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

Fernanda Nogueira Lotz[1,2#], Kétlyn Talise Knak Guerra[1,2], Ana Paula Crestani[3], Jorge Alberto Quillfeldt[1,2,4]

[1] Psychobiology and Neurocomputation Laboratory, Biophysics Department, Biosciences Institute, Federal University of Rio Grande do Sul, Porto Alegre - RS, Brazil.

[2] Graduate Program in Neuroscience, Institute of Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre - RS, Brazil.

[3] Department of Psychology, University of California Davis, 135 Young Hall, Davis, CA 95616, United States

[4] Brazilian Network of Memory Investigation (ReBraEM), Brazil.

Corresponding author.

Corresponding author: Fernanda Nogueira Lotz Alves, Laboratório de Psicobiologia e Neurocomputação, Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9500, Prédio 43422, Sala 208, CEP 91.501-970, Porto Alegre, Rio Grande do Sul, Brazil. E-mail: fernandanlalves@gmail.com

ABSTRACT

Memory generalization can be defined as the transference of conditioned fear responses to novel contexts. It happens in systems consolidation, a time-dependent reduction of discrimination precision due to the rearrangement of brain regions supporting memory retrieval. To better understand the fine temporal structure of this process in different sexes, young adult female and male rats were trained in a contextual fear conditioning task and tested in the conditioning or in three novel contexts either 2, 28 or 45 days later. A set of neutral contexts was selected and graded levels of fear expression compared to the training context was obtained, albeit observed only in males, not females, in a recent memory test - the first of a number of sexual dimorphisms detected. This asymmetry, however, disappeared over time due to partial generalization, with fear converging to similar, higher values, grouped in two levels, in both sexes. In all experiments, females were better discriminators and displayed lower fear responses than males, apparently valuing different sensory modalities. This is the first study to compare fear responses between adult female and male rats retrieving recent or remote memories in multiple neutral contexts, allowing several dimorphic findings.

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 behaviors to these new and potentially dangerous environments or situations based on similar cues (Bergstrom, 2020; Young, 2012).

Mammalian brains seem to have evolved to alternate between discrimination and generalization in the face of novel situations, yet we still do not fully understand the exact “boundary conditions” that trigger one or other outcome, especially when opting between slightly distinct contexts, and to which extent recognition of their features relies upon one or more sensory modality, or even a combination of these. Furthermore, it is also difficult to define which contextual elements (floor textures, lighting, size, chamber shape, odor, etc.) actually support discriminatory scalability, i.e., that allow for measurable, statistically significant increments in the responses, resulting in a staircase-like, step-wise comparison.

While generalization can have adaptive value, overgeneralization - an exaggerated fear response to neutral cues or contexts - can have maladaptive consequences, such as restricting important resource-seeking behaviors or resulting in mental disorders 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 signaling (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 behavioral 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 signaling safety can effectively induce learned fear inhibition (Asok et al., 2019), or straightforward time-dependent generalization.

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, for instance, 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) - a contextual memory generalization that increases as the interval between training (conditioning) and test sessions increases (Wiltgen & Silva, 2007).

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 in the conditioning context (A) or in any of three novel / neutral contexts aiming to obtain graded responses according to the different degrees of similarity in relation to the training context. To our knowledge, no previous study has compared fear responses between adult female and male rats in *multiple neutral contexts,* both in recent and remote test sessions.

RESULTS

PILOT STUDY

Subjects of both sexes were fear conditioned to context A, and, 2 days later, retrieval tests were performed in the same or in an alternative set of neutral contexts (P1, P2, P3 and P1' **Figure 1.A**), 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 4.A**) plus a version of P1 without wood shavings on the floor (Context P1') 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 1.B**: According to the ω^2, all detected effects were large and robust. Number of subjects per context (A, P1, P2, P3 and P1') respectively: females, 8, 15, 9, 9, 10 and males, 9, 10, 10 and 7.

