Differential effects of chronic restraint stress on two active avoidance tasks in rats

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Abstract

While avoidance can be a useful coping mechanism for those with anxiety, excessive or persistent avoidance can become a debilitating symptom of anxiety-related disorders. The transition from adaptive to maladaptive avoidance remains poorly understood, but stress is regarded as a potential contributing factor. This study investigates the effects of chronic restraint stress on two active avoidance procedures: two-way active avoidance (2WAA) and platform-mediated avoidance (PMA). In the latter, avoidance competes with food-seeking behavior. Adding a cost to the avoidance response mirrors the clinical reality of anxious individuals who avoid threatening but also possibly rewarding situations. Conversely, in the 2WAA, rodents must cross to another compartment upon a warning signal to avoid a shock, which represents a low-cost avoidance response. We hypothesized that chronic restraint stress would hinder avoidance acquisition in the 2WAA task, but increase avoidance acquisition in the PMA task. In both experiments, male and female rats underwent the chronic restraint stress protocol or remained in the control condition. In Experiment 1, all rats (N = 31) were then trained in a 2WAA acquisition and extinction procedure, in two contexts. Stressed rats showed significantly reduced avoidance acquisition, while extinction was unaffected. In Experiment 2 (N = 32), stressed rats and controls were trained in a PMA acquisition and extinction procedure. We found group and sex differences in lever pressing suppression during PMA acquisition, but not in avoidance or freezing behaviors. Both male and female rats gradually extinguished defensive behaviors during extinction. Overall, chronic restraint stress had limited effects on PMA, but significantly impacted avoidance acquisition in the 2WAA without affecting its extinction. Our findings suggest that chronic restraint stress differentially affects these two active avoidance procedures, potentially depending on how costly they are.

Introduction

Although avoidance can serve as a helpful coping strategy for individuals with anxiety, in an excessive or persistent form it can be a crippling symptom of anxiety-related disorders. For example, avoiding dark alleys at night in a large city may be an adaptive avoidance response because it thwarts potential threats and ensures survival. However, avoiding a generally safe neighborhood by day is arguably maladaptive, because an objectively safe situation is treated as if it were dangerous (Arnaudova et al., 2017).

The specific factors underlying the transition from adaptive to maladaptive avoidance are not yet fully understood, but chronic stress is a key candidate (Daviu et al., 2019; López-Moraga et al., 2022; McEwen, 2004). Stressors activate the hypothalamic-pituitary-adrenal (HPA) axis and trigger the release of glucocorticoids (McEwen & Akil, 2020). These hormones are involved in short-term adaptive responses to threats (e.g., release of glucose into the bloodstream, heightened cardiovascular activity, suppression of hunger) and in long-term responses, including effects on learning and memory (e.g., enhanced consolidation and reduced expression of extinction of classically conditioned fear) (De Quervain et al., 2016). Healthy responding to acute stressors does not only imply rapid glucocorticoid synthesis, but also a quick termination of the stress response to limit the effects of these hormones, as prolonged responses that exceed the occurrence of the initial stressor can lead to disease (McEwen & Akil, 2020).

The stress response is highly conserved between rodents and humans, allowing us to study chronic stress and its effects in animal models (Watson et al., 2016). Gaining a deeper understanding of the potential dysregulation of avoidance by stress may help us better comprehend the pathophysiology of anxiety-related disorders and develop new interventions (Bale et al., 2019). Clinical and experimental observations suggest that stress may influence avoidance, but their relationship is not unequivocal. Clinical findings indicate that uncontrollable or unpredictable stress leads to increased avoidance (Zinbarg et al., 2022). Conversely, learned helplessness, which is typically obtained by extended exposure to strong stressors, is characterized by poor avoidance or escape responses (Maier, 2001).

Our recent review of the rodent literature regarding the effects of stress on subsequent avoidance tasks revealed conflicting results (López-Moraga et al., 2022). When focusing on the effects of chronic stress on active avoidance, we found that chronic restraint stress typically impairs the acquisition of active avoidance in male rats (Bravo et al., 2009; Dagnino-Subiabre et al., 2005; Ulloa et al., 2010) and female rats (Gamaro et al., 1999). However, in other studies, different stress induction procedures (chronic cold swim stress, shock and variable stress) did not affect active avoidance (Wakizono et al., 2007; Weiss et al., 1975), or

increased active avoidance acquisition (with chronic cold temperature stress; Hata et al., 1989).

There are multiple possible reasons for these conflicting findings, including differences in the strain, sex, and age of subjects, and in the type and duration of the stress induction procedure (López-Moraga et al., 2022). All studies did use the same type of avoidance task, i.e., two-way active avoidance (2WAA). This task is conducted in a shuttle box with two compartments, between which the animal can freely move. The rodent first learns the association between a warning signal (e.g., a tone) and a following aversive event (e.g., a foot shock). Subsequently, it learns that if it shuttles to the other compartment during the tone, the tone is terminated and the foot shock is omitted. This way, rodents learn to perform conditioned avoidance responses (Choi et al., 2010). Inherent to the 2WAA procedure, the animal repeatedly needs to return to a place where it has received a shock before, and therefore does not have a permanent safe place (Diehl et al., 2019). Over the past decade, an alternative active avoidance task has been developed that does not have this feature: the platform-mediated avoidance (PMA) task (C. Bravo-Rivera et al., 2014). In this task, rats learn that they can avoid a tone-signaled foot shock by stepping onto a platform during the tone, which is a location where the animal is always safe from shocks. Stepping onto the platform does come at the expense of lever pressing for food, which constitutes an approach-avoidance conflict because rats are food-deprived. Such cost is not an aspect of the 2WAA task. Costly avoidance, as in the PMA task, may reflect more accurately the behavior of individuals with clinical anxiety, who often exhibit avoidance behaviors that result in the loss of positive outcomes or potential rewards (Diehl et al., 2019). In humans, emerging evidence suggests that acute and chronic stress increases costly avoidance in anxious individuals (Vogel & Schwabe, 2019; Weaver et al., 2020). Published data in the PMA task are currently lacking, thus the effects of chronic stress on costly avoidance in rodents are yet to be investigated.

The 2WAA and PMA tasks differ in several key aspects as mentioned above. Therefore, it is possible that chronic stress has differential effects on platform-mediated avoidance, which involves costly avoidance, compared to two-way active avoidance, which is a low-cost avoidance procedure. In a first experiment, we examine the effects of chronic restraint stress on active avoidance in the 2WAA task, while the second one assesses this in the PMA task.

Experiment 1 (n = 31) aims to test what has previously been found in the literature (Bravo et al., 2009; Dagnino-Subiabre et al., 2005; Gamaro et al., 1999; Ulloa et al., 2010), i.e., chronic restraint stress hinders 2WAA acquisition. In addition, it examines, for the first time, the subsequent extinction of avoidance behavior that was acquired with or without a history of

chronic restraint stress, hypothesizing that extinction would be impaired as well. Experiment 2 (n = 32) evaluates, for the first time, the effects of chronic restraint stress on avoidance acquisition and extinction in the PMA task. Here, in line with preliminary clinical observations (Vogel & Schwabe, 2019; Weaver et al., 2020), we hypothesized that previously stressed rats would show more avoidance than control rats during avoidance acquisition and its early extinction.

Methods

Preregistration and data availability

Designs, procedures, sample sizes, and analysis plans were registered on the Open Science Framework (OSF) prior to the start of data collection: https://osf.io/qm6y7/. Data and scripts can also be found on OSF.

Subjects

All experiments were performed in accordance with Belgian and European laws (Belgian Royal Decree of 29/05/2013 and European Directive 2010/63/EU) and the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020) and were approved by the KU Leuven animal ethics committee (project license number: 177/2023). The number of animals used per experiment (N = 32) was calculated based on previous studies.

Both experiments were conducted using 7-8-week-old female (180-200 g at arrival) and male (250-280 g at arrival) Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France). Animals arrived in our facility 4 (Experiment 1) or 5 days (Experiment 2) before the start of the experiment. Animals were housed in groups of 4. The housing room has a 12-hour light-dark cycle (lights on at 7 am), and experiments were performed between 8 am and 6 pm. Animals of the stressed group were housed in the same room, but in a different ventilated cabinet than animals of the control group. The cages had bedding and cage enrichment in the form of a red polycarbonate tunnel hanging from the top of the grid. Water was available ad libitum for the entire experiment, except during behavioral testing. For Experiment 1, food was provided ad libitum, except during behavioral testing (see Figure 1a). For Experiment 2, food was provided ad libitum except one day before and during lever press training, immediately after the sucrose preference test, and during avoidance and extinction in the platform-mediated avoidance task (see Figure 1b). Animals of this experiment were handled for 2 days before the start of lever press training.

In Experiment 1, one male rat from the stress group died unexpectedly on day 6 of the stress induction and was excluded from all analyses.

Body weight measurement

Body weight was measured after every stress induction session (control animals were weighed in their housing room while the others were in the restraint stress holder) and after every behavioral session.

Previous research has shown that restraint stress negatively affects body weight gain (Jeong et al., 2013). As previous authors have reported (Dagnino-Subiabre et al., 2005; Ulloa et al., 2010), monitoring changes in body weight can serve as an effective manipulation check for stress induction. Weight change over the stress induction period was calculated by taking the weight of the rat on a given day and dividing it by its baseline weight. For Experiment 1, the baseline weight was obtained 1 day before the start of the stress induction period. For Experiment 2, the baseline weight was defined as the weight on the first day of the stress induction, due to food restriction until the day before the start of the stress induction.

Sucrose preference test

The sucrose preference test has been used to evaluate the effects of chronic stress (Katz, 1982; Willner et al., 1987). Two days before the start of the stress induction, rats from Experiment 1 were habituated to consume a 1% sucrose (Acros Organics) solution for 24 h, with two bottles of a 1% sucrose solution placed in their home cage and no access to regular water. Rats from Experiment 2 underwent this sucrose habituation one day before the lever press training started. For both experiments, one day after the stress induction period, all rats were housed separately for 15 hours in smaller, individual cages and subjected to a sucrose preference test. Food was provided ad libitum, and water was provided in two bottles: one with tap water and one with the 1% sucrose solution, from 18:30 to 9:30 (Berrio et al., 2023; Liu et al., 2018). Given that rats have a choice between 1% sucrose in tap water and pure tap water, a reduced preference for the sweet solution compared to tap water may be an indication of anhedonia-like behavior and has been observed after chronic stress induction in rodents (Berrio et al., 2024; Mao et al., 2022). The placement of the two different bottles was randomized across cages. After the test, rats were again housed in their usual group home cages. The weight change of the bottles was used as a measure of intake. The sucrose preference ratio was calculated using the following formula: Sucrose Preference ratio = Sucrose solution intake (g) / [Water intake (g) + Sucrose solution intake (g)].

Chronic restraint stress induction

Rats from the stress group were brought to the hallway where the behavioral testing rooms are located on a cart in groups of two cages (one with 4 males and one with 4 females), a few minutes before the start of each stress induction session. Rats were placed in a cylindrical restrainer (males: #51335; females: #51336, Stoelting Europe, Dublin, Ireland) for 2 h daily for 10 consecutive days. Half of the rats underwent restraint before noon and the

other half in the afternoon. During the restraint sessions, the cylinders were placed in individual cages (type III) with a metal grid on top. For purely exploratory purposes, an ultrasound microphone (Avisoft-Bioacoustics CM16/CMPA, Glienicke, Germany) was attached with zip ties to the metal grid to record the occurrence of any aversive 22-kHz ultrasonic vocalizations (USV) emitted by the male rats (Wöhr & Schwarting, 2013). After each stress induction session, the cages, restrainers, and surfaces of the room were cleaned with Ecover All Purpose Cleaner, Lemongrass & Ginger (5%). Control rats remained undisturbed in their home cages, handled only to perform daily weighing and to obtain fecal samples.

