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Parametric modulators of sex-biased conditioned fear responding

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Abstract: Pavlovian fear conditioning is a widely used behavioral paradigm for studying associative learning in rodents. Despite early recognition that subjects may engage in a variety of behaviors that reflect the experimental parameters of a given protocol, the last several decades have seen the field narrow its focus to measure freezing as the sole indicator of conditioned fear. Additionally, unconditioned responses such as shock-related activity are rarely considered. We previously reported that female Sprague Dawley rats are more likely than males to engage in darting, an escape-like conditioned response that is associated with heightened shock reactivity, but we did not establish whether darting was sensitive to manipulations of factors such as chamber size, shock intensity, or number of trials. Our goal here was to address these questions by defining parametric and phenotypic predictors of darting in both sexes. To better capture fear-related behavioral repertoires in our animals, we developed ScaredyRat, a custom Python tool that analyzes Noldus Ethovision-generated raw data files to identify Darters and quantify both conditioned and unconditioned responses. We find that like freezing, darting probability scales with experimental alterations in multiple dimensions. In most cases, the sex bias towards females persists, but males will transition to darting in extended, “overtraining” fear conditioning protocols.

Introduction

Pavlovian fear conditioning is an experimental paradigm used to study associative learning in rodents (Fanselow, 1984). A neutral conditioned stimulus (CS)—usually an auditory tone—is paired with an aversive unconditioned stimulus (US), usually an electric foot shock. The US evokes an unconditioned response (UR) in the rodent, which learns to associate the CS with the US and ultimately exhibits a conditioned response (CR) when presented with the CS. The most commonly studied CR in Pavlovian fear conditioning is freezing, defined as the total lack of all movement except that required by respiration (Fanselow, 1980). For decades, Pavlovian fear conditioning studies have relied solely on the amount of time an animal spends freezing as an indicator of both the level of fear that the animal is experiencing, and of the strength of the CS-US association. This reliance on a single behavior excludes from analysis any other CRs that the animal might engage.

In 2015, our lab identified another CR: darting (Gruene et al., 2015a). Darting is characterized by a quick movement across a fear conditioning chamber and occurs in about 40% of female rats and about 10% of males. Using a seven CS-US auditory fear conditioning paradigm, we found that darting emerges during the later tones of the trial, often following earlier freezing responses. This pattern suggests that darting reflects a switch in conditioned responding that is more common in females. Since this initial report, other labs (and our own) have corroborated the finding that conditioned darting occurs primarily in female rats (Pellman et al., 2017; Colom-Lapetina et al., 2019; Greiner et al., 2019; Morena et al., 2021), but the significance of darting as a sex-biased conditioned fear response and the factors that modulate it have not been studied systematically. In contrast, a great deal of work has been done to examine the factors that affect conditioned freezing, including shock intensity (Fanselow, 1982), context cues (Bolles and Collier, 1976; Fanselow, 1980), amount of pre-exposure to the context (Fanselow, 1990), length of CS (Fanselow et al., 2019), and number of CS-US pairings (Maren, 1998). However, these seminal studies did not consider the sex of the subjects as a potentially data-driving variable.

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The goal of the current study was to determine the phenotypic scope and situational modulators of conditioned darting in both males and females. In other words, we asked whether the propensity to dart is pre-determined and fixed in a sex-dependent manner, or whether—like freezing—it can be manipulated by altering experimental parameters. We therefore investigated the influence of context, space, time, and shock intensity on conditioned fear behaviors in both male and female rats. To perform automated, unbiased detection of darting, freezing, and shock reactivity, our lab developed ScaredyRat, an open-access, custom Python tool that analyzes Noldus Ethovision-generated raw data files to identify Darters and quantify behavior throughout a trial. Our findings advance our previous work by illuminating both the experimental conditions under which darting is more or less likely to occur, as well as individual behavioral predictors of darting in both males and females. Overall, this work broadens the field's understanding of the multidimensional ways that male and female rodents can show conditioned fear, and underscores the need for more diverse behavioral considerations in classic learning paradigms (Shansky, 2018).

Introducing ScaredyRat

ScaredyRat is a custom Python tool that we developed to assist Ethovision users in evaluating darting, freezing, and shock response behavior in rodent fear conditioning experiments. Graphical User Interfaces (GUIs) for both Windows and Mac users, detailed user manuals, as well as code for Python-proficient users can be found at https://github.com/trettels/ScaredyRat_ver2. ScaredyRat processes raw data files exported from Ethovision and generates spreadsheets for freezing, darting, and velocity metrics, organized according to user-defined Epochs within an experimental session. These Epochs can include, but are not limited to: CS presentation, US presentation, and any pre- or post-Epoch periods of time in which behavior may be of interest. In addition, ScaredyRat generates individual velocity plots (Figure 1) for each subject with color-coded Epochs and bouts of darting and freezing indicated.

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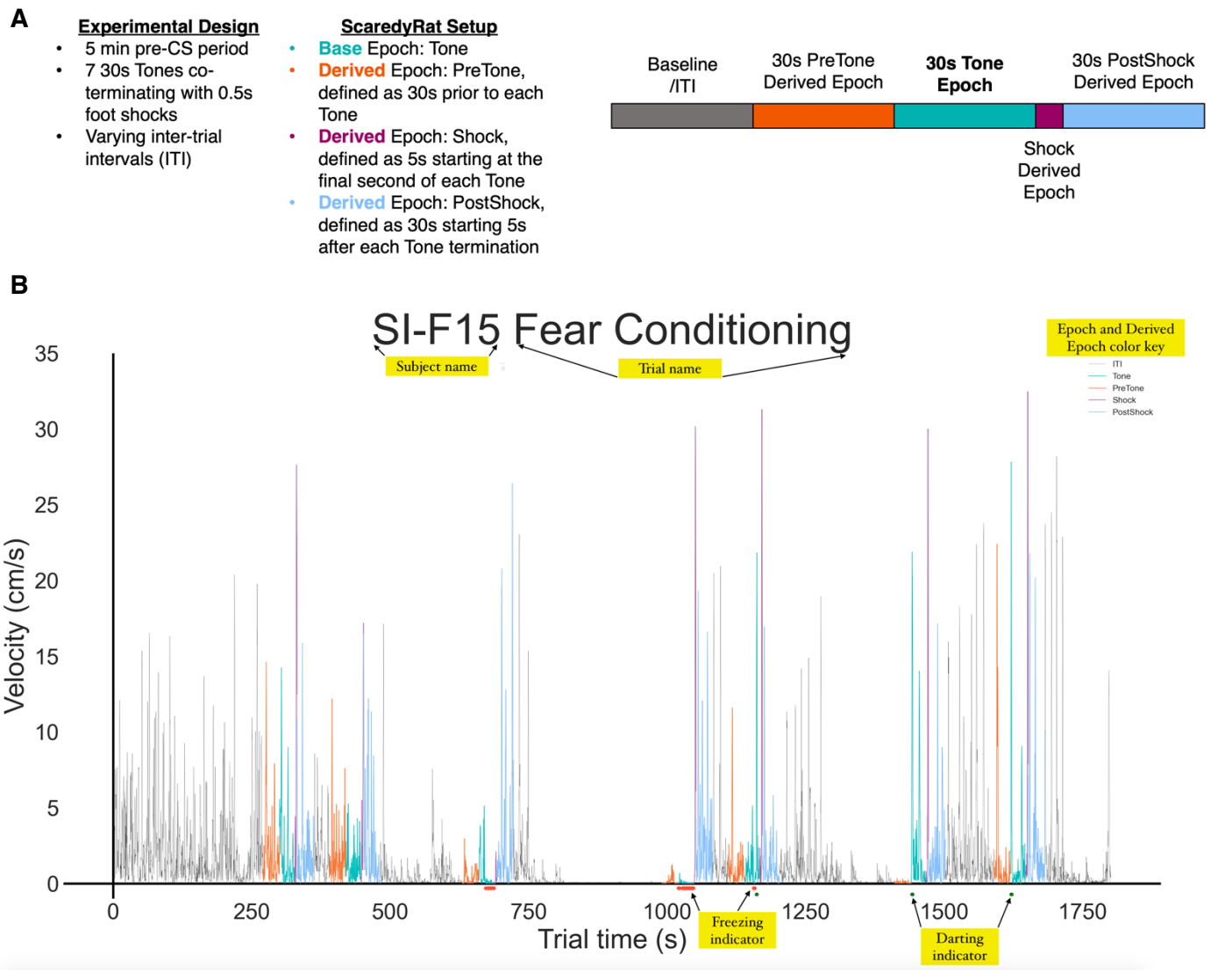

Figure 1. How does ScaredyRat work? A) Example Experimental Design, ScaredyRat setup, and Epoch/Derived Epoch schematic for the plot generated in B). Our experimental design consisted of a 5 min exploration period, followed by 5 30s Tones that co-terminated with a 0.5s footshock, with varying ITIs. ScaredyRat identifies Ethovision-defined Base Epochs (here, the Tone), and then the ScaredyRat user defines Derived Epochs of interest that are time-linked to the Base Epoch (here, PreTone, Shock, and PostShock). B) Representative ScaredyRat-generated plot for a single subject across time in an entire fear conditioning session. Yellow labels indicate notable features—these labels do not appear in actual plots. This animal would be classified as a Darter, because CS (tone)-related darting can be observed in CS 5, 6, and 7. Note earlier periods of freezing on CS 3 and 4. ScaredyRat also generates spreadsheets for Freezing, Darting, and Velocity during each Epoch and Derived Epoch. Behavior during ITIs is not reported.