In females (**Figure 1.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 P1, $g = 1.896$; A x P2, $g = 4.55$; A x P3, $g = 3.848$; A x P1', $g = 2.069$), though freezing levels did not differ significantly between the novel contexts P1, P2, P3 and P1' ($P > 0.90$). For males (also **Figure 1.B**), Tukey *post hoc* test showed groups differed significantly between Context A and novel contexts P2 ($P < 0.0001$, $g= 2.49$) and P3 ($P < 0.0001$, $g = 2.962$), but not between A and P1 ($P = 0.98$), A and P1' ($P = 0.57$) or P1 and P1' ($P =0.90$). Also, context P1 differed from P2 ($P = 0.0011$, $g = 1.727$) and P3 ($P = 0.0021$, $g = 1.872$). Male and female groups did not differ in freezing levels in the conditioning context A ($P >0.99$), context P2 ($P = 0.324$), P3 ($P = 0.675$) and P1' ($P = 0.12$), but females displayed less freezing than males in context P1 ($P < 0.0001$, $g = 1.656$) (**Figure 1.C**).

In order to estimate the degree of discrimination, we plotted the individual and average Z-scores for every neutral context (P1, P2, P3 or P1'). For females, all data points have significantly lower values compared to A, showing that females were better discriminators across all the novel contexts compared to males (**Figure 1.D**). Also different from males, females were able to discriminate between the conditioning context and P1 (and its modified version P1'), while males displayed high levels of fear in these contexts.

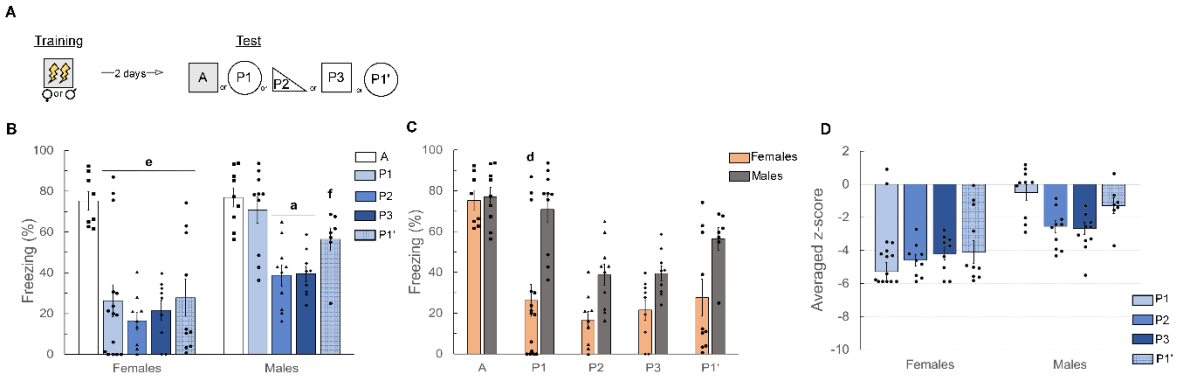


Figure 1. Contextual discrimination of female and male rats trained in a fear conditioning task and tested 2 days later.

(**A**) Experimental protocol: all subjects were trained in Context A and subsequently tested either in A, P1, P2, P3 or P1' 2 days after training (P1' being a slight modification of context P1); (**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 / P1), $P < 0.0055$, **f** (different from P2 / P3), $P < 0.032$, **d** (females differ from males tested in the same context), $P < 0.0001$, and **e** (all groups differ from A), $P < 0.0001$; (**D**) Individual (and the average) Z-scores for contexts P1, P2, P3 and P1' (standard deviations, all compared to A).

MAIN EXPERIMENT

Subjects of both sexes were fear conditioned to context A, and 2, 28 or 45 days later, different groups were submitted to retrieval tests in this or three other neutral contexts (B, C and D, **Figure 2.A**), sorted according to multidimensional criteria (chamber size and odor).

When animals were tested 2 days after training in one of four distinct contexts (See **Figure 4.B**), independent two-way ANOVA 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, 2.E and 2.H**): 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 ($P = 0.572$) (**Figure 2.B**).

For rats tested at 28 days in one of four distinct contexts, independent two-way ANOVA 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 2.C, 2.F and 2.I**): 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 2.C**).

Finally, for animals tested 45 days after training, independent two-way ANOVA 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 2.D, 2.G and 2.J**): 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 2.D**).