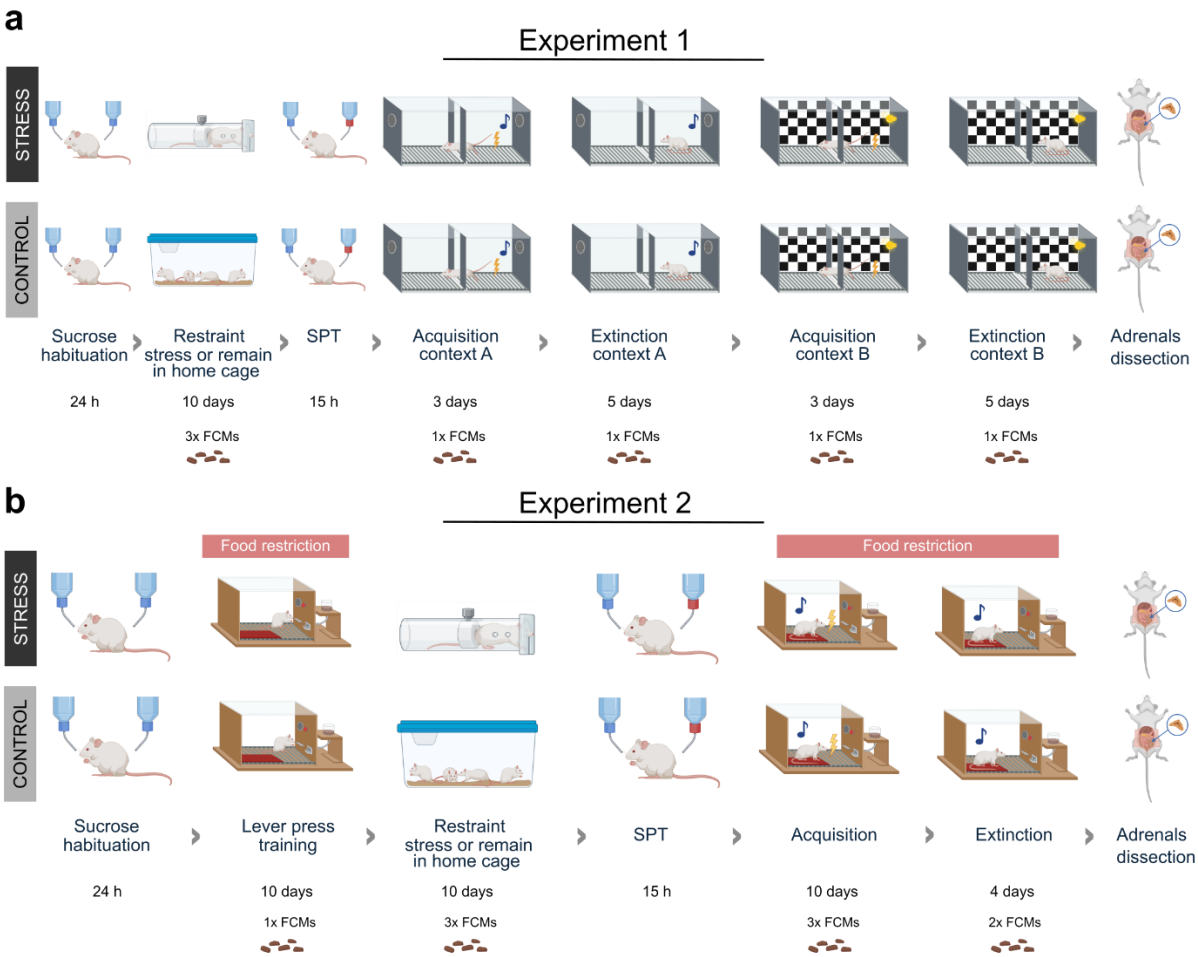

Figure 1. The experimental timeline of Experiment 1 (**a**) and Experiment 2 (**b**). SPT: sucrose preference test. FCMs: fecal corticosterone metabolites.

Two-way active avoidance (Experiment 1)

The effects of prior chronic restraint stress on 2WAA acquisition and extinction in two contexts were tested in 15 male and 16 female rats (see figure 1a).

<u>Apparatus</u>

192 Four identical operant chambers (28.7 width, 24.13 cm depth and 28.7 cm height; ENV-
193 010MXL, Med Associates Inc, Vermont, USA) were used simultaneously and were enclosed
194 in sound-attenuating cubicles (ENV-020M, Med Associates). Each operant chamber was
195 divided into two identical compartments by custom doorway panels. Behavior was recorded
196 with an infrared IP camera (Foscam C1, Shenzhen, China) attached to the ceiling of the
197 sound-attenuating cubicle. Tones were generated by a multiple tone generator (ENV-223,
198 Med Associates) and speaker (ENV-224AM-3, Med Associates). The shock floor (ENV-
199 005FPU-R, Med Associates) of each compartment consisted of 19 stainless steel rods 0.47
200 cm in diameter and spaced 1.59 cm apart, center to center. Each side of the compartment
201 contained four independent infrared photobeams (ENV-256-8S, Med Associates) to track
202 where the rat was located. All experimental sessions were programmed using Trans™ editor
203 and were carried out by MED-PC™ software.

204 Acquisition and extinction

205 Rats were tested in batches of four. The order of testing was counterbalanced for
206 experimental group and sex. All animals first underwent 3 days of avoidance acquisition and
207 5 days of avoidance extinction in context A, and then 3 days of acquisition and 5 days of
208 extinction in context B.

209 *Context A*
210 The shuttle box was not illuminated and was cleaned with a pine-scented cleaning product
211 (econom Allesreiniger dennenfris, 5%). The CS was a tone with an intensity of 75 dB and a
212 frequency of 3kHz. First, rats underwent 3 days of avoidance training in context A
213 (Vercammen et al., 2024) . On each day, avoidance training started with a 5-min acclimation
214 period, whereafter 30 CSs of maximum 20 s were presented, with an ITI averaging 70 s (40 s
215 to 100 s). Each CS co-terminated with a foot shock US of maximum 10 s and an intensity of
216 0.4 mA. All rats had the possibility to avoid or escape the US by shuttling to the opposite
217 compartment during or right after the tone, respectively. If the rat shuttled to the other
218 compartment during the presentation of the CS, the US was avoided, the CS was terminated
219 and the trial was labeled as an avoidance response. If the rat shuttled to the other
220 compartment during US presentation, the US was terminated and the trial was labeled as an
221 escape response. After 3 days of avoidance training, rats went through extinction training for
222 5 days, where each day started with a 5-min acclimation period, whereafter 24 CSs of the
223 same characteristics as above were presented per day, without foot shock. Note that for
224 video synchronizing purposes, the onset of the tone CS used in context A was accompanied
225 by a dim 2-s light cue (a partially covered house light at the center of the back wall) oriented
226 towards the camera.

227 *Context B*

228 Forty-eight hours after the last extinction session in context A, all rats were again subjected
229 to 3 days of avoidance training and 5 days of extinction, however this time in a different
230 context B, to further evaluate the longevity of any effects of the chronic restraint stress
231 induction. To change the context, a checkerboard pattern was added to the outside of the
232 long transparent sides of the box. The inside of the box was very dimly lit using two
233 houselights (3.7 lux, measured at the center of each compartment's grid). The scented
234 cleaning product was changed to Instanet (5%). And lastly, the CS used in context B was a
235 light stimulus (round yellow LED, 3 cm above the grid floor, MedAssociates, ENV-221M-LED,
236 40 lux) with a maximum duration of 20 s.

237 **Platform-mediated avoidance (Experiment 2)**

238 The effects of prior chronic restraint stress on PMA acquisition and extinction were tested in
239 16 male and 16 female rats (see Figure 1b).

240 Apparatus

241 Eight identical operant chambers (30.5 cm width, 25.4 cm depth and 30.5 cm height; Rat Test
242 Cage, Coulbourn Instruments, Pennsylvania, USA) were used simultaneously and were
243 enclosed in individual sound-attenuating boxes. A 12.8 cm by 15.2 cm platform made of a
244 non-transparent red plastic 3-mm sheet was used in all sessions. It approximately covered ¼
245 of the grid floor, and was placed in the corner opposite to the lever. Experiments were
246 conducted using Graphic State 4 (Coulbourn Instruments) with the house light off. Behavior
247 was continuously recorded during the experimental sessions using an IP camera (Foscam
248 C2M, Shenzhen, China).

249 Lever press training

250 Lever press training consisted of 45 min of training for 10 consecutive days. Rats were
251 gradually shaped to lever press for grain-based pellets (Grain-Based Dustless Precision
252 Pellets® for Rodent, Bio-Serv, Frenchtown, NJ, USA)..The first training phase consisted of
253 one magazine training session with the lever retracted, during which food pellets were
254 delivered at fixed 2-min intervals. The second phase of lever press training consisted of
255 magazine training with the lever extended, during which food pellets were delivered at fixed
256 2-min intervals, supplemented with direct food pellet delivery upon each lever press. This
257 phase lasted a minimum of 1 day and required at least 1 lever press/min on average to pass
258 on to the next phase. If there were no lever presses during the first half of the third day of the
259 training phase, hand shaping was performed. The next phases consisted of lever press
260 training on increasing variable ratio (VR) schedules, progressing from VR 3 through VR 5

261 and VR 15 to VR 30, where pellets were delivered after a variable number of lever presses,
262 with an increasing average reinforcement criterion per session. For VR 3, a criterion of at
263 least 3 lever press/min was set to pass to the next schedule, a criterion of at least 5 lever
264 press/min for VR 5, a criterion of at least 10 lever press/min for VR 15, and a criterion of at
265 least 15 lever press/min for VR 30. The final training phase consisted of training on a variable
266 interval (VI) schedule of reinforcement averaging 30 seconds (VI 30). The first lever press
267 after a variable refractory period averaging 30 s yielded pellet delivery.
268 Rats were included if they met at least one of two preregistered criteria: 10 lever press/min
269 after averaging the first 10 min of all lever-pressing training sessions in VI 30 or 10 lever
270 press/min after averaging the complete duration of all lever-pressing training sessions in VI
271 30. All rats met at least one of the two learning criteria; therefore all 32 rats were included.

272 Acquisition and extinction

273 Acquisition of platform-mediated avoidance started 1 day after the end of the sucrose
274 preference test. Avoidance acquisition lasted for 10 days in accordance with previous
275 research (C. Bravo-Rivera et al., 2014, 2015; Diehl et al., 2018; Martínez-Rivera et al., 2019).
276 Each day, 9 30-s CSs were presented, with an intertrial interval (ITI) averaging 180 s (150 s
277 to 210 s). This ITI was also used in subsequent sessions. The CS consisted of a pure 3-kHz
278 tone co-terminating with a 2-s, 0.4-mA foot shock US, which was delivered through the grid
279 floor. The plastic platform in one corner constituted a safe space where the rat would not
280 experience the foot shock during US presentation. The experiment ended with four daily
281 extinction training sessions in accordance with previous research (López-Moraga et al.,
282 2024a). Each extinction session consisted of the 30-s CS being presented nine times, as in
283 avoidance acquisition, but without US delivery.

284 **Fecal corticosterone metabolites**

285 At several time points, we collected fecal boli to determine fecal corticosterone metabolites
286 (FCMs) as a proxy for systemic corticosterone levels. This non-invasive method has
287 previously been validated in rats (Lepschy et al., 2007; Palme, 2019). In Experiment 1, fecal
288 boli were collected at the following time points (Figure 1a): immediately before sucrose
289 habituation, on days 2, 6 and 10 of restraint stress, day 2 of 2WAA acquisition in context A,
290 day 2 of 2WAA extinction training in context A, day 2 of 2WAA avoidance acquisition in
291 context B and day 2 of 2WAA extinction training in context B. In Experiment 2 (Figure 1b),
292 fecal boli were collected on day 10 of lever press training, days 2, 6 and 10 of restraint
293 stress, days 2, 6 and 10 of platform-mediated avoidance acquisition and days 2 and 4 of
294 extinction training. Fecal boli were collected from the restraint cylinder or behavioral testing
295 setup immediately after the end of the session. On days 2, 6 and 10 of the stress induction

296 period, fecal boli of the control group were collected during their daily weighing
297 measurements.