Results

To investigate the influence of dimensions such as context, space, time, and shock intensity on fear conditioning behavior in male and female rats, we performed four main experiments and used ScaredyRat to collect freezing, darting, and shock response data.

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1. Darting in a context conditioning experiment

Our initial report on darting used an auditory cued fear conditioning paradigm, in which only darts that occurred during the conditioned stimulus (CS) were used to classify animals as Darters. To investigate whether darting could be observed in the absence of a discrete CS, we performed a 2-day context conditioning experiment, in which adult Male (n=12) and Female (n=14) Sprague Dawley rats were exposed to seven footshocks with varying inter-trial intervals, followed by a 6-minute context exposure 24 hours later (Figure 2A). Darting for each trial was defined as any instance of a discrete movement that a) occurred outside the 30s "PostShock" Derived Epoch (See Fig 1 for Epoch illustration), and b) reached the velocity threshold for darting (20 cm/s). The percentage of animals darting for each trial is shown in Figure 2B (left). Animals that exhibited at least one dart outside the PostShock Derived Epoch and after the first shock presentation were classified as Darters. With these criteria, 57% of Females were classified as Darters whereas no Males were (Figure 2B, right).

We next measured the shock response in all animals, defined as the maximum velocity with which the animal moves at the time of shock delivery. Although Females appeared to increase their shock response as trials progressed (Figure 2C, left), effects of sex on shock response did not reach statistical significance when evaluated either as Trial Blocks ($F_{1,24}$=3.2, p=0.08) or as an average across all trials (Figure 2C, right; 2-tailed unpaired ttest: t= 1.9, df=24, p=0.07). Freezing levels for Day 1 were sampled by assessing % time freezing during the 30s prior to each shock presentation, to match the timing of freezing measurements taken in our standard cued conditioning experiments. A 2-way ANOVA of freezing behavior during fear conditioning (Figure 2D, left) revealed a significant time x sex interaction ($F_{2,48}$=5.6, p=0.006), as well as significant main effects of time ($F_{2,44}$=117.2, p<0.0001) and sex ($F_{1,24}$=4.7, p=0.039), with Females freezing significantly less than Males. This sex effect appears to be driven by Darters (Figure 2D, right: filled circles), as a one-way ANOVA of average freezing in Males, Darters, and Non-darters during Trial Blocks 2-3 revealed a significant group effect ($F_{2,23}$=7.2, p=0.004). An adjusted Dunnett's post-hoc test resulted in a significant difference between Males and Darters (p=0.002) but not Males and Non-darters (p=0.36).

On Day 2, no darting was observed in either sex. Freezing behavior was assessed across the entire 6-minute session in 90s Time Blocks (Figure 2E, left), and we again found significant main effects of time ($F_{3,63}$=11.7, p<0.0001) and sex ($F_{1,24}$=9.5, p=0.005). To assess the contribution of Darters to this effect, we performed a one-way ANOVA of average freezing across the session (Figure 2F, right) and found a significant group effect ($F_{2,23}$=11.8, p=0.0003). Adjusted Dunnett's post-hocs once again resulted in a significant difference between Males and Darters (filled circles; p=0.0002) but not Males and Non-darters (p=0.61).

Together, these data suggest that putative sex differences in context fear conditioning experiments may be driven by a subset of females that engage in darting behavior during training. We demonstrate that the engagement of a single darting response during fear conditioning is sufficient to predict reduced conditioned freezing across multiple days.

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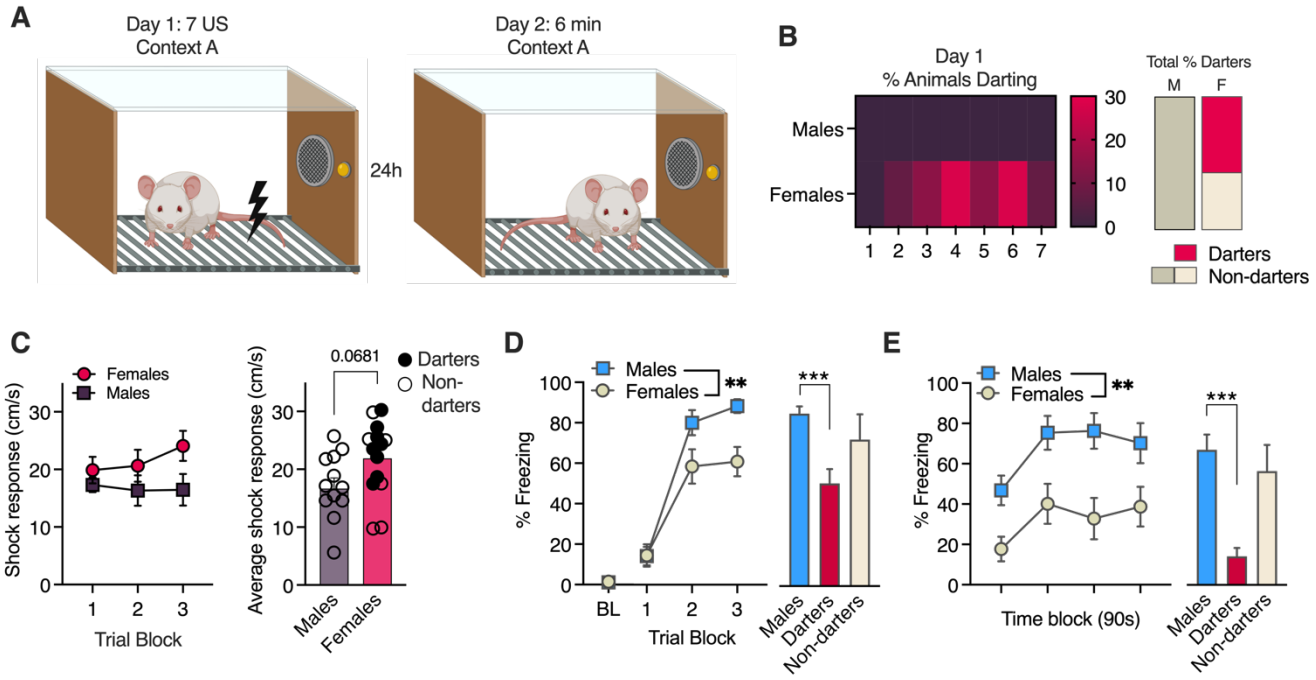

Figure 2. Influence of darting in a context conditioning experiment. A) Experimental design for Days 1 & 2. B) Percentage of Male and Female Darters for each time block across the session (left), and for the entire session (right). C) Shock response in Males and Females. D) Sex differences in freezing during fear conditioning were driven by Darters. E) Sex differences in freezing during the memory test were driven by Darters, who froze less than Males. ** p<0.01 Males vs. Females. *** p<0.001, Males vs. Darters. Fear conditioning Trial Blocks (Panels C-D) represented as averages of Trials 1-2, 3-4, 5-7. BL: baseline freezing, defined as the first 2 minutes of the session. Panel A created with BioRender.

2. Darting as a function of chamber size

Rodents can alter conditioned freezing behavior based on the size of the chamber (Bolles and Collier, 1976). Whether darting is also susceptible to such spatial manipulations is not known, nor have potential sex differences in the propensity to adapt conditioned responding as a function of space been investigated. To address these questions, we performed a two-day experiment in which adult Male (n=16) and Female (n=24) Sprague Dawley rats underwent a 7 CS-US cued fear conditioning session in standard 20cm x 20cm chambers (Figure 3A). Twenty-four hours later, they were placed in a 1m^2 Open Field arena and exposed to seven CS presentations. In half of each cohort, animals' movement was restricted by an interior arena with 20cm x 20cm dimensions (Small Field, SF), while the other half were given free range of the Open Field arena (OF).

We first evaluated shock response in all animals on Day 1 and found a significant main effect of sex ($F_{1,36}$=22.7, p<0.0001) but no effect of (future) field size assignment. Adjusted Sidak's multiple comparisons tests revealed that in both SF and OF cohorts, shock response in Females was greater than in Males (p=0.02 and p=0.0005, respectively; Figure 3B). We then evaluated conditioned freezing on both days as a factor of SF/OF assignment within each sex (Figure 3C). In Males, no effect of group was observed on either day (Day 1: $F_{1,14}$=0.02, p=0.87; Day 2: $F_{1,14}$=1.3, p=0.27). In contrast, while the two Female groups' freezing did not differ on Day 1 ($F_{1,22}$=1.8, p=0.27), Females exhibited a robust difference in freezing on Day 2 (Figure 3D; $F_{1,22}$=36.6, p<0.0001), with OF Females exhibiting less freezing than their SF counterparts.

Finally, we examined darting in all animals on both days. During fear conditioning (Figure 3E), darting followed a pattern consistent with our previous findings: specifically, darting emerged as the session progressed and was observed in a greater proportion of Females (38%) than in Males (6%). On Day 2,

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we did not observe any conditioned darting in SF Males or Females (data not depicted), consistent with previous observations that in our standard size chambers, darting on Day 2 is extremely rare (Gruene et al., 2015a). In contrast, we observed conditioned darting in OF Males and Females during early tones (Figure 3F, left), and overall 75% of Females and 33% of Males exhibited darting (Figure 3F, right).