In order to estimate the degree of discrimination, we plotted the individual and average Z-scores for every neutral context (B, C or D). For rats tested at 2 days (**Figure 2.H**), 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. For animals tested at 28 days (**Figure 2.I**), 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. The same convergence of responses (% freezing level) between contexts B and A verified at 28 days, was observed at 45 days (**Figure 2.J**). Discrimination of contexts C and D, however, is much less evident now, suggesting that, at this timepoint, all neutral contexts tend to evoke a fear response similar to A.

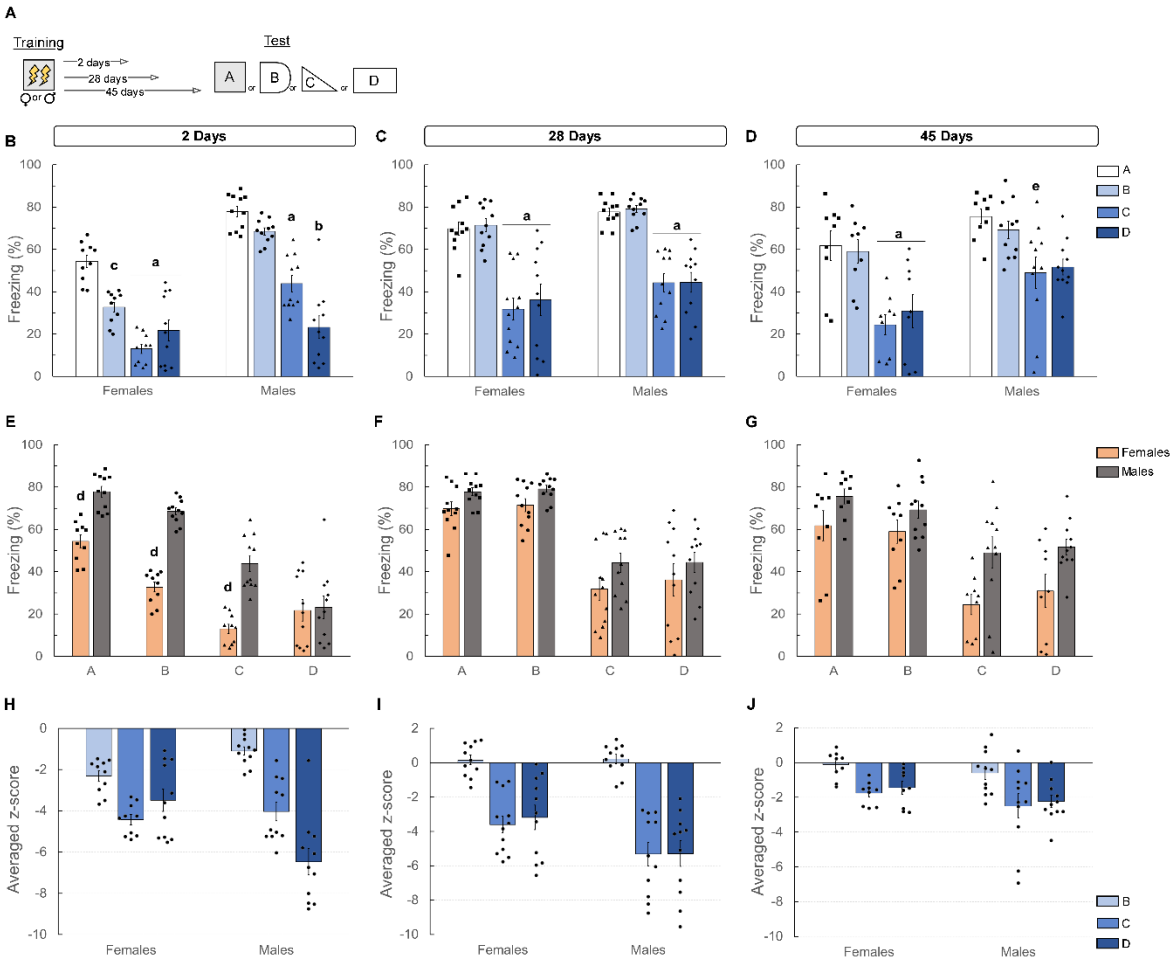


Figure 2. Context discrimination of female and male rats 2, 28 or 45 days after training in a fear conditioning task. (**A**) Experimental protocol: all subjects were trained in Context A and subsequently tested either in A, B, C or D 2, 28 or 45 days later; (**B, C and D**) Histograms showing mean±SEM of the % freezing time in each context grouped by sex with individual dots overlayed representing individual animals; (**E, F and G**) Same data (and statistical analysis) grouped by context. B - G: data analyzed by independent two-way ANOVA followed by Tukey *post hoc* test. **a** (different from A / B), P < 0.015, **b** (differs from same sex groups tested in all other contexts), P < 0.002, **c** (different from A), P = 0.002, **d** (females differ from males in this specific context E), P < 0.0005, and **e** (different from A), P = 0.03; (**H, J and I**) Individual (and average) Z-scores for contexts B, C and D (standard deviations compared to A).

DISCUSSION

We began by adjusting a selection of multiple novel contexts in order to obtain different levels of fear expression, i.e., a statistically significant step-wise reduction of freezing levels in relation to the conditioning context. Then, we aimed to observe and quantify changes in freezing levels over time to investigate the fine temporal structure of systems consolidation in rats of both sexes. As is well-known, this phenomenon is characterized by a time-dependent increase in fear to novel contexts, which is interpreted as memory generalization. With such an experimental setup we aimed to verify how generalization evolves over time in each distinct context, and whether this occurs continuously or discontinuously.

In planning our *graduated contexts*, instead of simply varying one sensory criterion as is usually done (Shepard, 1987; Young, 2012), we tried a *multidimensional approach*, since discrimination between distinct contexts must rely on multiple sensory modalities. At least seven factors were listed (see **Figure 4** for more details): (1) chamber shape, (2) size, (3) floor texture, (4) wall texture/color, (5) illumination, (6) odor and (7) the room used. Of course, this is not an exhaustive list of all possible sensory criteria available to describe the contextual elements of each experimental chamber, however it is experimentally difficult to take them all into consideration simultaneously, in all possible combinations, but even restricting ourselves to the more salient sensory modalities - such as olfactory, tactile or visual features - represents a huge challenge in trying to classify different contexts.