298 Fecal boli were immediately stored at -70°C until processing. Feces were dried in an oven at
299 70 °C (COOK-IT Mini Oven 21L, Media Evolution B.V, Eindhoven, The Netherlands) for 4 h.
300 Afterwards, they were reduced to powder using a mortar and pestle, and 0.05 g was weighed
301 and mixed with 1 ml of 80% methanol (Sigma-Aldrich, Schnelldorf, Germany) in deionized
302 water. Samples were placed in a multivortex (Thermomixer comfort, Eppendorf, Hamburg,
303 Germany) at 21 °C for 30 minutes at 1400 rpm and then centrifuged (Centrifuge 5424,
304 Eppendorf) for 10 minutes at 2500 g. Next, 0.5 ml of the supernatant was collected and
305 stored at -70 °C until analysis. Samples were shipped and a group-specific 5α-pregnane-
306 3β,11β,21-triol-20-one enzyme immunoassay, which measures a group of corticosterone
307 metabolites with a 5a-3ß,11ß-diol configuration (Palme, 2019), was performed in the
308 Laboratory of Experimental Endocrinology at the University of Veterinary Medicine Vienna as
309 previously described (Lepschy et al., 2007, 2010).

310 **Adrenal gland dissection**

311 One to two days after the last behavioral test session, rats were injected intraperitoneally
312 with an overdose of sodium pentobarbital (200 mg/ml solution for injection, vetoquinol B.V.).
313 In Experiment 1, the adrenal glands were extracted immediately after pentobarbital injection.
314 In Experiment 2, rats were first transcardially perfused with 1% phosphate-buffered saline
315 followed by 4% paraformaldehyde solution before dissecting the adrenals. Any visible
316 adipose tissue was surgically removed, and the glands were weighed. Chronic stress can
317 cause the enlargement of the adrenal glands, resulting in an increased organ weight (Ulrich-
318 Lai et al., 2006), making adrenal gland/body weight ratio a valuable chronic stress
319 manipulation check that other authors previously used (Dagnino-Subiabre et al., 2005).
320 Adrenal gland/body weight ratio was calculated by averaging the weight of the two adrenal
321 glands and dividing this by the body weight of the rat prior to euthanasia.

322 **Behavioral outcomes**

323 <u>Experiment 1</u>

324 Avoidance responses were measured via the infrared beams in each compartment and
325 collected by MED-PC software. Additionally, freezing during the 5-min acclimation period was
326 scored from video recordings. Freezing was defined as full immobility except for the minimal
327 movements associated with breathing. Scoring was done manually by the experimenter, who
328 was blinded to group allocation during scoring. The experimenter could not be blinded to the
329 phase of the experiment (avoidance training or extinction, and context A or B) and sex during

scoring, because this information is visible on the videos.

Experiment 2

Lever presses were recorded using Graphic State 4 software (Coulbourn Instruments, Pennsylvania, USA) and further processed using an in-house R script available on OSF. Lever presses produced 1 min before the tone (pretone) and during the tone were aggregated per block (each block consisting of 3 tones), after which the following formula was applied to calculate the suppression of lever pressing: (pretone rate - tone rate)/(pretone rate + tone rate) x 100 (Quirk et al., 2000). The obtained values ranged from no suppression of lever pressing (0%) to full suppression of lever pressing (100%). For blocks in which pretone and tone values were both 0, the rat's suppression was substituted by the mean group average for that block.

Time spent on the platform (i.e., avoidance) and freezing during CS presentations were scored using a custom automated scoring pipeline that relied on DeepLabCut and simBA (see below). To check its validity, we compared 16 videos (8 from acquisition and 8 from extinction sessions) analyzed using our automated scoring pipeline with manual scoring by trained observers and obtained an intraclass correlation coefficient (ICC) of 0.91, 95% CI [0.88, 0.94] for the avoidance measurement and an ICC of 0.88, 95% CI [0.83,0.91] for the freezing measurement (see Validation folder in OSF). Given the high ICC, we proceeded with automated scoring only.

We processed our videos using DLC and SimBA to obtain speed traces of each rat in cm/s. As previously described (Gruene et al., 2015), we considered a discrete darting bout when an animal moves with a minimum velocity of 23.5 cm/s during any CS presentation. An animal is considered a darter if it exhibits at least one dart between the 3rd CS-US pairing and the 7th CS-US pairing of the first avoidance training session (Gruene et al., 2015; López-Moraga et al., 2024b).

DeepLabCut

We used DeepLabCut (version 2.3.9) for body part tracking (Mathis et al., 2018; Nath et al., 2019). We labeled 196 frames taken from 10 videos, each video originating from a different animal; 95% of these frames were used for training. Five body points were selected: nose, left ear, right ear, centroid, and tail base. We used a RestNet-50 neural network (He et al., 2015; Insafutdinov et al., 2016) with default parameters for 300000 training iterations. We validated with 1 shuffle and found that the test error was 4.78 pixels, and the training error was 1.88 pixels (image size: 455 x 256 pixels, downsampled from original 1280 x 720 pixels).

simBA

To further analyze pose estimation data obtained using DeepLabCut, we used SimBA (Goodwin et al., 2024) to automate the analysis of different behaviors in the PMA as described previously (López-Moraga et al., 2024b). We created a single animal project configuration with user-defined body points. We defined pixels per mm by measuring the width of the bottom of the fear conditioning box.

To analyze avoidance behavior, we defined the platform and adjacent wall sections as the region of interest (ROI). If the centroid body part was inside the ROI, this was considered as avoidance behavior.

To analyze darting behavior, we used the Analyze Distances/Velocities module with a 1-second time bin. We then used our own scripts to identify time bins with CS presentations (see OSF). The last 2 s of the CS presentations were excluded, as the CS co-terminated with the foot shock, and darting could be confounded with a US escape response.

We constructed a random forest classifier in simBA to assess freezing. Eight videos were manually annotated; 22505 frames (750.17 s) were annotated with freezing present and 137638 frames (4587.93 s) with freezing absent. The start frame was defined as the first frame in which the animal stopped moving, and the end frame was defined as the last frame before the animal started moving again. We built the model with the following settings: n_estimators = 2000, RF_criterion = gini, RF_max_features = sqrt, RF_min_sample_leaf = 1, under_sample_ratio = 4, class_weights = custom: Freezing present = 2; Freezing Absent = 1, with an 80% training split. The evaluation performance of the freezing classifier had a precision score of 0.87, recall 0.70, and F1 score of 0.78 for freezing present, and a precision score of 0.92, recall 0.97, and F1 score of 0.94 for freezing absent. We set the discrimination threshold at Pr = 0.65, and a minimum duration of 500 ms. We used our own scripts to identify time bins with CS presentations to further analyze freezing instances (see OSF).

Data analyses

Statistical analyses were preregistered, and any deviations are specified below. Data were analyzed using two-way, repeated, or mixed ANOVAs, as appropriate. Negative effect sizes of ANOVA tests (ω_p^2) are reported as 0 (Kroes & Finley, 2023). Post hoc tests with multiple test corrections followed if significant main effects or interactions were found. If assumptions were violated, nonparametric statistical analyses were performed. For the nonparametric mixed RM ANOVAs, we applied an aligned rank transform ANOVA using the ARTool (Elkin et al., 2021) package from R (Version 4.1.3; R Core Team, 2022) and R Studio (Version 4.2.764; RStudio Team, 2021). All other statistical analyses were performed using performance (Lüdecke et al., 2021), afex (Singmann et al., 2023), emmeans (Lenth et al.,

2024) and effectsize (Ben-Shachar et al., 2024) R packages. Data were processed and plotted using the tidyverse (Wickham & RStudio, 2022) and zoo (Zeileis & Grothendieck, 2005) packages. The USV recordings were analyzed using Avisoft SASLabPro Software (Avisoft-Bioacustics, Glienicke/Nordbahn, Germany).

Stress induction

To evaluate the effectiveness of the chronic restraint stress induction, we assessed its effect on four variables: weight change, sucrose preference ratio, adrenal glands weight and FCM levels.

We performed a mixed ANOVA with day as a within factor, group as a between factor and weight change as a dependent variable. To analyze the sucrose preference ratio, we used a two-way ANOVA with the sucrose preference ratio as a dependent variable and sex and group as between factors. Adrenal gland/body weight ratio was used as a dependent variable in a two-way ANOVA with sex and group as factors. Differences in FCM levels (log-transformed) were analyzed with a mixed ANOVA with day (baseline, day 2, 6 and 10) as within factor, and sex and group as between factors. The inclusion of the baseline in FCM and weight was not preregistered.

2WAA analyses

To evaluate avoidance behavior in Experiment 1, we conducted between-group, between-sex and within-day comparisons of number of avoidance responses during 3 days of acquisition in context A and 5 days of extinction in context A with RM ANOVAs (Group x Sex x Day). In addition, we carried out separate analyses between groups and sex on the first day of acquisition in context A and on the first day of extinction in context A, in which we divided the 30 CSs of day 1 acquisition into 3 blocks of 10 CSs and the 24 CSs of day 1 extinction into 3 blocks of 8 CSs. RM ANOVAs (Group x Sex x Block) were then performed on these datasets. The same analyses repeated for acquisition and extinction in context B. To test a secondary hypothesis, we conducted a two-way ANOVA (Sex x Group) analysis of freezing during the acclimation of day 1 acquisition in context A. Because no freezing was observed during this period, we exploratively analyzed (non-preregistered analysis) freezing during acclimation of day 2 acquisition in context A, using a two-way ANOVA (Sex x Group).

PMA analyses

We conducted three mixed ANOVA analyses to examine the percentage of avoidance, freezing, and suppression of lever pressing behavior between stressed and control rats. We included days 1 to 3 as within-subject factors and sex and group as between-subject factors.

Similarly, we performed three mixed ANOVA analyses to investigate differences in the percentage of avoidance, freezing, and suppression of lever pressing behavior across the three blocks (3 CSs per block) of extinction (days 1-2), with sex and group as between-subject factors.

In addition, we evaluated persistent avoidance behavior. A rat was considered a persistent avoider when the time on the platform during the CS was more than 50% during the first block of the third extinction session. We compared the % of persistent avoider rats in the stress group versus the control group with a Chi-square test. Additionally, we performed four two-way ANOVAs with sex and persistent avoidance as between-subjects factors to analyze differences in freezing and suppression of lever pressing during the last avoidance acquisition day and the third extinction day. Given that there were no differences between sex or group in a Chi-square test, we investigated if there were differences between persistent avoiders and non-persistent avoiders regardless of group allocation on the last day of acquisition and on the third extinction session as preregistered.

Finally, we assessed darting behavior in this task. The classification of darters and non-darters was performed as preregistered and as reported by other authors (Gruene et al., 2015). We preregistered a comparison of the % of darter rats in females versus male rats during avoidance training using a Chi square test. We exploratorily investigated the % of darter rats in control versus stressed rats during avoidance training using a Chi square test. Given that the assumption of more than five counts per cell was violated, we used the Fisher's exact test as well, which yielded similar results (see OSF). We preregistered that if the Chi-square test comparison between male and female rats was significant, we would conduct further analyses. Since this was not the case, we did not perform these analyses. Additionally, due to the low number of stressed darter rats, it was not possible to run further exploratory analyses comparing darters vs non-darters between the stress and control conditions.

Results

Experiment 1

Chronic restraint stress affects body weight but not sucrose preference, relative adrenal weight or fecal corticosterone metabolites

At the start of the experiment, rats were divided into two groups: stress and control. Rats in the stress group were subjected to 2 hours of restraint stress on 10 consecutive days. Rats in the control group were handled only. After every session, rats were weighed and their weight change relative to the day before stress induction or control treatment was assessed

(Figure 2a). We found a significant interaction between group and day ($F(3.84, 111.44) = 56.71$, $p < 0.001$, $\omega_p^2 = 0.46$, see Table 1 for further statistics). Upon further investigation, we found significant differences between control and stressed rats early on (Day 2: $t(29) = 6.50$, $p < 0.001$, $d = 2.36$), in the middle (Day 6: $t(29) = 10.74$, $p < 0.001$, $d = 3.88$), and at the end (Day 10: $t(29) = 10.49$, $p < 0.001$, $d = 3.79$) of the 10-day stress induction.

Fecal samples were collected at baseline, at day 2, 6 and 10 of the stress induction or control treatment. The concentration of FCMs was analyzed in each sample (Figure 2b). A strong sex effect was found, with female rats having significantly lower FCM concentrations than males ($F(1,26) = 146.53$, $p < 0.001$, $\omega_p^2 = 0.84$). We also found a significant main effect of Day ($F(3, 78) = 6.09$, $p < 0.001$, $\omega_p^2 = 0.09$), where post-hoc testing revealed a significant increase in FCM concentration between baseline and day 2 ($p = 0.01$) and between baseline and day 6 ($p = 0.01$). We did not find any differences between groups ($F(1, 26) = 0.16$, $p = 0.67$, $\omega_p^2 = 0$).