The results here demonstrate that while both Males and Females are more likely to exhibit conditioned darting when the space available to them increases, the Female-biased nature of darting persists. Furthermore, our freezing data indicate that Females are much more likely than Males to adapt their conditioned fear behavior according to their environment, an important consideration for future fear conditioning studies in which spatial parameters are manipulated.

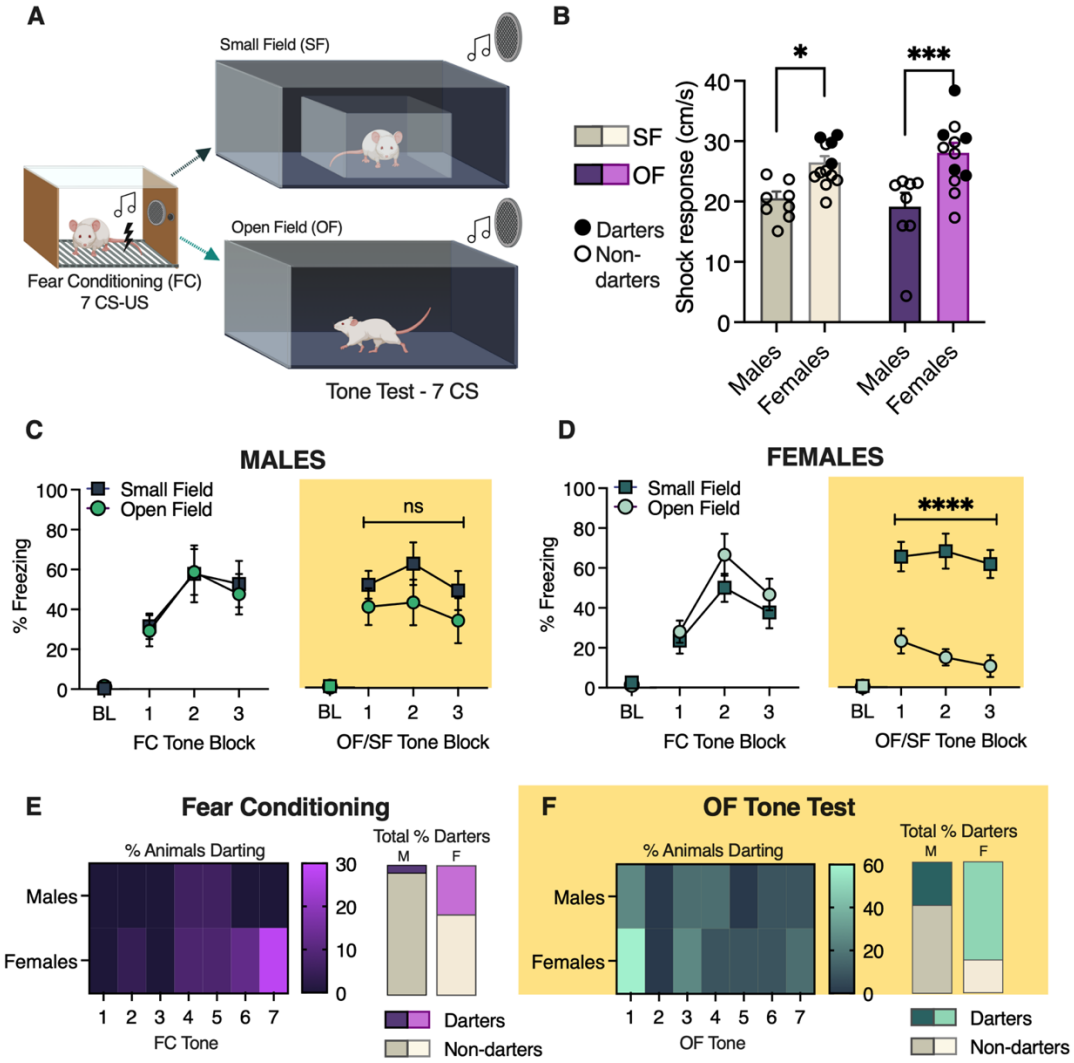

Figure 3. Effects of chamber size on conditioned darting and freezing. A) Experimental design. B) Sex differences in shock response on Day 1 for both future SF and OF cohorts C) Conditioned freezing in Males during fear conditioning (standard chamber) and on Day 2 (either SF or OF). D) Conditioned freezing in Females during fear conditioning (standard chamber) and on Day 2 (either SF or OF). E) Darting prevalence during fear conditioning tones. F) Darting prevalence during the tone test in the Open Field. All Tone Blocks (Panels C-D) represented as averages of Tones 1-2, 3-4, 5-7. BL: baseline freezing, defined as the first 2 minutes of the session, pre-CS. Panel A created with BioRender.

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3. Darting as a function of fear conditioning trial number

We reliably observe that darting appears to emerge as CS-US trials progress—often after brief bouts of conditioned freezing—suggesting a switch in conditioned response strategy (see Figure 1 for example). If this is the case, then the animals that engage in darting during a 7 CS-US session may be "early adopters," and we may thus observe darting in more animals, including Males, if CS-US presentations continue. To answer this question empirically, we performed a 2-day overtraining fear conditioning experiment (20 CS-US presentations, followed by 2 CS presentations in a new context on Day 2) in adult Male (n=19) and Female (n=20) Sprague Dawley rats (Figure 4A).

When we evaluated the prevalence of darting across fear conditioning trials (Figure 4B, left), we observed that as in previous studies, darting in Females emerged around CS 5-7. In Males, we observed an increase in darting shortly after that, with nearly 30% of Males darting during tones 11-12. Overall, 45% of Males and 50% of Females were classified as Darters (Figure 4B, right), suggesting that sex differences in darting prevalence can be eliminated with extended protocols.

The near 50/50 split in both sexes allowed us to conduct within-sex comparisons of other behaviors in Darters vs. Non-darters. In Males, Darters exhibited reduced freezing during fear conditioning but not the tone test on Day 2 (Figure 4C). A 2-way ANOVA for freezing on Day 1 revealed a significant darting x trial interaction ($F_{9,153}$=2.1, p=0.04) and a significant main effect of darting ($F_{1,17}$=26.1, p<0.0001). Analysis of shock responding (Figure 4D) also revealed a significant darting x trial interaction ($F_{9,153}$=6.9, p<0.0001) and a significant main effect of darting ($F_{1,17}$=42.0, p<0.0001), driven by an increase in shock response velocity that intriguingly appeared *before* the emergence of darting itself. Similarly, evaluation of PostShock activity (Figure 4E), defined as the maximum velocity reached in the 30s PostShock Derived Epoch (see Figure 1), revealed a significant darting x trial interaction ($F_{9,153}$=2.6, p=0.008) and a significant main effect of darting ($F_{1,17}$=16.3, p=0.0009). We again note that Darter and Non-darter populations appeared to diverge in this measure before most darting could be observed.

Surprisingly, Female Darters and Non-darters did not significantly differ in conditioned freezing during fear conditioning or the tone test (Figure 4F; 2-way ANOVA effect of darting: $F_{1,18}$=1.87, p=0.19). However, analysis of shock responding (Figure 4G) revealed a significant darting x trial interaction ($F_{9,162}$=2.6, p=0.007) and a significant main effect of darting ($F_{1,18}$=10.8, p=0.004). As in Males, this effect was driven by a progressive increase in Female Darters' shock response velocity that again appeared to emerge before darting did. We observed a similar pattern in analysis of PostShock activity (Figure 4H), finding a significant darting x trial interaction ($F_{9,162}$=2.3, p=0.02) and a significant main effect of darting ($F_{1,18}$=12.0, p=0.003). Interestingly, the significant effect of darting in both Males and Female PostShock responding appears to be primarily driven by a rapid drop in PostShock activity exhibited by Non-darters after the first two shocks, while Darters maintain or increase their activity levels.

We conclude from these analyses that with an extended fear conditioning session, similar proportions of Males and Females will engage in conditioned darting. These findings support the idea that darting reflects a switch in conditioned fear behavior that occurs earlier in Females. Moreover, darting in both sexes is correlated with an increase in shock response and PostShock locomotor activity that often precede the emergence of darting itself, suggesting that individual differences in shock perception may be an important predictor of an animal's propensity to dart.