In terms of chamber shape, cleaning agent odor or floor texture, the pilot study explored a randomly ordered set of neutral contexts, while in the main experiment, distinct neutral contexts were tried, sorted loosely based on chamber size and cleaning agent odor. **Figure 4** shows the chosen contexts.

SORTING OF MULTIPLE CONTEXTS: RECENT MEMORY VS. SEX

As mentioned above, we tried to obtain graded responses across successive neutral contexts, but those chosen for the pilot study - P1, P2, P3 and P1’ – mostly grouped into two levels and did not meet our goal (**Figure 1.B**). However, there was a noticeable difference between sexes: females were able to clearly discriminate context P1 from A, while males were not. This was surprising because P1 looked,

intuitively, to be the most different context compared to the conditioning one. Since context P1 had wood chips spread on its smooth floor, we suspected this could have acted as a reminder of their homecage, in which wood chips are used as bedding, somehow signaling safety in an otherwise very different environment - or even acting as a distractor. Therefore, we tested the animals in a cleaner variant, P1', devoid of wood chips, but the freezing response of males remained higher to this context, while females had, again, lower levels, discriminating it from A just as they had with P1.

In sum, females appear to be better discriminators than males, being able to discriminate all four neutral contexts used in this pilot study equally well, all with significantly lower freezing performances compared to expression of fear in the conditioning context A. This dimorphic behavioral response suggests each sex values different parameters in different ways.

It is possible that the abnormally higher fear response of males in such dissimilar contexts P1 / P1' just two days after training might be due either to the novelty component itself, either a case of *novelty-induced fear* (Maren, 2014) or *neophobia* (Greggor et al., 2015). We did not pursue this avenue of investigation, but in order to avoid this kind of interference in recent memory and verify which contextual features might endure across a time-dependent generalization, a different set of neutral contexts was chosen for the main experiment (B, C and D – see **Figure 4**) to compare female and male fear responses at different training-test intervals.

These contexts, sorted in a way loosely based on chamber size and cleaning agent odor, allowed to successfully obtain a "staircase-like" series of decreasing freezing values in males (see **Figure 2.B**, right panel). Females, however, displayed a completely different pattern of fear expression, evincing another important difference between sexes (**Figure 2.B**, right panel).