Shortly after the last session of stress induction or control treatment, we conducted a sucrose preference test (Figure 2c). In this experiment, chronic restraint stress did not have an effect on the sucrose preference ratio ($F(1,27) = 2.38$, $p = 0.134$, $\omega_p^2 = 0.04$).

At the end of all experimental manipulations, we dissected the adrenal glands to assess the adrenal gland-to-body weight ratio (Figure 2d). While we did not find any significant differences between the stress and control groups ($F(1, 27) = 0.81$, $p = 0.376$, $\omega_p^2 = 0$), we did observe a strong sex dimorphism ($F(1, 27) = 94.63$, $p < 0.001$, $\omega_p^2 = 0.75$), where regardless of group, females had a higher adrenal-to-body-weight ratio than males.

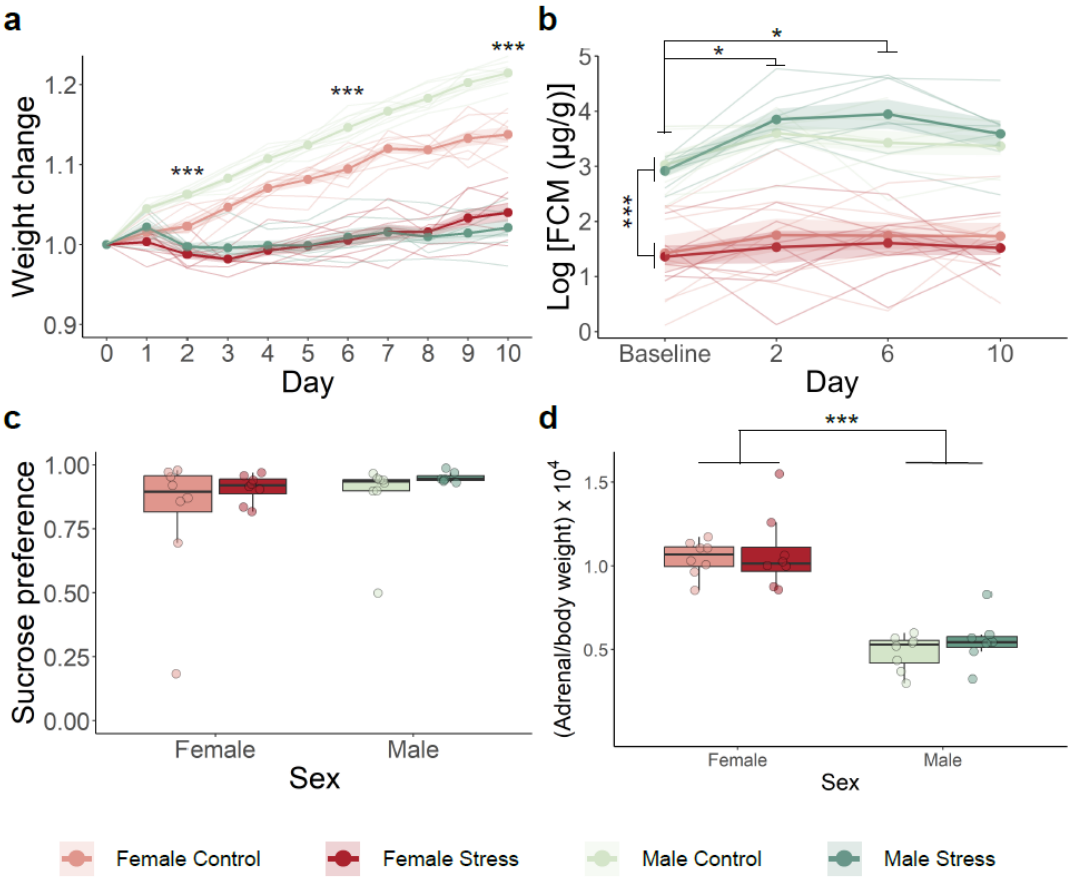

Figure 2. Stress manipulation checks. The bold lines represent the mean, the surrounding shades are the standard error of the mean and thinner lines represent individual results. **a**. A significant interaction between day and group ($F(3.55, 102.87) = 73.42$, $p < 0.001$) in body weight change was detected. There were significant differences in body weight change between the groups starting on day 2 ($t(29) = 6.5066$, $p < 0.001$). **b**. Males show higher concentrations of fecal corticosterone metabolites (FCMs) than females ($F(1,26) = 146.53$, $p < 0.001$). No group differences were detected ($F(1, 26) = 0.16$, $p = 0.696$). **c**. Both groups and both sexes presented a similar preference for the sucrose solution. **d**. Females had a higher adrenal-to-body-weight ratio than males ($F(1, 27) = 94.63$, $p < 0.001$), but no differences were found between the control and stress group.

<u>Chronically stressed rats show an impairment in acquiring avoidance in the two-way active avoidance procedure in context A.</u>

To evaluate whether chronic restraint stress has an effect on active avoidance behavior, we subjected control rats and rats that underwent chronic restraint to the two-way active avoidance task. During avoidance acquisition in context A, chronically restrained rats showed a significantly lower number of avoidance responses compared to control rats ($F(1, 27) = 9.79$, $p < 0.01$, $\omega_p^2 = 0.23$, see Table 2 for further statistics). A significant main effect of Day showed that all rats learned the avoidance response over the days ($F(2, 54) = 29.31$, $p < 0.001$, $\omega_p^2 = 0.23$, Figure 3a). When looking at the number of avoidance responses during

510 the 3 blocks of day 1, a main effect of both Group ($F(1, 27) = 5.39$, $p = 0.028$, $\omega_p^2 = 0.13$) and
511 Block ($F(2, 54) = 11.67$, $p < 0.001$, $\omega_p^2 = 0.13$) was found (Figure 3b). Rats in the stress
512 group overall thus showed less avoidance responses than rats in the control group on day 1
513 across all blocks. Rats showed no freezing during acclimation on day 1 of acquisition in
514 context A.

515 No differences in avoidance between stressed and control rats during extinction.

516 After acquisition, rats underwent 5 daily sessions of extinction training in context A. Rats from
517 both groups and sexes equally extinguished their avoidance responses over the days
518 ($F(2.79, 75.22) = 55.67$, $p < 0.001$, $\omega_p^2 = 0.48$, Figure 3c). A significant reduction of
519 avoidance responses was already observed from day 1 to day 2 ($t(135) = 4.11$, $p < 0.001$, d
520 $= 1.05$). Analysis on block level during day 1 of extinction did not reveal any group or sex
521 differences (Figure 3d).

522 Stressed females show reduced avoidance on the final acquisition day in Context B

523 In order to determine the longevity of any effects of chronic restraint stress, all rats again
524 went through avoidance training, but this time in a new context B and with a different warning
525 signal (Figure 3e). Analysis revealed a significant Sex by Day by Group interaction ($F(2, 54)$
526 $= 5.95$, $p = 0.005$, $\omega_p^2 = 0$). Follow-up analyses showed that males increased their avoidance
527 responses across both groups over test sessions ($F(2, 26) = 45.08$, $p < 0.001$, $\omega_p^2 = 0.19$),
528 whereas females showed an interaction between group and day ($F(2, 28) = 11.57$, $p < 0.001$,
529 $\omega_p^2 = 0.02$). Particularly, stressed females showed a significantly lower number of avoidance
530 responses than control females on the last day of avoidance acquisition in context B ($W =$
531 57.50, $p = 0.021$, $r = 0.69$). Zooming in on the first day of avoidance training in context B,
532 only a significant main effect of Block was found ($F(2, 54) = 6.01$, $p = 0.004$, $\omega_p^2 = 0.09$,
533 Figure 3f), meaning that regardless of sex or group, rats learned to acquire avoidance
534 responses. Follow-up analysis showed that a significant increase of avoidance responses
535 occurred from block 1 to 3 ($t(81) = -3.21$, $p = 0.005$, $d = -0.82$).

536 Male rats surpass female rats in extinction of their avoidance responses in context B

537 Analysis of extinction in context B revealed a sex difference (Figure 3g). A significant main
538 effect of sex showed that female rats had an overall higher number of avoidance responses
539 throughout all extinction sessions, compared to male rats ($F(1, 27) = 7.34$, $p = 0.012$, $\omega_p^2 =$
540 0.18). A significant main effect of Day revealed that rats from both groups and sexes learned
541 to extinguish their avoidance responses ($F(4, 108) = 24.56$, $p < 0.001$, $\omega_p^2 = 0.22$). A
542 significant reduction of avoidance responses was observed already early on (e.g., from day 1
543 to day 3 ($t(135) = 3.04$, $p = 0.023$, $d = 0.77$)). At the block level on day 1, we found a

significant Sex by Block interaction (F(2, 54) = 3.63, p = 0.033, ω_p^2 = 0, Figure 3h). A significant effect of Block was found only in male rats ($\chi^2(2)$ = 12.61, p = 0.002, W = 0.42), with males significantly reducing their avoidance responses from block 1 to 3 (p = 0.01, r = 0.54) and from block 2 to 3 (p = 0.04, r = 0.46).

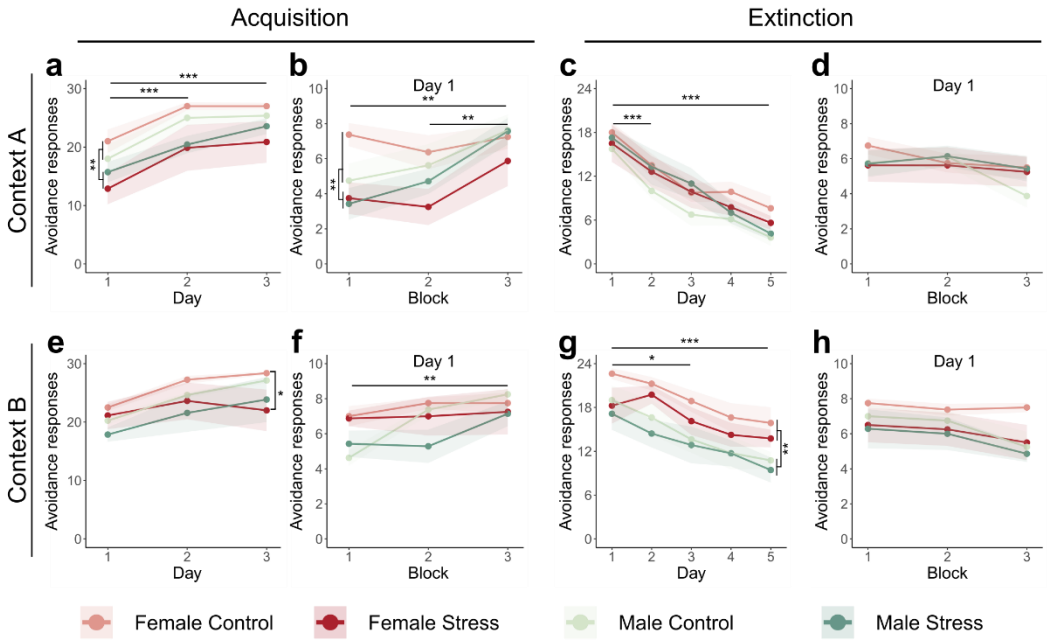

Figure 3. Avoidance responses during two-way active avoidance acquisition and extinction across two distinct contexts. The lines represent the mean number of avoidance responses, the surrounding shaded area represents the standard error of the mean. Results are expressed in the absolute number of avoidance responses. Each acquisition day consisted of 30 trials, and each extinction day consisted of 24 trials. **a**. Avoidance acquisition in context A. Rats that were subjected to chronic restraint stress showed significantly fewer avoidance responses than rats in the control group throughout all days of acquisition (F(1, 27) = 9.79, p < 0.01). **b**. Results of day 1 avoidance acquisition in context A show that rats in the stress group overall showed less avoidance responses than rats in the control group across all blocks (F(1, 27) = 5.39, p = 0.03) **c**. Extinction in context A. All rats, regardless of group or sex, demonstrated a similar rate of extinction of avoidance responses (F(2.79, 75.22) = 55.67, p < 0.001). **d**. Analysis on block level during day 1 of extinction in context A did not reveal any group or sex differences. **e**. Avoidance acquisition in context B. Female rats in the chronic restraint stress group displayed a significantly lower number of avoidance responses compared to female rats in the control group on day 3 of acquisition in context B (W = 57.5, p = 0.02). **f**. Analysis on block level during day 1 of acquisition in context B revealed a significant main effect of Block (F(2, 54) = 6.01, p < 0.01) meaning that regardless of sex or group, rats learned to acquire avoidance responses. **g**. Extinction in context B. Female rats, regardless of group, showed a higher number of avoidance responses than male rats across all days of extinction in context B (F(1, 27) = 7.34, p = 0.01). **h**. Analysis on block level during day 1 of extinction in context B revealed a significant effect of Block, but only in male rats ($\chi^2(2)$ = 12.61, p <0.01).