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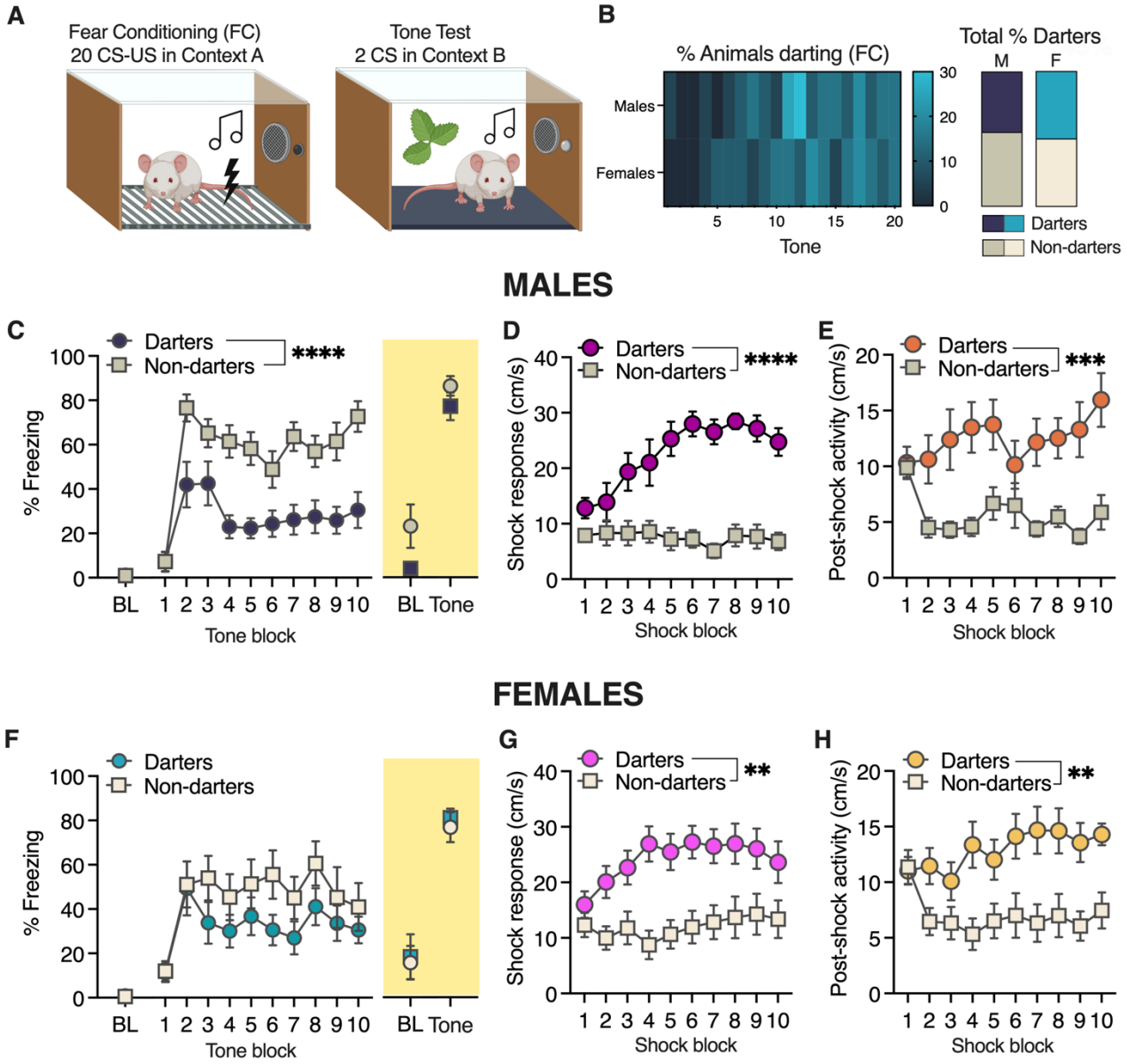

Figure 4. Conditioned darting and freezing in an overtraining paradigm. A) Experimental design. B) Proportion of animals darting during each CS (left) and overall for the entire session C) Conditioned freezing in Male Darters and Non-darters during fear conditioning and on Day 2. D) Shock responses diverged in Male Darters and Non-darters as trials progressed. E) After the first two trials, PostShock activity rapidly diverged in Male Darters and Non-darters. F) Conditioned freezing in Females during fear conditioning and on Day 2. G) Shock responses diverged in Female Darters and Non-darters as trials progressed. H) After the first two trials, PostShock activity rapidly diverged in Female Darters and Non-darters. All statistical significance noted represents within sex Darters vs. Non-darters. ****p<0.0001, **p<0.01. Panel A created with BioRender. BL: baseline freezing, defined as the first 2 minutes of the session, pre-CS. All Tone and Shock blocks represented as averages of every two trials.

4. Darting as a function of shock intensity and CS pre-exposure

Our previous and current results suggest that darting may arise in animals that exhibit an amplified and protracted behavioral response to the shock. To investigate the role that shock intensity might play in modulating the prevalence of darting, we performed a 2-day auditory cued fear conditioning experiment

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in which animals (total Males: n=48; total Females: n=47) were exposed to seven presentations of our standard 30-s CS, which co-terminated with either a 0.3mA or 1.0mA foot shock (Figure 5A). A two-CS tone test was performed in a new context the following day. In a second round of experiments, all parameters were identical to the first, but animals were first exposed to five unreinforced CSs (Figure 5B), as in previous reports from our lab and others (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2007; Rey et al., 2014; Gruene et al., 2015b; a).

With no CS pre-exposure, Males showed a more rapid increase in CS-elicited freezing when the US was 1.0mA compared to 0.3mA (Figure 5C, left; main effect of intensity: $F_{1,22}$=7.4, p=0.01). This discrimination was also evident in CS-elicited freezing on Day 2 (Figure 5C, right; Mann Whitney U=16; p=0.0007). In contrast, Males receiving 0.3mA vs. 1.0mA shocks did not exhibit different freezing levels on either day when the CS-US pairs were preceded by 5 unreinforced CSs (Figure 5D). As assessed by shock response (Figure 5E), Males clearly discriminated between the two intensities in both conditions (main effect of intensity: $F_{1,44}$=31.8, p<0.0001). In addition, we observed a significant main effect of CS pre-exposure ($F_{1,44}$=5.0, p=0.03) suggesting that shock responses were higher in the CS pre-exposed group. However, adjusted Sidak's post-hoc tests only reached significance for within condition effects of shock intensity. Darting prevalence in Males was characteristically low—no animals at either shock intensity in the no CS pre-exposure experiment reached the Darter criteria (Figure 5F). In CS pre-exposed Males (Figure 5G), 17% of animals in the 0.3mA group and 8% of animals in the 1.0mA group were classified as Darters.

In Females with no CS pre-exposure (Figure 5H), animals in the 1.0mA group exhibited greater levels of CS-elicited freezing compared to 0.3mA (Day 1, effect of shock: $F_{1,22}$=8.4, p=0.008; Day 2, unpaired t-test = 3.4, p=0.002). In contrast, CS-elicited freezing in CS pre-exposed Females (Figure 5I) differed as a function of shock intensity only on Day 2 (unpaired t-test t=3.2, p=0.004). Interestingly, baseline freezing (pre-CS) on Day 2 was also higher in the 1.0mA group compared to 0.3mA (Mann-Whitney U = 24.5, p=0.009). A 2-way ANOVA of shock response in all Females (Figure 5J) revealed main effects of both shock intensity ($F_{1,43}$=37.1, p<0.0001) and CS pre-exposure ($F_{1,44}$=32.3, p<0.0001). As expected, Females in both experimental conditions clearly discriminated between the two shock intensities (adjusted Sidak's multiple comparison tests for 1.0mA vs. 0.3mA: p=0.0007 without CS pre-exposure; p=0.0004 with CS pre-exposure). However, we were surprised to find that CS pre-exposure appeared to induce a dramatic increase in shock response at both intensities (adjusted Sidak's multiple comparison tests for with vs. without CS pre-exposure: p=0.002 for 0.3mA; p=0.0008 for 1.0mA).

Examination of darting in Females revealed an interesting pattern. In both experimental conditions, darting emerged in response to later tones, as we have previously observed (Figure 5K-L, left). In addition, a greater proportion of the 0.3mA group in both experimental conditions were classified as Darters compared to the 1.0mA group (Figure 5K-L, right). We also observed, as with the Males, a slight uptick in Darter proportions in the CS pre-exposure group compared to the no pre-exposure group.

Together, these data tell an intriguing and somewhat paradoxical story about the interactions between shock intensity, shock response, conditioned freezing, and the propensity to engage darting behavior. We found that shock response is a reliable measure to demonstrate discrimination between shocks of different intensities in both sexes, and that shock response in Darters is generally found to be at the upper end of a cohort's range. However, we also found that CS-elicited darting is more likely to be observed when shock intensities are *lower*. In addition, CS pre-exposure appears to 1) amplify shock reactivity in Females regardless of shock intensity, 2) prevent shock intensity-based differences in CS-elicited freezing in both sexes, and 3) increase darting during fear conditioning in both sexes. We discuss the potential implications of these data and look forward to investigating the mechanistic basis of these surprising findings in future work.