Employing this protocol, with this selection of contexts, 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.B**, **C** and **D**). Of note, two days after training, males were definitely not able to distinguish context B from A, while females were, displaying a (statistically significant) lower freezing level in B compared to A (c in **Figure 2.B** and d in **2.E**).

Of relevance, context B is actually a modified version of the original conditioning chamber A, with the grid floor covered by a flat linoleum and a curved wall added

inside the chamber, which may be the main reason for the high expression of fear by males in this context. We suspect the scent of the original training context was not sufficiently masked (plus, the same cleaning agent was used in A and B), thus the higher fear response might be because males, somehow, value olfactory cues more than females. The presence of the original conditioning scent might have transferred enough 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 when tested at this recent timepoint. This behavioral 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 only 2 days after training, while females keep discriminating B from A? Would this be an actual case of generalization? If generalization can be accurately defined as “transference of conditioned responding to stimuli that perceptually differ from the original one” (Bergstrom, 2020), this higher response at 2 days could be considered a case of “rapid generalization”. However, at such a recent timepoint it might hardly be considered a “*bona fide* time-dependent loss of contextual precision” such as that resulting from neuroanatomical reorganizational processes.

This sex asymmetry, however, disappears at 28 days, when B becomes indistinguishable from A for both females and males (**Figures 2.C** and **2.F**), perseverating like this until 45 days (**Figure 2.D** and **2.G**). **Figure 3**, below, summarizes most of the similarities and differences between the pilot study and the main experiment.

Another noticeable fact is that, in the main experiment recent memory test, 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.E**), pointing again to a superior discriminative capacity of young adult females relative to males. This is consistent with some studies comparing conditioned fear between males and females (Colon et al.,

2018; Morena et al., 2021; Trott et al., 2022), but might differ from other findings (Lynch III et al, 2013).

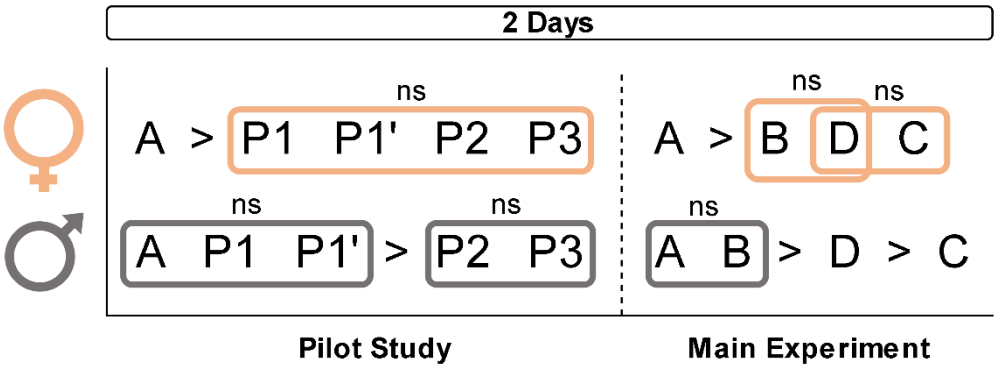


Figure 3. Graphic summary of the behavioral response of females and males to each context in both experiments (recent memory only). All subjects were equally trained in Context A (3 min habituation plus two 0.5 mA / 2 sec footshocks within 30 sec intervals) and, 2 days later, tested either in A, P1, P2, P3 or P1', or in A, B, C or D (Main Experiment). ">" or "<", statistically significant difference, "ns", non-significant difference.

TIME-DEPENDENT PROGRESSIVE GENERALIZATION: REMOTE MEMORY

In murine fear conditioning models, the most frequent indicator of poor context discrimination is expressing freezing in neutral contexts in levels close to that of the conditioning one. Despite a *partial* increase in this response being considered a *generalization* of sorts, only when they are statistically indistinguishable from the conditioning context freezing could it be considered a *full generalization*. Assaying multiple neutral contexts may be a way to quantify distinct levels or degrees of generalization.

The main experiment consisted of investigating how the discrimination of each context (of the second set in **Figure 4**) evolves over time for both sexes, testing two instances of remote memory compared to a recent memory performance.