571 **Experiment 2**

572 Chronic restraint stress affects body weight and FCM but not sucrose preference or
573 relative adrenal weight

574 In Experiment 2, rats were food-restricted and underwent lever press training (see Figure 1b
575 and Methods). Subsequently, rats were divided into two groups: stress and control. Rats in
576 the stress group were subjected to 2 hours of restraint stress on 10 consecutive days. Rats
577 in the control group were handled only. After every session, rats were weighed and their
578 weight change relative to the start of stress induction or control treatment was assessed
579 (Figure 4a). We found a significant interaction between group and day ($F(2.56, 76.81) =$
580 62.98, $p < 0.001$; for further statistics, see Table 3). Upon further investigation, we found
581 significant differences between control and stressed rats at the beginning (Day 2: $t(30) =$
582 6.09, $p < 0.001$, $d = 2.15$), middle (Day 6: $t(30) = 10.76$, $p < 0.001$, $d = 3.8$) and end (Day 10:
583 $t(30) = 10.22$, $p < 0.001$, $d = 3.61$) of treatment. Fecal samples were collected at the same
584 time points during the stress induction phase as in Experiment 1 (Figure 4b). We again found
585 a strong sex effect, with females showing lower FCM concentrations than males ($F(1, 28) =$
586 242.92, $p < 0.001$, $\omega_p^2 = 0.89$). In addition, we found an interaction between day and group
587 ($F(3, 84) = 3.15$, $p = 0.029$, $\omega_p^2 = 0.04$, see Table 2 for further analyses). Concretely, we did
588 not find any differences between groups at baseline ($t(112) = 1.12$, $p = 0.26$, $d = 0.4$), day 2
589 ($t(112) = -0.71$, $p = 0.479$, $d = -0.25$) and day 6 ($t(112) = -0.47$, $p = 0.637$, $d = -0.17$).
590 However, on day 10, the stress group showed higher concentrations of FCMs than the
591 control group ($t(112) = -2.78$, $p = 0.006$, $d = -0.98$).

592 Shortly after the last session of stress induction or control treatment, we conducted a sucrose
593 preference test (Figure 4c). However, we did not observe a significant group difference ($F(1,$
594 $28) = 0.01$, $p = 0.91$, $\omega_p^2 = -0.02$), although the stress group presented higher variability than
595 the control group.

596 At the end of all experimental manipulations, we perfused all rats and dissected their adrenal
597 glands to assess the adrenal gland-to-body weight ratio (Figure 4d). While we did not find
598 any significant differences between the stress and control groups ($F(1, 28) = 0.02$, $p = 0.900$,
599 $\omega_p^2 = 0$), we did observe a strong sex dimorphism ($F(1, 28) = 112.67$, $p < 0.001$, $\omega_p^2 = 0.81$),
600 where regardless of group, females had a higher adrenal-to-body weight ratio than males.

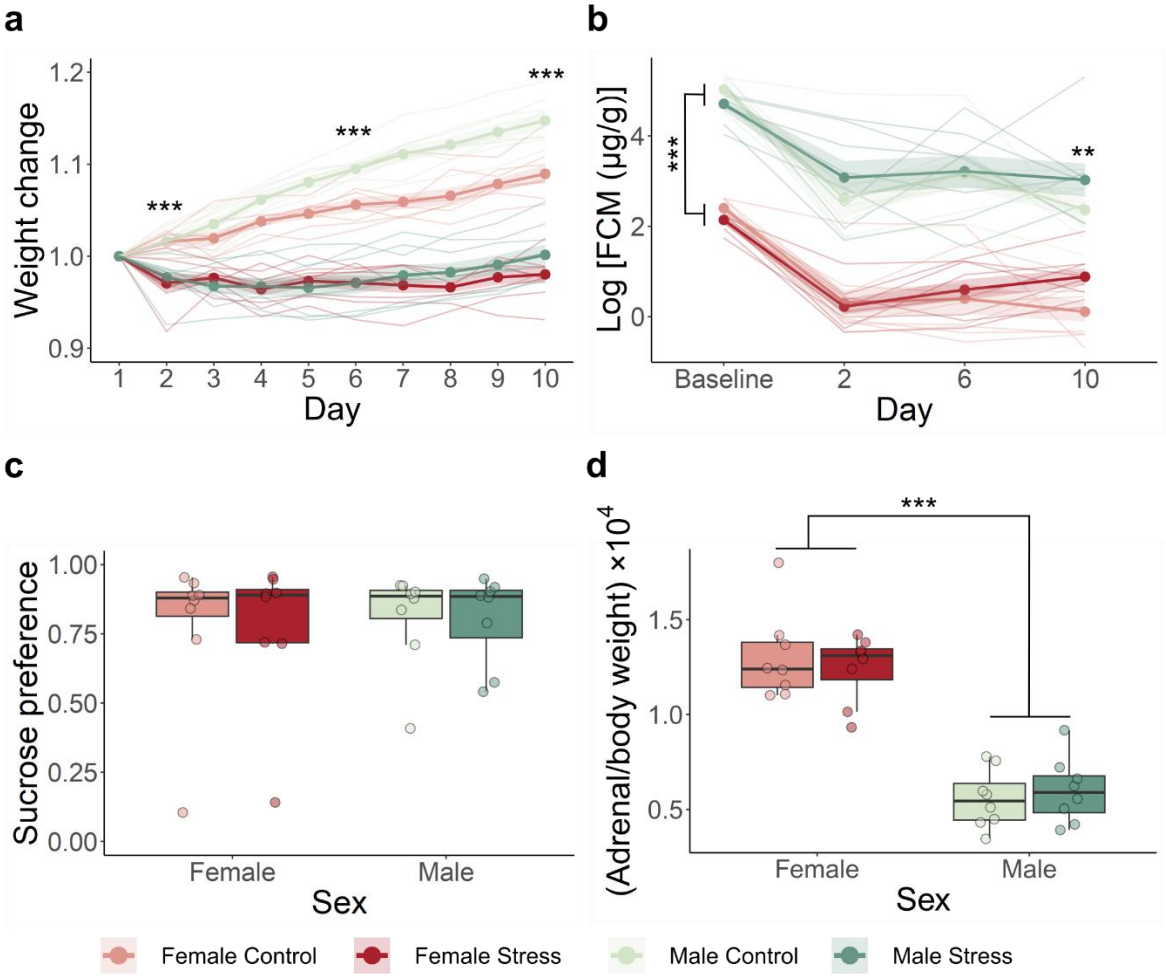

Figure 4. Stress manipulation checks. The bold lines represent the mean, the surrounding shaded area the standard error of the mean and thinner lines represent individual results. **a.** A significant interaction between day and group ($F(2.56, 76.81) = 62.98$, $p < 0.001$) in body weight change was detected. There were significant differences in body weight change between the groups starting on day 2 ($t(30) = 6.09$, $p < 0.001$). **b.** Males show higher concentrations of fecal corticosterone metabolites (FCMs) than females ($F(1, 28) = 242.92$, $p < 0.001$). At day 10, stress animals had higher concentrations of FCM than their control counterparts ($t(112) = -2.78$, $p = 0.006$). **c.** Both groups and both sexes showed a similar preference for the sucrose solution. **d.** Females had higher adrenals/body weight ratios than male animals ($F(1, 27) = 133.45$, $p < 0.001$), but no differences were observed between control and stress animals.

Subtle effects of chronic restraint stress on platform-mediated avoidance acquisition and extinction

After the sucrose preference test, we started platform-mediated avoidance acquisition for 10 days in both groups (Figure 5). On each day, 9 CS-US pairings were presented, and rats had the opportunity to avoid or escape from the foot shock US by stepping onto the platform, at the expense of the opportunity to lever press for food.

Although we hypothesized that stressed rats would show more avoidance than control rats, this hypothesis was not confirmed; all rats similarly acquired avoidance responding over the first three acquisition sessions ($F(2, 56) = 36.73$, $p < 0.001$, $\omega_p^2 = 0.43$; for further statistics, see Table 2). Freezing did not significantly increase over the first three acquisition sessions ($F(2, 56) = 1.59$, $p = 0.212$, $\omega_p^2 = 0$). Regarding suppression of lever pressing, we found a Day by Sex by Group interaction ($F(2, 56) = 7.33$, $p = 0.001$, $\omega_p^2 = 0.05$). Follow-up analyses showed that females increased their suppression of lever pressing similarly in both groups over the first three test sessions ($F(2, 28) = 8.84$, $p = 0.001$, $\omega_p^2 = 0.08$), whereas males showed an interaction between group and day ($F(2, 28) = 10.29$, $p < 0.001$, $\omega_p^2 = 0.01$). Stressed males showed significantly higher suppression of lever pressing than control males on day 1 ($W = 12$, $p = 0.04$, $r = -0.62$), but not on later sessions (see Table 4).

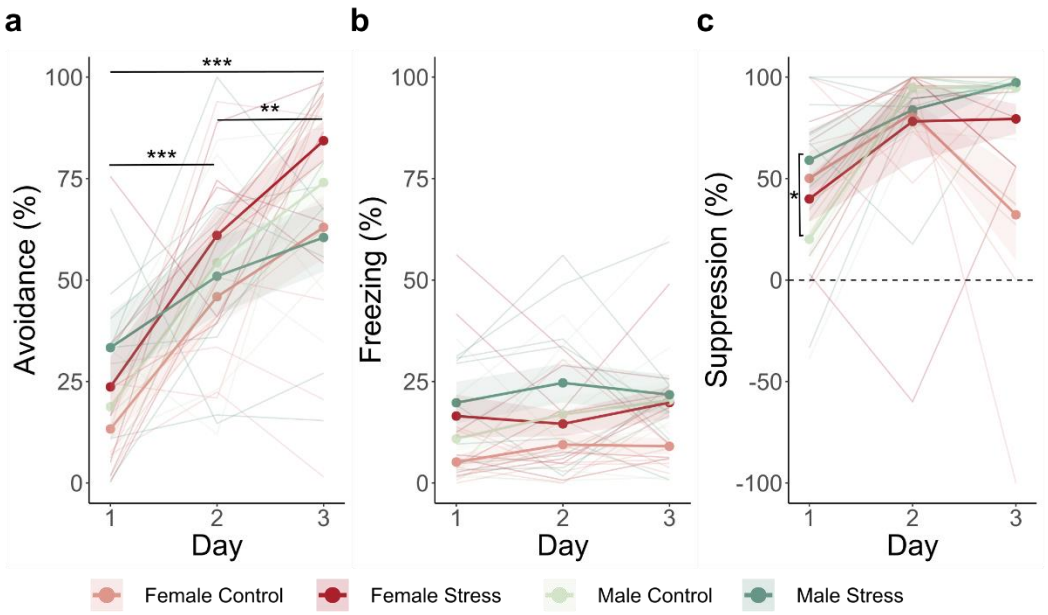

Figure 5. Avoidance acquisition results for Experiment 2. The bold lines represent the mean of the first block (first 3 CS) on each day, the surrounding shaded area the standard error of the mean and thinner lines represent individual results. **a.** Avoidance behavior significantly increased over acquisition sessions ($F(2, 56) = 36.73$, $p < 0.001$). **b.** Freezing did not significantly increase over acquisition sessions ($F(2, 56) = 1.59$, $p = 0.212$). **c.** Suppression of lever pressing showed a triple interaction of day, sex and group ($F(2, 56) = 7.33$, $p = 0.001$). Female rats showed a similar increase of suppression of lever pressing across groups ($F(2, 28) = 8.84$, $p = 0.001$), while data for male rats showed an interaction between day and group ($F(2, 28) = 10.29$, $p < 0.001$). Stressed males showed significantly higher suppression of lever pressing than control males on day 1 ($W = 12$, $p = 0.04$).