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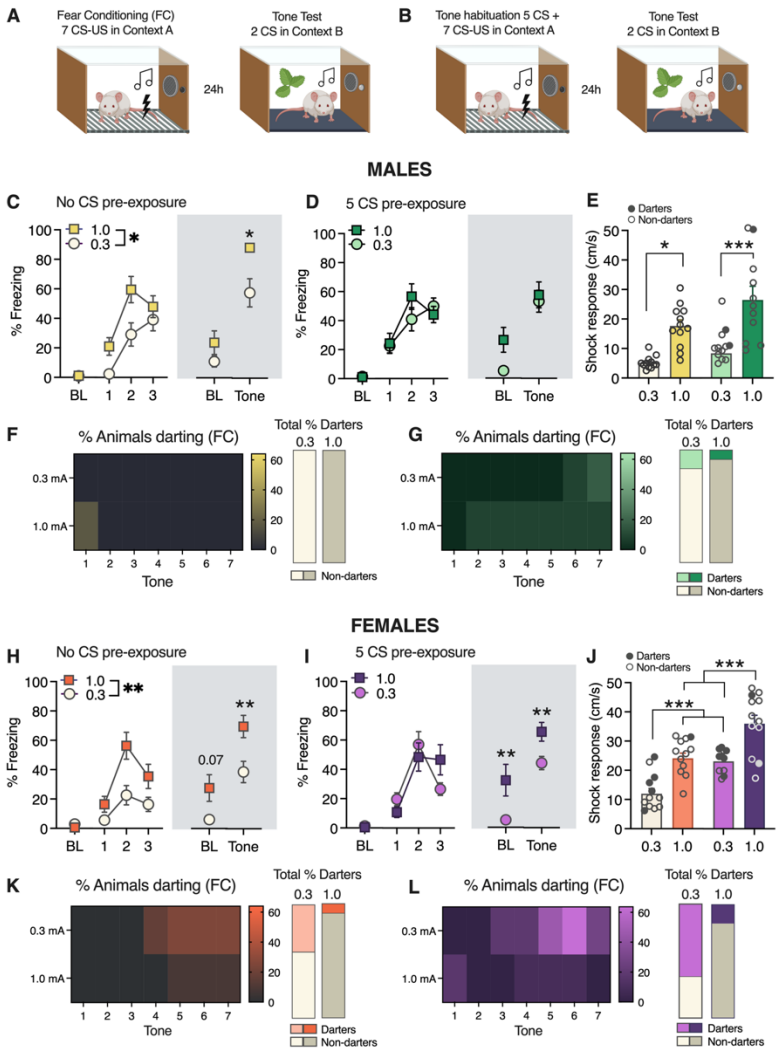

Figure 5. Conditioned darting and freezing as a function of shock intensity and CS pre-exposure. A) Experimental design without CS pre-exposure. B) Experimental design with CS pre-exposure. C) Freezing in Males during no CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. D) Freezing in Males during 5 CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. E) Shock response in Males for all experimental conditions . F) Proportion of Males in the no CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). G) Proportion of Males in the CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). H) Freezing in Females during no CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. I) Freezing in Females during 5 CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. J) Proportion of Females in the no CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). K) Proportion of Females in the CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). All statistical significance noted represents within sex and condition differences between 0.3mA and 1.0mA groups, except J), which additionally show significant within shock intensity effects of CS pre-exposure. *p<0.05, **p<0.01, ***p<0.001. Panels A & B created with BioRender. BL: baseline freezing, defined as the first 2 minutes of the session, pre-CS. All fear conditioning Tone Blocks (Panels C-D, H-I [left]) represented as averages of Tones 1-2, 3-4, 5-7. CS-elicited freezing on Day 2 is represented as the average of 2 CSs. All group n's=12, except for Female CS pre-exposure 0.3mA, n=11.

Discussion

The purpose of these experiments was to identify situational modulators and behavioral predictors of conditioned darting in Male and Female rats during Pavlovian fear conditioning. Manipulations of context, trial number, shock intensity, and CS exposure revealed that, like freezing, darting as a conditioned response is indeed sensitive to these experimental parameters; more space or CS-US trials led to an increase in the number of Darters, while increasing shock intensity decreased the number of Darters. ScaredyRat analysis also demonstrated that Darters of both sexes reliably show higher shock responses and protracted PostShock activity than Non-darters, suggesting that the propensity of an individual animal to engage in darting as a conditioned response is related to the magnitude of its unconditioned responses. Intriguingly, these phenotypic splits in shock reactivity emerge earlier than darting itself but are not evident in the very first trials, suggesting that the initial CS and US experiences trigger these divergent trajectories.

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Our findings build on decades of seminal single-sex work that defined how experimental parameters affect conditioned freezing, but we also make significant advances in several notable ways. First, ScaredyRat allowed us to examine not only freezing but also multiple conditioned and unconditioned fear behaviors across different experimental preparations. While freezing is a well-established and dependable conditioned fear response, the field's current reliance on it as the sole indicator of conditioned fear in rodents has given us an incomplete picture. For decades, any non-freezing behavior exhibited during classical fear conditioning has been excluded from analyses as not fear-related or not representative of learning. We see here that at least one of those behaviors, darting, is representative of learning and is a conditioned response that is affected by similar situational modulators that affect freezing. When designing and conducting experiments, therefore, the inclusion of both sexes will be critical to capturing the range of conditioned behaviors that may be exhibited, not simply those that are dominant in one sex (Shansky and Woolley, 2016; Shansky, 2018, 2019). Side-by-side assessment of conditioned fear in both Females and Males is still woefully rare (Lebron-Milad and Milad, 2012). Our work provides not only novel insight into the facets of the paradigm in which the sexes do and do not differ but also a novel tool with which to collect multidimensional behavioral data.

Some of the more thought-provoking aspects of our work arise when trying to reconcile the relationship between darting and shock reactivity. In Experiment 3, Darters of both sexes had a higher maximum shock response velocity than non-darters, suggesting that there may be a difference in pain sensitivity between Darters and Non-darters. We might then expect to observe more darting at higher shock intensities, which also elicit higher shock responses. However, Experiment 4 showed that when shock intensity increased, both Male and Female rats were *less* likely to dart, despite clear evidence that the shock was perceived as more intense. Bolles & Fanselow's perceptual-defensive-recuperative model of pain (Bolles and Fanselow, 1980) states that when a fearful and painful stimuli are both present, fear will "win out" over pain, leading to an opioid-mediated analgesic effect that allows the animal to respond to the fear-inducing stimulus (e.g., freeze). In this context, one possible explanation of our data is that darting indicates that conditioned analgesia has failed to occur. Supporting this hypothesis is evidence from Experiment 3, in which we found that shock responding in Non-Darters stayed relatively flat, while progressively escalating in Darters. Given known sex differences in pain processing and opioid signaling (Loyd et al., 2008; Fullerton et al., 2018; Mogil, 2020), we feel that future investigations into the neural circuits that might drive this possibility are likely to be fruitful. In particular, the periaqueductal gray (PAG) is a center for both pain and defensive responding in the rodent (de Oca et al., 1998; Vianna et al., 2001; Vanegas and Schaible, 2004) and is an attractive target for mechanistic inquiry.

We also found that brief pre-exposure to the CS affected darting, freezing, and shock response across the sexes. CS pre-exposure led to a slight increase in darting in both Males and Females as well as a robust increase in shock response in Females. These surprising changes in behavior are unlikely to reflect latent inhibition, which would predict compromised learning and reduced conditioned responding (Sotty et al., 1996; Maes, 2002), which we did not observe. In light of our comments above, one intriguing possibility is that CS pre-exposure can inhibit fear-conditioned analgesia. To our knowledge, this effect has not been previously demonstrated, but the dramatic effect on shock responding particularly in Females suggests that the mechanisms by which CSs and USs are processed and integrated across time in a given experiment may be sex-specific. These findings further underscore the need to consider a multitude of variables when designing and conducting fear conditioning experiments that include both sexes.

This work advances and expands upon decades of research on conditioned fear, the vast majority of which has been conducted only in Males and only reported freezing behavior. Although landmark papers from the 1970s and 1980s were more likely to include evaluation of both freezing and shock-related activity metrics (e.g. activity bursts or circa-strike behavior; reviewed in Fanselow, 1994), this practice has fallen out of fashion in more recent years, we believe to the detriment of the field of Behavioral Neuroscience. We are excited to share ScaredyRat, a novel tool that allows unbiased quantification of multiple CRs and URs, painting a more nuanced picture of behavior in a classic and

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foundational paradigm. Our work makes clear that sex is a key biological variable in fear conditioning outcomes, and we hope that as others move to include both sexes in their experimental designs, they will take advantage of ScaredyRat to analyze and identify individual differences in both unconditioned and conditioned fear responses.

Methods

Subjects

All experiments were conducted in young adult (8-10 weeks) Male (n=95) and Female (n=105) Sprague Dawley rats (Charles River), weighing 325-350g and 225-250g, respectively. See **Experimental Design Table** for group n's. Animals were same sex pair-housed in the Nightingale Hall Animal Facility at Northeastern University in a 12:12 light:dark cycle with access to food and water *ad libitum*, and were allowed to acclimate to the facilities undisturbed for at least one week prior to testing. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Northeastern University Institutional Animal Care And Use Committee. All experimenters were female.

Behavioral Apparatus and Fixed Experimental Parameters

Fear conditioning training (FC) and recall tone testing (except in Experiment 2) were conducted as described in Gruene et al., 2015, in one of eight identical 30cm^2 chambers constructed of aluminum and Plexiglass walls (Rat Test Cage; Coulbourn Instruments, Allentown Pennsylvania) with metal stainless steel rod flooring attached to a shock generator (Model H13-15; Coulbourn instruments). Each chamber was enclosed within a sound-isolation cubicle (model H10-24A; Coulbourn Instruments). An infrared digital camera allowed videotaping (30 frames per second) during behavioral procedures. Chamber grid floors, trays, walls, and ceilings were thoroughly cleaned with water and 70% ethanol and dried between sessions.

For context A, chambers were lit with a single house light. Context B testing was done in the same chambers, but the house light was off and a cue light was illuminated. In addition, plexiglass panels covered the metal grid floor, and a light peppermint scent (Dr. Bronner's) was applied to a removable tray under the chamber floor. Across experiments, animals were allowed 5 minutes to explore the chamber before CS or US (Experiment 1 only) presentation commenced. Mean intertrial interval for all experiments was 3 minutes with a range of 1.5-5 minutes. In all cued fear conditioning experiments, the CS was a 30s, 4 kHz, 80 dB SPL sine wave tone, which co-terminated with a 0.5-s footshock. Number of CS presentations and footshock intensity varied for each individual experiment and are noted below in the Experimental Design Table.