What we observed was that 28 and 45 days after training animals of both sexes expressed similar freezing values, grouped in two levels. At 28 days, discrimination of contexts C and D converged to a similar level, albeit significantly lower than the response to A and B (**Figure 2.C**), i.e., contexts C and D (and also between A and B) grouped two by two, effectively disrupting the multilevel pattern observed in the recent memory test, and, in particular, dismantling the "staircase-like" effect exhibited by males at 2 days (**Figure 2.B**). This new pattern, however, persisted up until 45 days, with a slight increase in the performance in context D in males (**Figure 2.D** and **G**). Importantly, all novel contexts displayed an increase in freezing levels over these

remote tests, which is consistent with a *partial time-dependent contextual generalization* process (Haubrich et al., 2016).

This relates to another open question of *whether context generalization increases continually or discontinuously over 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 performance plateau.

Our experiments, employing two sets of multiple contexts allowed the detection of a wide variety of behavioral responses, with different instances of sexual dimorphisms. These young adult subjects exhibited a diversity of sex- and time-dependent contextual fear responses, sorted according to multisensory criteria outside our capacity of anticipation (e.g., either visual or olfactory, or combined). Besides, there was a variety of distinct 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 ordering criteria. And it might explain the pervasive difficulties when trying to find the *right* neutral contexts when planning experimental setups to investigate memory generalization (Bouton et al., 2021; Giachero et al., 2024; Rosas & Alonso, 1997; Haubrich et al, 2016): specific animal preferences, including sexual differences, should be added to a much-needed list of *"Systems Consolidation Boundary Conditions"* (SCBC), since they can inadvertently interfere with such experiments.

MEMORY GENERALIZATION OR WHAT?

As Shepard (1987) explains, "generalization is thus 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. So far, our data might allow us to discard at least two confounding factors, *forgetting* of the aversive experience and *fear incubation*.

It is well established that high emotional arousal such as fear is important to consolidate long-lasting memories, and less emotionally charged memories tend to decay easier with time, i.e., to be *forgotten*. However, in the conditioning context freezing performance remained high for both sexes even 45 days after training – over 60% of the session time (see **Figure 2.D** and **G**) – indicating that the amount of forgetting of the aversive experience at our most remote test, if it exists, could be considered negligible.

In novel contexts, however, the specific contribution of forgetting to the retrieval of fear memories can be difficult to separate from a true generalization process. For instance, memory for contextual cues signaling safety might undergo gradual decay with time and give way to threat signals becoming more salient, such as touching a metal grid floor similar to the one in which footshocks were received. Indeed, one theory proposes that the main cause of generalization is *forgetting* of the stimulus attributes (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, however, none of these additional possibilities were examined here.

Another important confounding factor would be *fear incubation* - when fear responses increase over time or incubate 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 entirely clear where the dividing line lies between Fear Generalization (FG) and Fear Incubation (FI): experimental evidence that effectively distinguish the two is scarce in the literature. One procedural approach to this problem, however, would be to consider FI a time-dependent fear increase only if 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 **Supplemental Figure 1**). Of course, it is still possible that both processes interact synergically, but our 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, to begin with, this increase must *first be present in the conditioning context* – something verified only in females in our experiment. Another aspect to consider is that our experimental protocol is quite mild in terms of aversiveness – at least compared to the most reliable known protocols of FI induction, 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 be the case that our male subjects have reached a *ceiling* (see Quillfeldt, 2018) in terms of % freezing as an expression of fear: in fact, males displayed much higher levels of freezing than females in all training-test intervals – rendering us unable to observe fear incubation in those groups (**Supplemental Figure 1.B**), while females, tested in the conditioning context A and in B, showed a substantial increase in % freezing from day 2 to day 28, which then remained stable up to 45 days (**Supplemental Figure 1.A**), which perhaps suggests that at least females exhibit some degree of fear incubation. Actually, in females, the increase of fear in B was even steeper than in A, suggesting the interesting possibility that incubated fear might be generalized to neutral contexts depending on the degree of similarity to the conditioning one. This, however, was outside the scope of the present study.

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 - **Supplemental Figure 1.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 (**Supplemental Figure 1.C**). Taken together, these results suggest that while this salient aversive contextual cue became progressively generalized to a novel context, other background features signaling 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 behavioral differences. The CFC task is an inescapable, classical conditioning task, and even in auditory (tone) fear conditioning, subjects cannot escape the aversive context and tend to express a more 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 behavior 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 may discard the possibility of this defensive behavior interfering in our results. Interestingly, though infrequent, males in general displayed more *flight behavior* compared to females, depending on the context (data shown here): 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).