We also analyzed darting behavior during the acquisition sessions and found that 37.5% (6/16) females were classified as darters, while only 18.75% (3/16) males were classified as darters. Using a Chi-square test we observed that there was not a higher likelihood that females were classified as darters compared to males ($\chi^2(1) = 0.62$, $p = 0.432$). Additionally,

we exploratorily investigated whether stress or control rats were more likely to be classified as darters. We found that control rats were more likely to be darters ($\chi^2(1) = 5.57$, $p = 0.018$), as 93.75% (15/16) of the stressed rats were classified as non-darters while only 6.25% (1/16) of the stressed rats were classified as darter, while in control rats half were classified as darters (8/16) and the other half (8/16) as non-darters. Given that in the stress group only one rat was classified as a darter, it was not possible to run further statistics comparing control and stress groups over darting.

Next, we performed 4 sessions of extinction training, on consecutive days, with 9 CSs per session (Figure 6). Concretely, we examined how defensive responding to the CS changed over blocks in the first two sessions. Overall, avoidance behavior decreased from the first to the second extinction session ($F(1, 140) = 140.88$, $p < 0.001$, $\omega_p^2 = 0.27$; for further statistics, see Table 5). We found an interaction between sex and day in freezing ($F(1, 140) = 6.53$, $p = 0.012$, $\omega_p^2 = 0.01$). Concretely, females showed a significant reduction of freezing over days ($V = 705$, $p < 0.001$, $r = 0.53$), while males did not ($V = 644$, $p = 0.08$, $r = 0.26$). Suppression of lever pressing decreased over the two extinction sessions ($F(1, 28) = 26.69$, $p < 0.001$, $\omega_p^2 = 0.12$), without significant differences between sexes or groups.

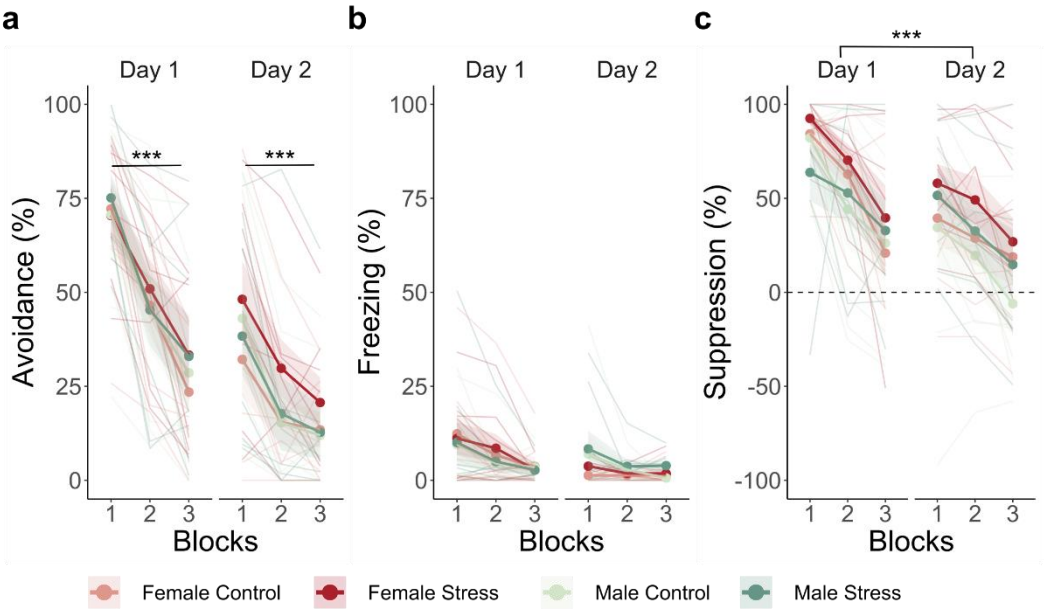

Figure 6. Extinction results for Experiment 2. The bold lines represent the mean of the blocks on each day, the surrounding shaded area the standard error of the mean and thinner lines represent individual results. **a.** Avoidance behavior significantly decreased over the blocks on day 1 ($\chi^2(2) = 51.94$, $p < 0.001$) and on day 2 ($\chi^2(2) = 37.1$, $p < 0.001$), without any group or sex differences. **b.** Freezing significantly decreased over extinction sessions for females ($V = 705$, $p < 0.001$), but not males ($V = 644$, $p = 0.08$), in the absence of group differences. **c.**

Suppression of lever pressing significantly decreased over extinction sessions ($F(1, 28) = 26.69$, $p < 0.001$), without any group or sex differences.

We investigated if there were differences in persistence of avoidance between control and stress groups. We classified a rat as persistent avoider if it presented more than 50% freezing during the first block of the third extinction session. We found that 18.75% of rats (6/32; 3 males and 3 females) were considered persistent avoiders and a Chi-square test ($\chi^2(1) = 0.2$, $p = 0.651$) determined neither control nor stressed rats had more chances of becoming persistent avoiders.

We did not find any differences in freezing between persistent and non-persistent avoiders on day 10 of avoidance acquisition ($W = 64$, $p = 0.514$, $r = 0.012$) nor in suppression of lever pressing ($W = 104.5$, $p = 0.202$, $r = 0.23$). However, we observed differences in defensive responding between persistent avoiders and non-persistent avoiders in freezing ($W = 22$, $p = 0.005$) and suppression of lever pressing ($W = 3$, $p < 0.001$, $r = 0.64$). Concretely, persistent avoiders showed higher freezing ($M = 6.75$, $SE = 2.65$) and higher suppression of lever pressing ($M = 92.4$, $SE = 3.86$) than non-persistent avoiders (freezing: $M = 2.20$, $SE = 1.11$; suppression of lever pressing: $M = 7.75$, $SE = 7.61$).

Discussion

We investigated the effects of chronic restraint stress on the acquisition and extinction of 2WAA (Experiment 1) and PMA (Experiment 2) in male and female rats. In both experiments, the chronic restraint stress procedure significantly diminished weight gain in stressed rats relative to controls, without affecting sucrose preference or relative adrenal gland weights. Regarding FCM levels, in Experiment 1 we did not observe group differences, whereas in Experiment 2 we observed that stressed rats showed increased levels of FCM compared to their control counterparts at the end of the stress induction procedure.

In Experiment 1, previously stressed male and female rats showed an impairment in two-way active avoidance acquisition compared to their control counterparts. During subsequent extinction of avoidance, no differences between stressed and control rats were observed. During the next phase of the experiment, which took place in a different context and with a different warning signal, female control rats showed higher avoidance responses than their control counterparts in the last acquisition session. This effect was not observed in male rats. During subsequent extinction, females showed overall higher avoidance responding than males.

In contrast with Experiment 1, Experiment 2 only showed subtle effects of the chronic stress induction on the acquisition of avoidance. Specifically, there was an effect on the suppression

of lever pressing during acquisition of platform-mediated avoidance, but not in avoidance or freezing behavior. Surprisingly, animals in the stress condition were less likely to be classified as darters. Both males and females readily extinguished avoidance and other defensive behaviors during the extinction phase, although males did not show a significant reduction in freezing over extinction. This lack of a reduction may, however, not be very informative as freezing levels remained rather low throughout the PMA procedure.

We have replicated findings from multiple authors (Bravo et al., 2009; Dagnino-Subiabre et al., 2005; Gamaro et al., 1999; Ulloa et al., 2010), which support that chronic restraint stress impairs the acquisition of active avoidance behavior in the shuttle avoidance task. This effect has been observed despite differences in chronic restraint stress procedures. Three earlier active avoidance studies reported stress manipulation checks (Gamaro et al. (1999) did not). Dagnino-Subiabre et al. (2005) conducted restraint stress for 2 h daily for 10 days, while Ulloa et al. (2010) and Bravo et al. (2009) reported 2.5 h of daily restraint stress for 13 and 14 days, respectively. The latter studies used plexiglass tubes, presumably similar to our own restraint tubes, and Dagnino-Subiabre et al. (2005) used their own custom immobilization cages. Dagnino-Subiabre et al. (2005) and Ulloa et al. (2010) reported significantly reduced weight gain in stressed rats and an increase in adrenal gland weight in stressed rats compared to controls. Ulloa et al. (2010) examined adrenal glands two days after the last session of chronic stress induction, compared to our 15-16 days after the last stress induction session. Dagnino-Subiabre et al (2005) observed that rats euthanized more than 15 days after stress induction showed similar adrenal weights as controls, congruent with our results. Like us, Bravo et al. (2009) observed significant differences in body weight gain but no significant differences in adrenal gland weights between control and stressed rats. Unlike us, Bravo et al. (2009) also found significant differences in a sucrose preference test, with stressed rats showing a reduced preference for the sucrose solution compared to controls. Likewise, a recent meta-analysis indicates that chronic restraint stress decreases sucrose preference in male rodents (Mao et al., 2022). It should be noted that the use of the sucrose preference test as a measure of anhedonia is not uncontested, especially when confounding variables such as metabolic factors are not controlled (Berrio et al., 2024).

It is worth noting that all three studies mentioned above performed the stress induction in the morning of a standard light-dark cycle, while half of our rats were immobilized in the morning and the other half in the early afternoon. Moreover, they all used male Sprague-Dawley rats, whereas we used male and female Wistar rats. All these differences, alongside the larger sample size in Dagnino-Subiabre et al. (2005) and the longer stress induction procedures in

Ulloa et al. (2010) and Bravo et al. (2009), may partially explain why we replicated the effect of chronic restraint stress on weight gain, but not on adrenal glands or sucrose preference. The lack of pervasive effects of chronic restraint stress in the platform-mediated avoidance task is surprising. We hypothesized that chronically stressed rats would show increased avoidance in the platform-mediated avoidance task. Previous research in humans found that acute stress increased costly avoidance in healthy humans (Vogel & Schwabe, 2019) and that patients suffering from PTSD were biased towards avoidance in an approach-avoidance conflict task compared to controls (Weaver et al., 2020). However, we did not observe significant differences in the acquisition of avoidance behavior between stressed and control rats. An open question for future research is whether such differences might emerge in a variation of the platform-mediated avoidance task that involves a higher conflict, where food rewards are only available during CS presentation (H. Bravo-Rivera et al., 2021)

In sum, while the platform-mediated avoidance task and the shuttle avoidance task are both active avoidance procedures, they exhibit crucial differences. In shuttle avoidance, there is no safe area, whereas in platform-mediated avoidance the platform is always safe. Additionally, shuttling during the tone in the shuttle avoidance task typically terminates the CS tone, which is not the case in the platform-mediated avoidance task (Diehl et al., 2019). Previous research has shown that stress controllability may recruit a different neurocircuitry than safety learning (Christianson et al., 2008). Shuttle avoidance may primarily tap into rats' sense of control, without affording strong safety learning, while platform-mediated avoidance may primarily tap into safety learning structures. This divergence may provide an alternative explanation for the lack of clear effects of chronic stress in Experiment 2 compared to Experiment 1 and prior studies using the two-way active avoidance task as they may be affected differently by stressors.

Additionally, avoidance in the platform-mediated avoidance task comes at a relatively high cost compared to avoidance in shuttle avoidance tasks, as rats in the platform-mediated avoidance task are food-restricted and have to forsake food-seeking when avoiding. Prior evidence suggests that acute stress exacerbates costly avoidance in individuals with anxiety (Vogel & Schwabe, 2019), but the effects of stress on costly avoidance in rodents has not been investigated so far. Previous research with rodents has shown that chronic stress can lead to reduced exploration of the open arms in the elevated plus maze (Bondi et al., 2008; Chiba et al., 2012), which is a low-cost approach-avoidance conflict task. However, other studies have reported no significant differences between chronically stressed and control animals in the elevated plus maze (Cox et al., 2011; Mitra et al., 2005).