Open Field Arena

For Experiment 2, Day 2 testing occurred in a 1m^2 wooden Open Field Arena with 30cm height walls, painted with matte black spray paint. For the Small Field condition, a 30cm^2 wooden box with open top and bottom (also painted with matte black spray paint) was placed inside the Open Field (see Figure 3A). The CS was generated with Garage Band software (Apple) and played at an ITI mimicking those in the standard chambers. Behavior was recorded with a Microsoft Kinect camera.

Behavior tracking and data processing

We used Ethovision software (Noldus; Leesburg, VA) to generate raw velocity data sheets from all video files at a sample rate of 15 frames per second (i.e. every other frame). These files were then fed to ScaredyRat, which extracted freezing, darting, and velocity data for each animal during defined Epochs and Derived Epochs as conveyed in Results.

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ScaredyRat settings

- The Time Bin Duration was set at 1s.
- The threshold for **freezing** for all experiments was set at <0.1 cm/s (average speed within a Time Bin).
- The threshold for **darting** for all experiments was set at >20 cm/s.
- **Baseline** (BL) measures reflect the first two minutes of all sessions.
- **Shock response** is defined as the maximum velocity reached within a 5 second Derived Epoch that begins with the shock presentation.
- **PostShock activity** is defined as the maximum velocity reached within a 30 second Derived Epoch that begins immediately after each Shock Epoch.

Experimental Design Table

Experiment Name	Male n	Female n	Day 1 (FC) context	Day 2 (Test) context	Trial info (Day 1/Day 2)	Shock intensity	5 CS pre-exposure
1. Context Conditioning	12	14	A	A	7 US/ NA	0.7 mA	---
2. Small/Open Field	8/8	12/12	A	Small/Open Field	7 CS-US/7CS	0.7 mA	yes
3. Overtraining	19	20	A	B	20 CS-US/2 CS	0.5 mA	no
4. Shock intensity/ CS pre-exposure	12/12/ 12/12	11/12 12/12	A	B	7 CS-US/2CS	0.3 or 1.0 mA	both

Statistical analysis

All statistical analyses were done with Graphpad Prism Software. Data sets with multiple time points (e.g., fear conditioning data) were analyzed with 2-way repeated measure ANOVAs, followed by Sidak's post-hoc tests adjusted for multiple comparisons when appropriate. Group differences in data sets with a single time point (e.g. Day 2 average freezing) were assessed with either unpaired t-tests or Mann Whitney tests if groups were determined to have unequal variances.

Contributions

JRM, RMS, and SW designed the experiments. AJL, SGT, and KAH developed ScaredyRat. JRM, SW, EG, and MF ran the experiments. JRM and RMS analyzed the data. JRM and RMS wrote the manuscript.

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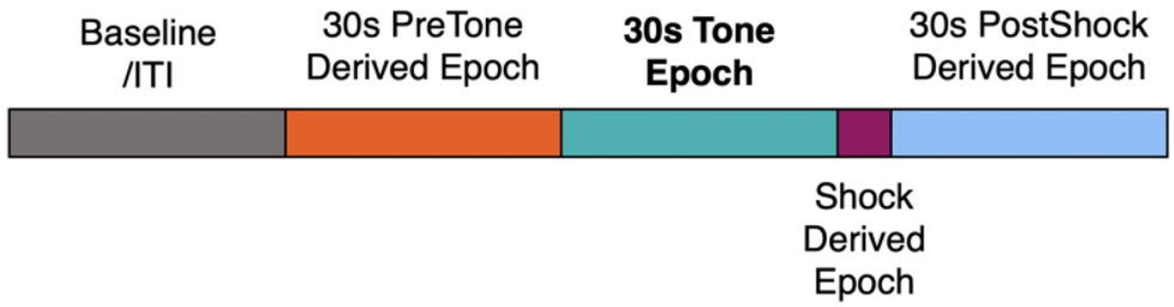
A

Experimental Design

- 5 min pre-CS period
- 7 30s Tones co-terminating with 0.5s foot shocks
- Varying inter-trial intervals (ITI)

ScaredyRat Setup

- **Base** Epoch: Tone
- **Derived** Epoch: PreTone, defined as 30s prior to each Tone
- **Derived** Epoch: Shock, defined as 5s starting at the final second of each Tone
- **Derived** Epoch: PostShock, defined as 30s starting 5s after each Tone termination



B

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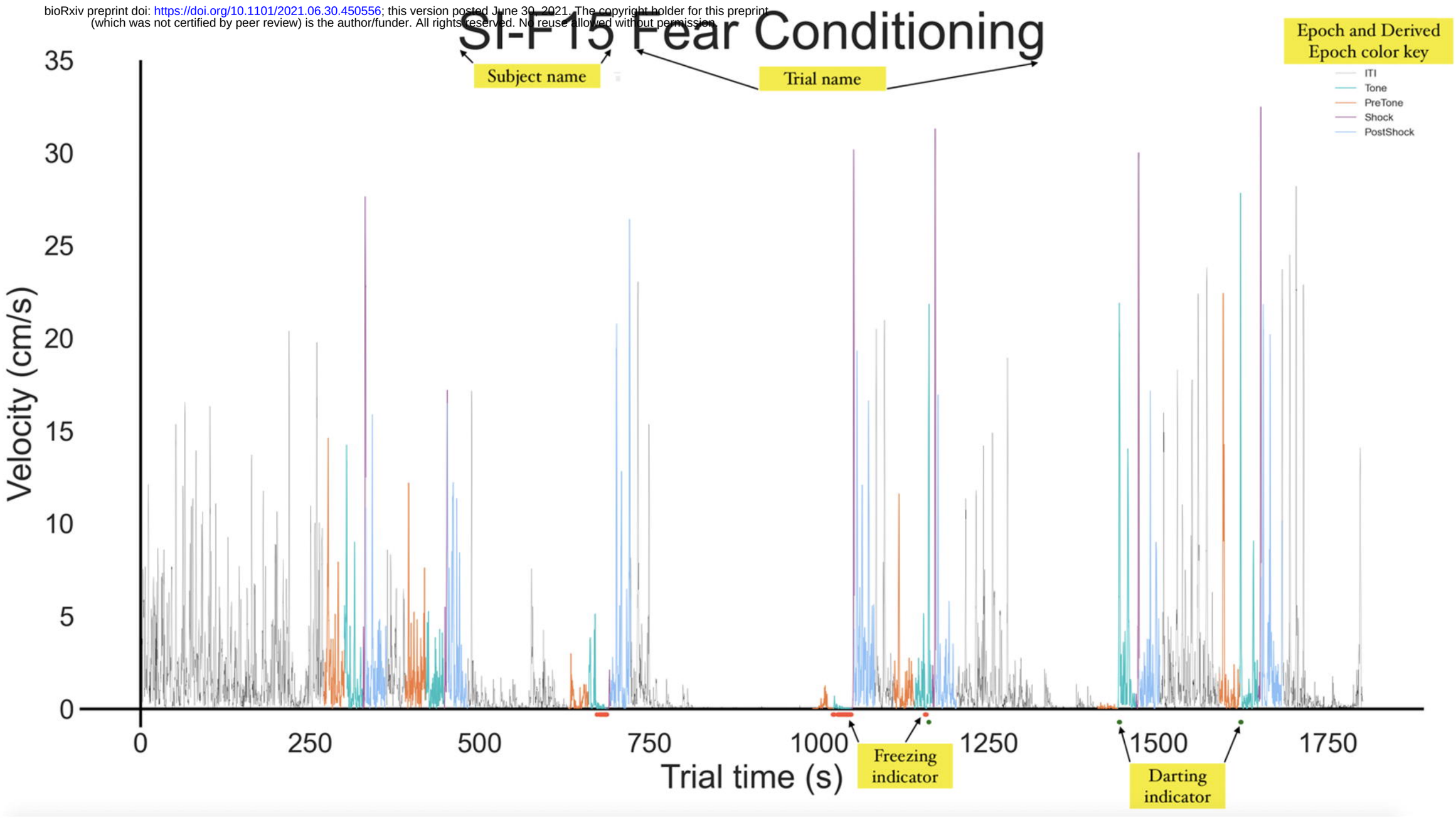

Figure 1. How does ScaredyRat work? A) Example Experimental Design, ScaredyRat setup, and Epoch/Derived Epoch schematic for the plot generated in B). Our experimental design consisted of a 5 min exploration period, followed by 5 30s Tones that co-terminated with a 0.5s footshock, with varying ITIs. ScaredyRat identifies Ethovision-defined Base Epochs (here, the Tone), and then the ScaredyRat user defines Derived Epochs of interest that are time-linked to the Base Epoch (here, PreTone, Shock, and PostShock). B) Representative ScaredyRat-generated plot for a single subject across time in an entire fear conditioning session. Yellow labels indicate notable features—these labels do not appear in actual plots. This animal would be classified as a Darter, because CS (tone)-related darting can be observed in CS 5, 6, and 7. Note earlier periods of freezing on CS 3 and 4. ScaredyRat also generates spreadsheets for Freezing, Darting, and Velocity during each Epoch and Derived Epoch. Behavior during ITIs is not reported.