Another factor that may play into sex differences in fear memory generalization is the fact that females 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). Previous unpublished results of our lab compared adult females who had undergone several days of previous handling with those who remained in their homecages, with handled females displaying significantly less fear when tested in the conditioning context 2 days after training (**Supplemental Figure 2.B**), but did not differ in anxiety behavior in the Elevated Plus-Maze compared to nonhandled females (**Supplemental Figure 2.C**), suggesting that frequent handling increased resilience to fear in these females while having no effect upon anxiety.

SEX-SPECIFIC COGNITIVE MAPS?

Our results suggest different factors might be at stake in fear memory discrimination, such as distinct safety signaling between males and females. Also, it is possible that the generalization process simply happens differently in females and males. 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 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 in our experiments females expressed lower freezing levels than males in the conditioning context A in the main experiment recent memory test, female groups tested later, at more remote timepoints, equaled males in terms of freezing behavior, indicating *there was no deficit in acquisition or consolidation of the fear conditioning experience*, but what was in fact observed was differences in the qualitative transformation of long-term memory, which is consistent with numerous other studies (Taxier et al., 2020).

Another possibility to explain our findings may be that, at least in part, females and males have different *cognitive contextual maps* (Posani et al., 2018), and 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 other 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, however, was also beyond the scope of the present work.

Roger Shepard (1987) is again relevant to be cited at this point (italics are ours): “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 behavioral 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”. At least when describing rat behavior, we can say exactly the same for conspecifics of distinct sexes.

MATERIALS AND METHODS

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 our pilot study (**Figure 4.A**) and main experiment (**Figure 4.B**) are depicted below.

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 caliber 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.

In the pilot study, Context P1 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 P1', identical to P1 but without wood shavings, was also assayed.

Context P2 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 P2 was cleaned with quaternary ammonium disinfectant cleaning solution before each animal.

Context P3 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.

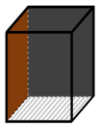
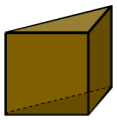
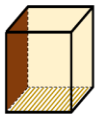
In our main experiment, Context B was a not-so-slightly modified version of Context A, in which the metal grid floor was covered with a smooth brown vinyl mat and a semi-circular rubber wall placed inside the conditioning chamber. Both Contexts A and B were cleaned with 70% ethanol before each session.

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 caliber stainless copper bars spaced 1 cm apart. The test chamber 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, P1, P1’ and P2 were located in separate rooms from that of the conditioning chamber.

A

Pilot Study				
				
Context	**A (conditioning)**	**P1 (neutral)**	**P2 (neutral)**	**P3 (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

B

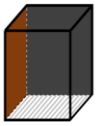
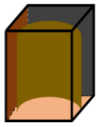
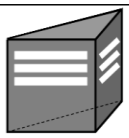
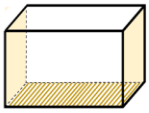
Main Experiment				
				
Context	**A (conditioning)**	**B (modified conditioning)**	**C (neutral)**	**D (neutral)**
Chamber shape	square cuboid	semicircle	triangular	rectangular cuboid
Size	22 x 22 x 33 (LxWxH cm)	22 x 22 x 33 (LxWxH cm)	34 ; 28 ; 48 x 19 (S1;S2; S3 x H cm)	50 x 25 x 29 (LxWxH cm)
Floor 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

Figure 4. Schematic illustration depicting the specific contexts used in this study. (**A**) Pilot study (2 days only); (**B**) Main experiment (2, 28 and 45 day training-test intervals). In our pilot groups we also included P1', a modified version of context P1, without wood shavings in the floor. For more details, please, see text.

Behavioral 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, P1/P1', P2 or P3 – for the pilot study (**Figure 1**) or A, B, C or D - for the main experiment (**Figure 2**). In both experiments, test sessions lasted 5 min during which freezing responses were manually scored by an experienced observer using a stopwatch. Defensive freezing behavior, 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 behavior. 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 1.D** and **2.D**, each point represents the behavioral response of one individual animal.

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