To conclude, our chronic restraint stress induction procedure primarily affected weight gain in both experiments. Chronic restraint stress impaired avoidance acquisition in the 2WAA but not in the PMA procedure. Extinction in both tasks was not impaired by the stress induction in any of the tasks.

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Tables

Table 1. Stress induction outcomes – Experiment 1

Measured outcome	Statistical test	Result	Effect size
Weight gain	Mixed ANOVA (Greenhouse-Geisser sphericity correction)	Group: F(1, 29): = 149.98, **p < 0.001** Day: F(3.55, 102.87) = 139.05, **p < 0.001** G*D: F(3.55, 102.87) = 73.42, **p < 0.001**	ω_p^2 = 0.83 [0.72, 1] ω_p^2 = 0.62 [0.56, 1] ω_p^2 = 0.46 [0.39, 1]
Weight gain	Simple effects by day: two-sample t-test with Bonferroni correction	Day 2 – Group: t(29) = 6.50, **p < 0.001** Day 6 – Group: t(29) = 10.74, **p < 0.001** Day 10 – Group: t(29) = 10.49, **p < 0.001**	d = 2.34 d = 3.86 d = 3.77
Sucrose preference ratio	two-way parametric ANOVA	Sex: F(1, 27) = 1.02, p = 0.321 Group: F(1,27) = 2.38, p = 0.134 S*G: F(1, 27) = 0.07, p = 0.788	ω_p^2 = 0 [0, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0 [0, 1]
Adrenal-gland (avg)-to-body-weight-ratio	Two-way parametric ANOVA	Sex: F(1, 27) = 94.63, **p < 0.001** Group: F(1, 27) = 0.81, p = 0.376 S*G: F(1, 27) = 0.11, p = 0.74	ω_p^2 = 0.75 [0.6, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Logarithm of the concentration of fecal corticosterone metabolites	Mixed ANOVA	Sex: F(1,26) = 146.53, **p < 0.001** Group: F(1, 26) = 0.16, p = 0.696 Day: F(3, 78) = 6.09, **p < 0.001** S*G: F(1, 26) = 2.03, p = 0.166 S*D: F(3, 78) = 1.42, p = 0.243 G*D: F(3, 78) = 0.44, p = 0.726 S*G*D: F(3, 78) = 0.57, p = 0.6637	ω_p^2 = 0.84 [0.73, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.09 [0, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Logarithm of the concentration of fecal corticosterone metabolites	Pairwise comparisons with Tukey correction	Day baseline-2: t(107) = -3.16, **p = 0.01** Day baseline-6: t(107) = -3.13, **p = 0.01** Day baseline-10: t(107) = -2.30, p = 0.104 Day 2-6: t(107) = 0.03, p = 1 Day 2-10: t(107) = 0.83, p = 0.841 Day 6-10: t(107) = 0.8, p = 0.853	d = -0.80 [-1.32, -0.29] d = -0.80 [-1.31, -0.28] d = -0.59 [-1.11, -0.08] d = 0.01 [-0.5, 0.51] d = 0.21 [-0.3, 0.72] d = 0.21 [-0.3, 0.72]

Table 2. Avoidance responses during acquisition and extinction in contexts A and B – Experiment 1

Test phase	Statistical test	Result	Effect size
Acquisition in context A	Non-parametric mixed ANOVA	Sex: F(1, 27) = 0.04, p = 0.846 Group: F(1, 27) = 9.79, **p < 0.01** Day: F(2, 54) = 29.31, **p < 0.001** S*G: F(1, 27) = 0.79, p = 0.382 S*D: F(2, 54) = 0.40, p = 0.67 G*D: F(2, 54) = 1.32, p = 0.277 S*G*D: F(2, 54) = 1.05, p = 0.355	ω_p^2 = 0 [0, 1] ω_p^2 = 0.23 [0.04, 1] ω_p^2 = 0.23 [0.07, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Acquisition in context A	Pairwise comparisons with tukey correction	Day 1-2: t(54) = -5.74, **p < 0.001** Day 1-3: t(54) = -7.23, **p < 0.001** Day 2-3: t(54) = -1.49, p = 0.304	r = -0.57 [-0.74, -0.35] r = -0.71 [-0.82, -0.53] r = -0.07 [-0.35, 0.21]
Acquisition in context A day 1	Mixed ANOVA	Sex: F(1, 27) = 0, p = 0.972 Group: F(1, 27) = 5.39, **p = 0.028** Block: F(2, 54) = 11.67, **p < 0.001** S*G: F(1, 27) = 1.70, p = 0.204 S*B: F(2, 54) = 3.11, p = 0.053 G*B: F(2, 54) = 1.54, p = 0.224 S*G*B: F(2, 54) = 0.14, p = 0.873	ω_p^2 = 0 [0, 1] ω_p^2 = 0.13 [0, 1] ω_p^2 = 0.13 [0.01, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 = 0.03 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Acquisition in context A day 1	Pairwise comparisons with tukey correction	Block 1-2: t(81) = -0.24, p = 0.968 Block 1-3: t(81) = -3.33, **p = 0.004** Block 2-3: t(81) = -3.10, **p = 0.008**	d = -0.06 [-0.57, 0.44] d = -0.85 [-1.37, -0.32] d = -0.78 [-1.30, -0.26]
Extinction in context A	Mixed ANOVA (Greenhouse-Geisser sphericity correction)	Sex: F(1, 27) = 2.06, p = 0.163 Group: F(1, 27) = 0.13, p = 0.719 Day: F(2.79, 75.22) = 55.67, **p < 0.001** S*G: F(1, 27) = 2.24, p = 0.146 S*D: F(2.79, 75.22) = 0.51, p = 0.664 G*D: F(2.79, 75.22) = 1.09, p = 0.355 S*G*D: F(2.79, 75.22) = 0.09, p = 0.956	ω_p^2 = 0.04 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.48 [0.36, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Extinction in context A	Pairwise comparisons with tukey correction	Day 1-2: t(135) = 4.11, **p < 0.001** Day 1-3: t(135) = 6.84, **p < 0.001** Day 1-4: t(135) = 8.35, **p < 0.001** Day 1-5: t(135) =10.56, **p < 0.001** Day 2-3: t(135) = 2.73, p = 0.055	d = 1.05 [0.53, 1.57] d = 1.74 [1.20, 2.29] d = 2.12 [1.56, 2.69] d = 2.69 [2.09, 3.28] d = 0.70 [0.19, 1.21]

		Day 2-4: t(135) = 4.23, **p < 0.001** Day 2-5: t(135) = 6.44, **p < 0.001** Day 3-4: t(135) =1.50, p = 0.562 Day 3- 5: t(135) = 3.71, **p < 0.01** Day 4- 5: t(135) = 2.21, p = 0.183	d = 1.08 [0.56, 1.60] d = 1.64 [1.10, 2.18] d = 0.38 [-0.12, 0.89] d = 0.94 [0.43, 1.46] d = 0.56 [0.05, 1.07]
Extinction in context A Day 1	Mixed ANOVA (Greenhouse-Geisser sphericity correction)	Sex: F(1, 27) = 0.20, p = 0.660 Group: F(1, 27) = 0, p = 0.991 Block: F(1.6, 43.17) = 3.40, p = 0.052 S*G: F(1, 27) = 0.85, p = 0.365 S*B: F(1.6, 43.17) = 1.13, p = 0.323 G*B: F(1.6, 43.17) = 1.15, p = 0.317 S*G*B: F(1.6, 43.17) = 0.52, p = 0.558	ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Acquisition in context B	Non-parametric mixed ANOVA	Sex: F(1, 27) = 4.48, **p = 0.044** Group: F(1, 27) = 0.12, p = 0.732 Day: F(2, 54) = 73.10, **p < 0.001** S*G: F(1, 27) = 0.07, p = 0.789 S*D: F(2, 54) = 11.13, **p < 0.001** G*D: F(2, 54) = 1.05, p = 0.356 S*G*D: F(2, 54) = 5.95, **p = 0.005**	ω_p^2 = 0 [0, 1] ω_p^2 = 0.06 [0, 1] ω_p^2 = 0.12 [0.01, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Acquisition in context B	Effects by sex (females) - non-parametric mixed ANOVA	Group: F(1, 14) = 0.22 , p = 0.647 Day: F(2, 28) = 30.74, **p < 0.001** G*D: F(2, 28) = 11.57, **p < 0.001**	ω_p^2 = 0.04 [0, 1] ω_p^2 = 0.07 [0, 1] ω_p^2 = 0.02 [0, 1]
Acquisition in context B	Simple effects by day (females): Wilcoxon rank sum test with Bonferroni correction	Day 1 – Group: W = 26.5, p = 1 Day 2 – Group: W = 33, p = 1 Day 3 – Group: W = 57.5, **p = 0.021**	r = 0.15 [0, 0.62] r = 0.03 [0, 0.6] r = 0.69 [0.4, 0.86]
Acquisition in context B	Effects by sex (males) - non-parametric mixed ANOVA	Group: F(1, 13) = 0.27, p = 0.614 Day: F(2, 26) = 45.08, **p < 0.001** G*D: F(2, 26) = 1.26, p = 0.3	ω_p^2 = 0.03 [0, 1] ω_p^2 = 0.19 [0, 1] ω_p^2 = 0 [0, 1]
Acquisition in context B	Pairwise comparisons with tukey correction (males)	Day 1-2: t(26) = -6.1, **p < 0.001** Day 1-3: t(26) = -9.42, **p < 0.001** Day 2-3: t(26) = -3.32, **p = 0.007**	r = -0.8 [-0.91, -0.6] r = -0.87 [-0.94, -0.72] r = -0.67 [-0.84, -0.37]
Acquisition in context B Day 1	Mixed ANOVA	Sex: F(1, 27) = 3.92, p = 0.058 Group: F(1, 27) = 1.83, p = 0.188 Block: F(2, 54) = 6.01, **p = 0.004** S*G: F(1, 27) = 0.13, p = 0.718	ω_p^2 = 0.09 [0, 1] ω_p^2 = 0.03 [0, 1] ω_p^2 = 0.09 [0, 1] ω_p^2 = 0 [0, 1]

		S*B: F(2, 54) = 2.58, p = 0.085 G*B: F(2, 54) = 1.83, p = 0.170 S*G*B: F(2, 54) = 0.77, p = 0.468	ω_p^2 = 0.03 [0, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 = 0 [0, 1]
Acquisition in context B Day 1	Pairwise comparisons with tukey correction	Block 1-2: t(81) = -1.73, p = 0.2 Block 1-3: t(81) = -3.21, **p = 0.005** Block 2-3: t(81) = -1.48, p = 0.305	d = -0.44 [-0.95, 0.07] d = -0.82 [-1.34, -0.3] d = -0.38 [-0.89, 0.13]
Extinction in context B	Mixed ANOVA	Sex: F(1, 27) = 7.34, **p = 0.012** Group: F(1, 27) = 1.71, p = 0.203 Day: F(4, 108) = 24.56, **p < 0.001** S*G: F(1, 27) = 0.22, p = 0.642 S*D: F(4, 108) = 0.80, p = 0.53 G*D: F(4, 108) = 0.37, p = 0.083 S*G*D: F(4, 108) = 0.33, p = 0.854	ω_p^2 = 0.18 [0.02, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 = 0.22 [0.09, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Extinction in context B	Pairwise comparisons with tukey correction	Day 1-2: t(135) = 0.97, p = 0.868 Day 1-3: t(135) =3.04, **p = 0.023** Day 1-4: t(135) = 4.44, **p < 0.001** Day 1-5: t(135) = 5.33, **p < 0.001** Day 2-3: t(135) = 2.07, p = 0.24 Day 2-4: t(135) = 3.47, **p = 0.006** Day 2-5: t(135) = 4.35, **p < 0.001** Day 3-4: t(135) = 1.4, p = 0.630 Day 3- 5: t(135) = 2.29, p = 0.156 Day 4- 5: t(135) = 0.89, p = 0.901	d = 0.25 [-0.26, 0.75] d = 0.77 [0.26, 1.28] d = 1.13 [0.61, 1.65] d = 1.36 [0.83, 1.88] d = 0.53 [0.02, 1.03] d = 0.88 [0.37, 1.4] d = 1.11 [0.59, 1.63] d = 0.36 [-0.15, 0.86] d = 0.58 [0.07, 1.09] d = 0.23 [-0.28, 0.73]
Extinction in context B Day 1	Non-parametric mixed ANOVA	Sex: F(1, 27) = 3.66, p = 0.066 Group: F(1, 27) = 1.55, p = 0.224 Block: F(2, 54) = 12.50, **p < 0.001** S*G: F(1, 27) = 0.01, p = 0.944 S*B: F(2, 54) = 3.63, **p = 0.033** G*B: F(2, 54) = 1.03, p = 0.363 S*G*B: F(2, 54) = 0.06, p = 0.945	ω_p^2 = 0.02 [0, 1] ω_p^2 = 0.05 [0, 1] ω_p^2 = 0.05 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Extinction in context B Day 1	Friedman rank sum test (female)	Block: $\chi^2(2)$ = 1.15, p = 0.562	W = 0.04 [0, 0.36]
Extinction in context B Day 1	Friedman rank sum test (male)	Block: $\chi^2(2)$ = 12.61, **p = 0.002**	W = 0.42 [0.09, 0.82]