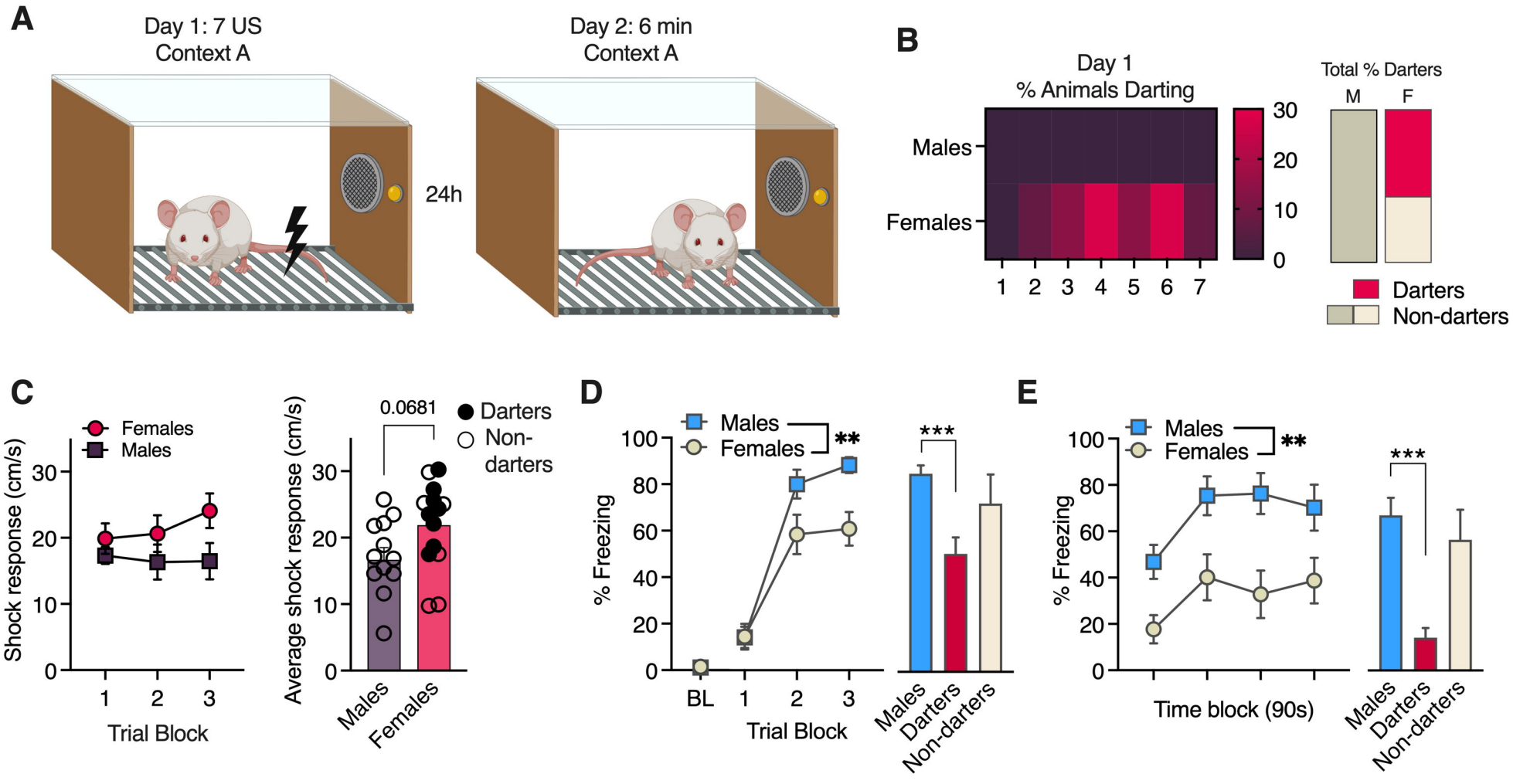

Figure 2. Influence of darting in a context conditioning experiment. A) Experimental design for Days 1 & 2. B) Percentage of Male and Female Darters for each time block across the session (left), and for the entire session (right). C) Shock response in Males and Females. D) Sex differences in freezing during fear conditioning were driven by Darters. E) Sex differences in freezing during the memory test were driven by Darters, who froze less than Males. ** $p<0.01$ Males vs. Females. *** $p<0.001$, Males vs. Darters. Fear conditioning Trial Blocks (Panels C-D) represented as averages of Trials 1-2, 3-4, 5-7. BL: baseline freezing, defined as the first 2 minutes of the session. Panel A created with BioRender.

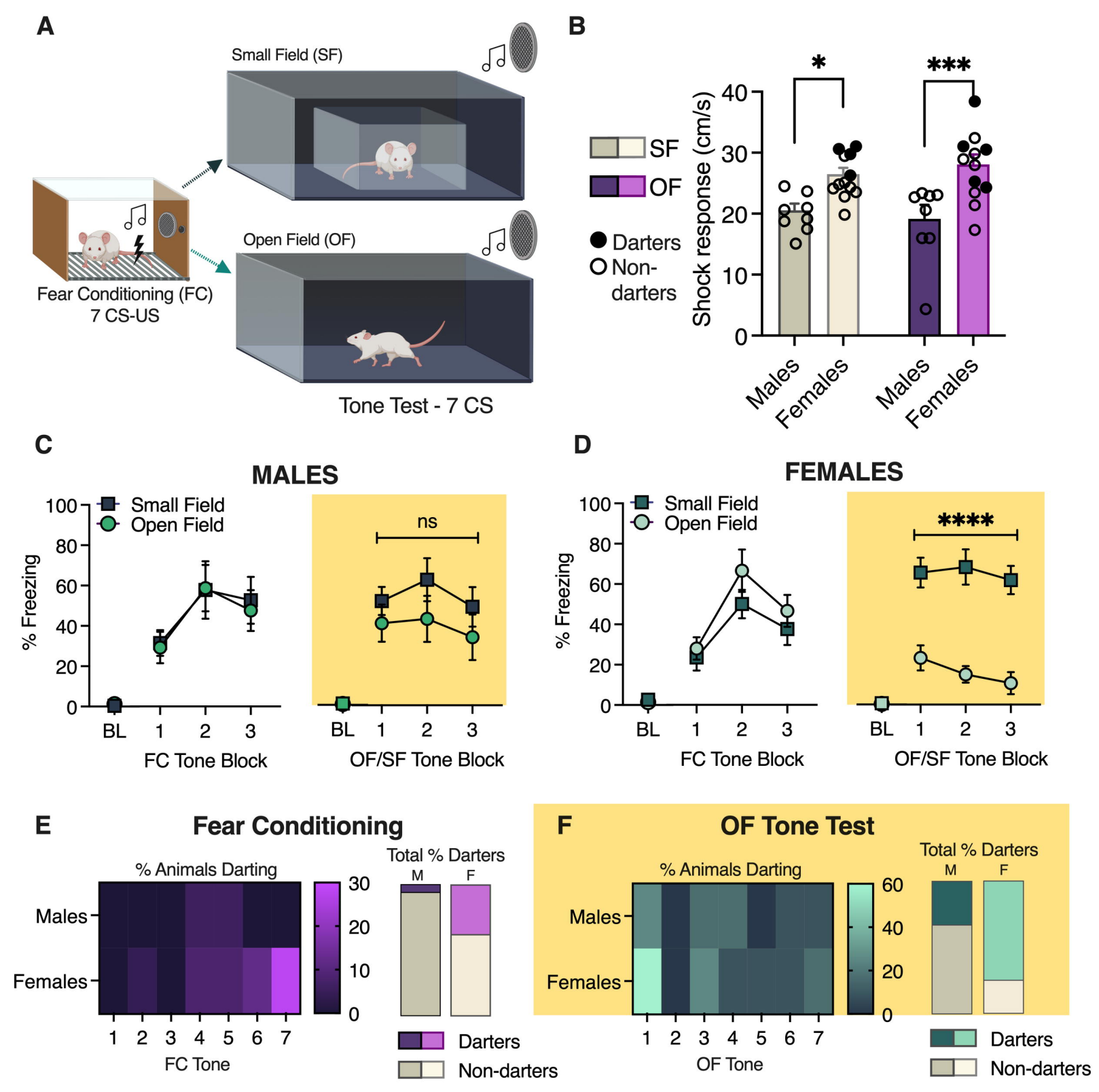

Figure 3. Effects of chamber size on conditioned darting and freezing. A) Experimental design. B) Sex differences in shock response on Day 1 for both future SF and OF cohorts C) Conditioned freezing in Males during fear conditioning (standard chamber) and on Day 2 (either SF or OF). D) Conditioned freezing in Females during fear conditioning (standard chamber) and on Day 2 (either SF or OF). E) Darting prevalence during fear conditioning tones. F) Darting prevalence during the tone test in the Open Field. All Tone Blocks (Panels C-D) represented as averages of Tones 1-2, 3-4, 5-7. BL: baseline freezing, defined as the first 2 minutes of the session, pre-CS. Panel A created with BioRender.

