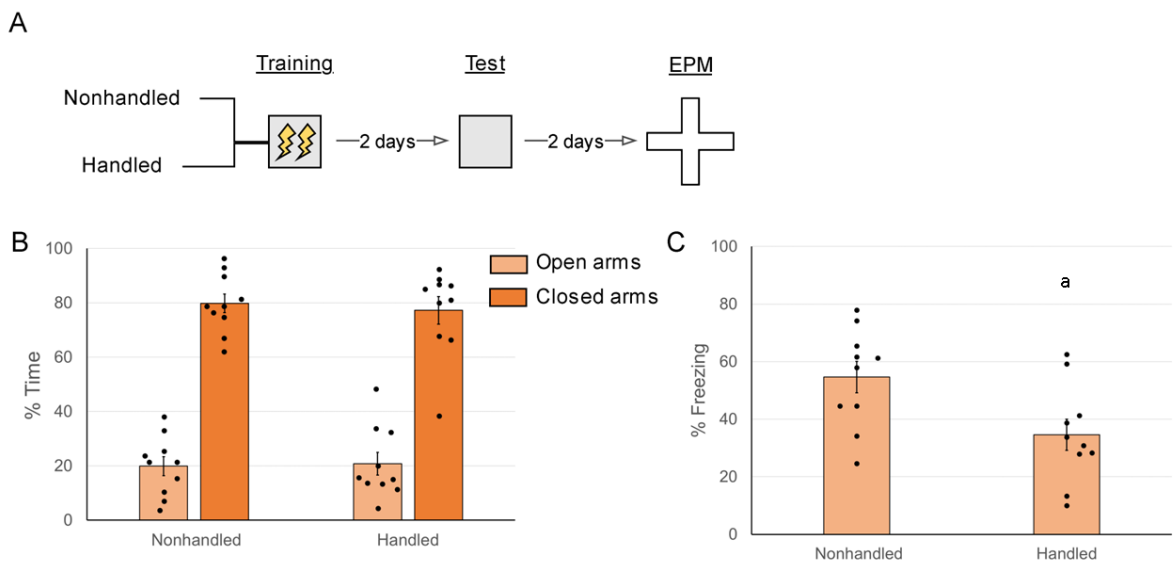


Figure 8. Comparison of conditioned fear and anxiety behaviour in previously handled vs. nonhandled young adult female rats. (A) Experimental protocol: the handled group underwent daily handling (up to 3 min) for 7 days while the nonhandled group remained in their homecages. Subjects were trained and 2 days later tested in Context A. After another 2 days all rats were tested in the Elevated Plus Maze. (B) Histogram showing mean±SEM of the % time spent in open and closed arms, with individual dots overlayed representing individual animals. Data analyzed by independent one-way ANOVA followed by Tukey post hoc test; no differences were found for time spent in open and closed arms for both groups (C) Histogram showing mean±SEM of % freezing, with individual dots overlayed representing individual animals. Data analyzed by unpaired bicaudal Student's t-test. **a** (different from Nonhandled females), $t_{(18)} = 2.62$; $P < 0.0174$.

Besides differences in stress responses, another factor that could explain the variability of results in females is the natural fluctuation of sex hormones levels: it was found that gonadectomized males and females showing conditioned inhibition have this behaviour disrupted only in females that had undergone estradiol replacement, suggesting a particular role of this hormone in safety signalling (Toufexis et al., 2007). In our results, cycle phase is a likely explanation for the observed variability in the novel contexts C and D, especially at 45 days (**Figure 4.A**).

SEX-SPECIFIC COGNITIVE MAPS?

Our results suggest different factors influencing fear memory discrimination, such as differences in safety signalling between males and females, and even that the

generalization process in males and females does not proceed the same way. Dimorphic strategies have been observed in other hippocampus-dependent tasks, such as spatial navigation, in which circulating estrogen levels along with volumetric differences in the hippocampus and entorhinal cortex did influence the extent to which females adopt an allocentric as opposed to an egocentric navigation strategy (Keeley et al., 2013). In the case of auditory cued fear memories, contextual foreground cues are more salient in generating a fear response in females compared to males, while males in general use more background cues when retrieving contextual fear memories (Cossio et al., 2016), suggesting increased recruitment of the amygdala in females, while males may rely more on hippocampus-dependent processes.

While our females expressed lower freezing levels than males in the conditioning context in the recent memory test, subsequent female groups tested at more remote timepoints equalled males in freezing behaviour, indicating there was no deficit in acquisition or consolidation of the fear conditioning experience, but in fact differences in the qualitative transformation of long-term memory, in line with numerous other studies (Taxier et al., 2020).

Another possibility to explain our findings may rest, at least in part, to differences in *cognitive contextual maps* (Posani et al., 2018) between males and females, where relevant features, such as the aversive metal grid floor from which animals received footshocks, become increasingly salient and more easily dissociated from contextual safety signals for male rats as time passes, while females make use of several contextual features to determine how safe or threatening a context is in its entirety, resulting in better context discrimination for longer periods of time. This hypothesis was not explored here also.

As Roger Shepard (1987) comments, “Because any object or situation experienced by an individual is unlikely to recur in exactly the same form and context (...) a full characterization of the change that even a single environmental event induces in an individual must entail a specification of how that individual's behavioural dispositions have been altered relative to any ensuing situation. Differences in the way individuals of different species represent the same physical situation implicate, in each individual, an internal metric of similarity between possible situations.” We could say the same for individuals of distinct sexes of the same species.

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