Extinction in context B Day 1	Nemenyi's all-pairs comparisons test (males)	Block 1-2: p = 0.848 Block 1-3: **p = 0.007** Block 2-3: **p = 0.037**	r = 0.20 [-0.22, 0.55] r = 0.54 [0.18, 0.77] r = 0.46 [0.08, 0.73]

Table 3. Stress induction outcomes – Experiment 2

Measured outcome	Statistical Test	Result	Effect size
Weight change	Mixed ANOVA (Greenhouse-Geisser sphericity correction)	Group: F(1, 30) = 123.64, **p < 0.001** Day: F(2.56, 76.81) = 61.1, **p < 0.001** G*D: F(2.56, 76.81) = 62.98, **p < 0.001**	ω_p^2 = 0.79 [0.67, 1] ω_p^2 = 0.36 [0.27, 1] ω_p^2 = 0.37 [0.28, 1]
Weight change	Simple effects by day: two-sample t-test	Day 2 – Group: t(30) = 6.09, **p < 0.001** Day 6 – Group: t(30) = 10.76, **p < 0.001** Day 10 – Group: t(30) = 10.22, **p < 0.001**	d = 2.15 [1.63, 3.24] d = 3.8 [3.03, 5.15] d = 3.61 [2.1, 5.43]
Sucrose preference ratio	Two-way non-parametric ANOVA	Sex: F(1, 28) < 0.01, p = 0.943 Group: F(1, 28) = 0.01, p = 0.91 S*G: F(1, 28) < 0.01, p = 0.942	ω_p^2 = 0.03 [0, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 = 0.03 [0, 1]
Logarithm of the concentration of fecal corticosterone metabolites	Mixed ANOVA (Greenhouse-Geisser sphericity correction)	Group: F(1, 28) = 1.29, p = 0.265 Sex: F(1, 28) = 242.92, **p < 0.001** G*S: F(1, 28) = 0.03, p = 0.868 Day: F(3, 84) = 67.8, **p < 0.001** G*D: F(3, 84) = 3.15, **p = 0.029** S*D: F(3, 84) = 0.89, p = 0.45 G*S*D: F(3, 84) = 0.48, p = 0.694	ω_p^2 < 0.01 [0, 1] ω_p^2 = 0.89 [0.82, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.58 [0.47, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Logarithm of the concentration of fecal corticosterone metabolites	Simple effects by day: two-sample t-test	Baseline – Group: t(112) = 1.12, p = 0.26 Day 2 – Group: t(112) = -0.71, p = 0.479 Day 6 – Group: t(112) = -0.47, p = 0.637 Day 10 – Group: t(112) = -2.78, **p = 0.006**	d = 0.4 [-0.31, 1.1] d = -0.25 [-0.95, 0.45] d = -0.17 [-0.87, 0.53] d = -0.98 [-1.69, -0.27]
Averaged weight adrenal glands to body weight ratio	Two-way parametric ANOVA	Sex: F(1, 28) = 112.67, **p < 0.001** Group: F(1, 28) = 0.02, p = 0.900 S*G: F(1, 28) = 0.64, p = 0.432	ω_p^2 = 0.78 [0.64, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]

Table 4. Statistical test results Acquisition – Experiment 2

Measured outcome	Statistical Test	Result	Effect size
Avoidance	Mixed ANOVA	Group: F(1, 28) = 2.02, p = 0.166 Sex: F(1, 28) = 0, p = 0.984 G*S: F(1, 28) = 2.47, p = 0.128 D: F(2, 56) = 36.73, **p < 0.001** G*D: F(2, 56) = 0.31, p = 0.735 S*D: F(2, 56) = 0.76, p = 0.471 G*S*D: F(2, 56) = 1.48, p = 0.235	ω_p^2 = 0.03 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.05 [0, 1] ω_p^2 = 0.43 [0.26, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.01 [0, 1]
Avoidance	Pairwise comparisons with Tukey correction	Day 1 – 2: t(84) = -5.18, **p < 0.001** Day 1 – 3: t(84) = -8.12, **p < 0.001** Day 2 – 3: t(84) = -2.94, **p = 0.012**	d = -1.3 [-1.83, -0.76] d = -2.03 [-2.62, -1.44] d = 0.74 [-1.24, -0.22]
Freezing	Non-parametric mixed ANOVA	Day: F(2, 56) = 1.59, p = 0.212 Sex: F(1, 28) = 2.44, p = 0.129 Group: F(1, 28) = 3.26, p = 0.081 D*S: F(2, 56) = 0.05, p = 0.952 D*G: F(2, 56) = 0.08, p = 0.925 S*G: F(1, 28) = 0.03, p = 0.871 D*S*G: F(2, 56) = 0.64, p = 0.529	ω_p^2 = 0 [0, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0.05 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Suppression of lever pressing	Non-parametric mixed ANOVA	Sex: F(1, 28) = 3.28, p = 0.081 Group: F(1, 28) = 7.96, **p = 0.008** Day: F(2, 56) = 28.11, **p < 0.001** S*G: F(1, 28) = 0.39, p = 0.539 S*D: F(2, 56) = 8.51**, p < 0.001** G*D: F(2, 56) = 2.62, p = 0.082 S*G*D: F(2, 56) = 7.33, **p = 0.001**	ω_p^2 = 0.03 [0.11, 1] ω_p^2 = 0.01 [0, 1] ω_p^2 = 0.21 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.05 [0, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 = 0.05 [0, 1]
Suppression of lever pressing	Effects by sex (males) – non-parametric mixed ANOVA	Day: F(2, 28) = 37.69, **p < 0.001** Group: F(1, 14) = 14.33, **p = 0.002** D*G: F(2, 28) = 10.29**, p < 0.001**	ω_p^2 = 0.18 [0.07, 1] ω_p^2 = 0.01 [0, 1] ω_p^2 = 0.01 [0, 1]
Suppression of lever pressing	Simple effects by day (males): Wilcox rank sum test	Day 1 – Group: W = 12, **p = 0.04** Day 2 – Group: W = 43.5, p = 0.216 Day 3 – Group: W = 35.5, p = 0.732	r = -0.62 [-0.87, -0.15] r = 0.36 [-0.2, 0.74] r = 0.11 [-0.44, 0.6]
Suppression of lever pressing	Effects by sex (females) – non-parametric mixed ANOVA	Day: F(2, 28) = 8.84, **p = 0.001** Group: F(1, 14) = 1.72, p = 0.211 D*G: F(2, 28) = 2.24**,** p = 0.125	ω_p^2 = 0.08 [0.01, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.05 [0, 1]

Suppression of lever pressing	Pairwise comparisons with Tukey correction (females)	Day 1 – 2: t(28) = -4.07, **p = 0.001** Day 1 – 3: t(28) = -1.12, p = 0.821 Day 2 – 3: t(28) = 2.95, **p = 0.019**	r = -0.64 [-0.82, -0.34] r = -0.26 [-0.59, 0.14] r = 0.39 [0, 0.67]

Table 5. Statistical test results Extinction – Experiment 2

Measured outcome	Statistical Test	Result	Effect size
Avoidance	Mixed ANOVA	Group: F(1, 28) = 0.39, p = 0.539 Sex: F(1, 28) = 0.22, p = 0.643 Day: F(1, 140) = 140.88, **p < 0.001** Block: F(2, 140) = 81.77, **p < 0.001** G*S: F(1, 28) = 0.29, p = 0.593 G*D: F(1, 140) = 0.4, p = 0.528 S*D: F(1, 140) = 0.7, p = 0.403 G*B: F(2, 140) = 0.01, p = 0.988 S*B: F(2, 140) = 0.74, p = 0.48 D*B: F(2, 140) = 7.34, **p < 0.001** G*S*D: F(1, 140) = 2.64, p = 0.106 G*S*B: F(2, 140) = 0.12, p = 0.89 G*D*B: F(2, 140) = 0.49, p = 0.612 S*D*B: F(2, 140) = 0.01, p = 0.985 G*S*D*B: F(2, 140) = 0.84, p = 0.435	ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.27 [0.06, 1] ω_p^2 = 0.33 [0.16, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.02 [0, 1] ω_p^2 < 0.01 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]
Avoidance	Simple effects by block: Day 1 – Friedman test	$X^2(2)$ = 51.94, **p < 0.001**	W = 0.81 [0.66, 1]
Avoidance	Simple effects by block: Day 2 – Friedman test	$X^2(2)$ = 37.1, **p < 0.001**	W = 0.58 [0.37, 1]
Freezing	Non-parametric mixed ANOVA	Group: F(1, 28) = 0.48, p = 0.49 Sex: F(1, 28) = 0.26, p = 0.614 Day: F(1, 140) = 15.92, **p < 0.001** Block: F(2, 140) = 4.85, **p = 0.009** G*S: F(1, 28) = 0.03, p = 0.86 G*D: F(1, 140) = 0.01, p = 0.924 S*D: F(1, 140) = 6.53, **p = 0.012** G*B: F(2, 140) = 0.14, p = 0.872 S*B: F(2, 140) = 2.05, p = 0.132	ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.04 [0, 1] ω_p^2 = 0.06 [0.01, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0.01 [0, 1] ω_p^2 = 0 [0, 1] ω_p^2 = 0 [0, 1]

		D*B: F(2, 140) = 4.14, **p = 0.018** G*S*D: F(1, 140) = 0.77, p = 0.381 G*S*B: F(2, 140) = 0.41, p = 0.661 G*D*B: F(2, 140) = 0.76, p = 0.468 S*D*B: F(2, 140) = 0.92, p = 0.4 G*S*D*B: F(2, 140) = 0.16, p = 0.853	$\omega_p^2 < 0.01$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1]
Freezing	Simple effects by Day: Wilcoxon signed rank test (males)	V = 644, p = 0.08	r = 0.26 [0.03, 0.53]
Freezing	Simple effects by Day: Wilcoxon signed rank test (females)	V = 705, **p < 0.001**	r = 0.53 [0.29, 0.71]
Suppression	Mixed ANOVA	Group: F(1, 28) = 0.87, p = 0.358 Sex: F(1, 28) = 1.06, p = 0.312 G*S: F(1, 28) = 0.06, p = 0.811 Day: F(1, 28) = 26.69, **p < 0.001** G*D: F(1, 28) = 1.28, p = 0.268 S*D: F(1, 28) = 0.01, p = 0.92 G*S*D: F(1, 135) = 0.5, p = 0.486 Block: F(1.63, 45.56) = 41.17, **p < 0.001** G*B: F(1.63, 45.56) = 0.35, p = 0.661 S*B: F(1.63, 45.56) = 0.24, p = 0.745 D*B: F(1.63, 45.56) = 0.34, p = 0.672 G*S*B: F(1.54, 43.25) = 2.06, p = 0.149 G*D*B: F(1.54, 43.25) = 0.7, p = 0.466 S*D*B: F(1.54, 43.25) = 1.3, p = 0.276 G*S*D*B: F(1.54, 43.25) = 0.56, p = 0.533	$\omega_p^2 = 0$ [0, 1] $\omega_p^2 < 0.01$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0.12$ [0, 1] $\omega_p^2 < 0.01$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0.19$ [0.05, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 < 0.01$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 = 0$ [0, 1] $\omega_p^2 < 0.01$ [0, 1] $\omega_p^2 = 0$ [0, 1]