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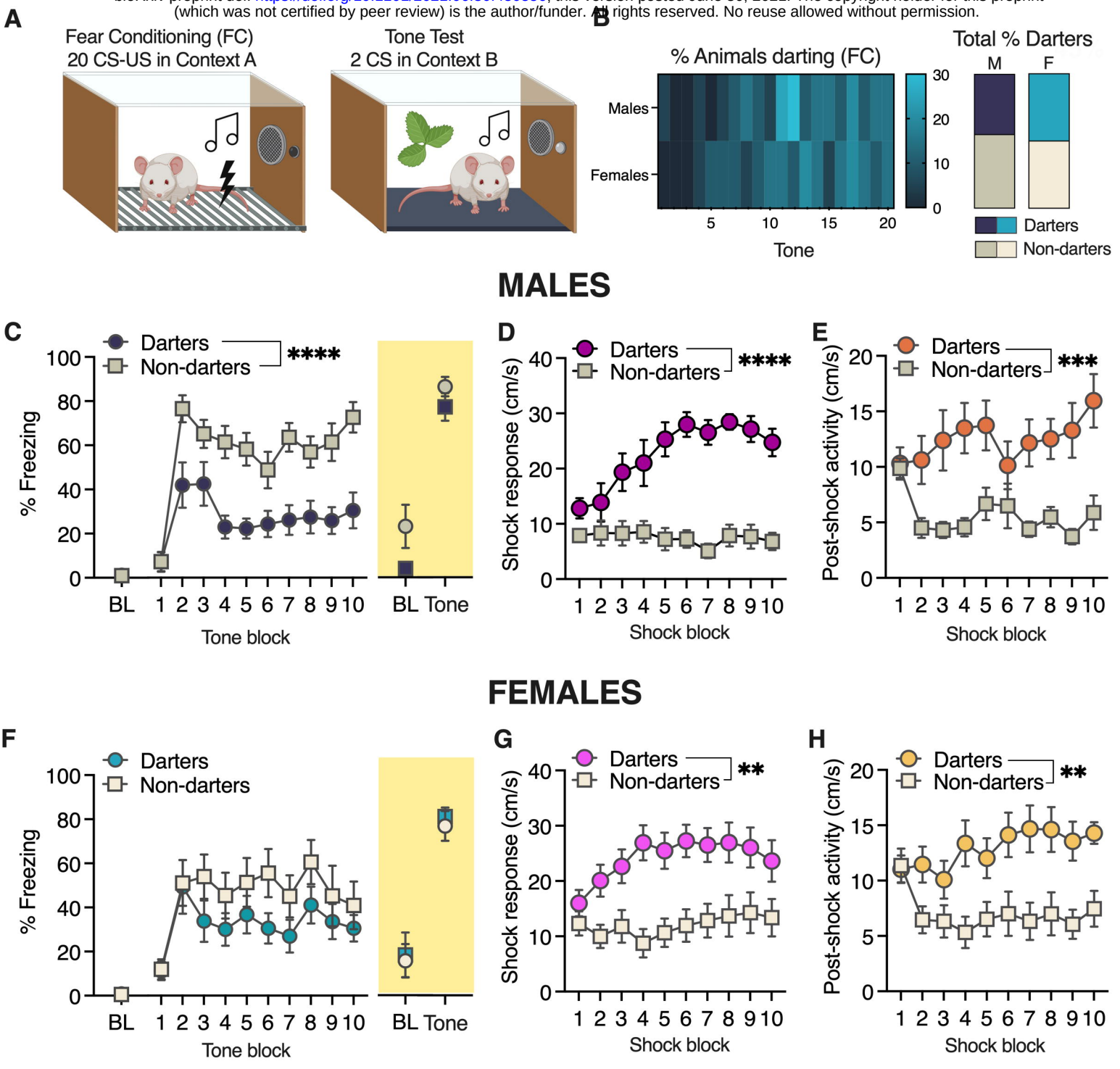

Figure 4. Conditioned darting and freezing in an overtraining paradigm. A) Experimental design. B) Proportion of animals darting during each CS (left) and overall for the entire session C) Conditioned freezing in Male Darters and Non-darters during fear conditioning and on Day 2. D) Shock responses diverged in Male Darters and Non-darters as trials progressed. E) After the first two trials, PostShock activity rapidly diverged in Male Darters and Non-darters. F) Conditioned freezing in Females during fear conditioning and on Day 2. G) Shock responses diverged in Female Darters and Non-darters as trials progressed. H) After the first two trials, PostShock activity rapidly diverged in Female Darters and Non-darters. All statistical significance noted represents within sex Darters vs. Non-darters. ****p<0.0001, **p<0.01. Panel A created with BioRender. BL: baseline freezing, defined as the first 2 minutes of the session, pre-CS. All Tone and Shock blocks represented as averages of every two trials.

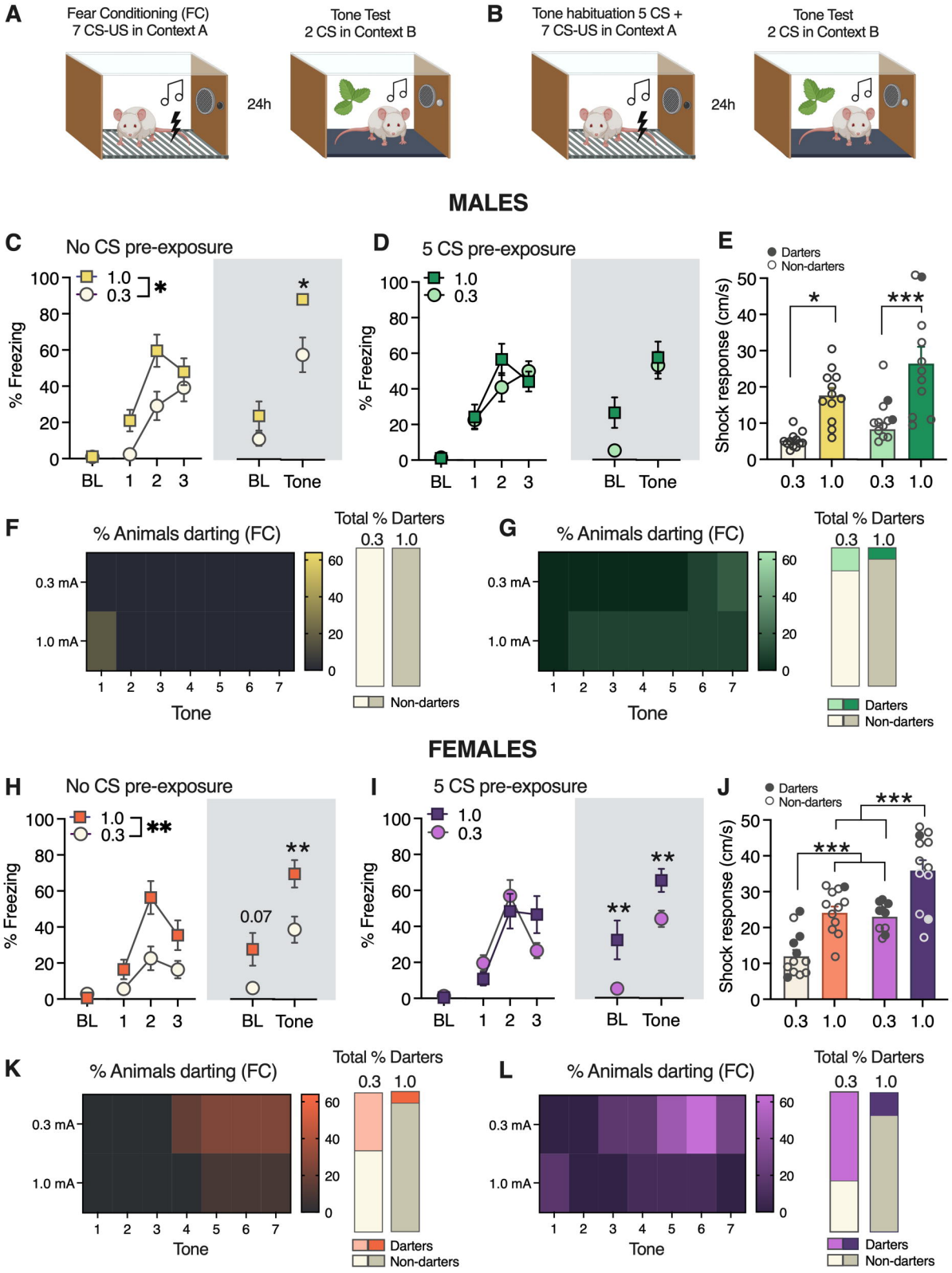

Figure 5. Conditioned darting and freezing as a function of shock intensity and CS pre-exposure. A) Experimental design without CS pre-exposure. B) Experimental design with CS pre-exposure. C) Freezing in Males during no CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. D) Freezing in Males during 5 CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. E) Shock response in Males for all experimental conditions . F) Proportion of Males in the no CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). G) Proportion of Males in the CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). H) Freezing in Females during no CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. I) Freezing in Females during 5 CS pre-exposure fear conditioning (left) and a 2-CS test in a new context 24h later. J) Proportion of Females in the no CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). K) Proportion of Females in the CS pre-exposure condition that darted during each CS (left) and overall for the entire fear conditioning session (right). All statistical significance noted represents within sex and condition differences between 0.3mA and 1.0mA groups, except J), which additionally show significant within shock intensity effects of CS pre-exposure. *p<0.05, **p<0.01, ***p<0.001. Panels A & B created with BioRender. BL: baseline freezing, defined as the first 2 minutes of the session, pre-CS. All fear conditioning Tone Blocks (Panels C-D, H-I [left]) represented as averages of Tones 1-2, 3-4, 5-7. CS-elicited freezing on Day 2 is represented as the average of 2 CSs. All group n's=12, except for Female CS pre-exposure 0.3mA, n=11.