

1 Pavlovian fear conditioning does not readily occur in rats in naturalistic
2 environments

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13 **Author Contributions:**

14 P.R.Z, E.J.K., and J.J.K. conceived the study. P.R.Z., B.P.S., B.E.L., and A.S. performed surgery,
15 behavioral experiments and analyses. P.R.Z., B.P.S., E.J.K. and J.J.K. wrote the manuscript. J.J.K.
16 supervised all aspects of the study.

17

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22 Main Text

23 Figures 1 to 3

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25

26 **Abstract**

27 Pavlovian fear conditioning, which offers the advantage of simplicity in both the control of conditioned
28 and unconditioned stimuli (CS, US) presentation and the analysis of specific conditioned and
29 unconditioned responses (CR, UR) in a controlled laboratory setting, has been the standard model in
30 basic and translational fear research. Despite 100 years of experiments, the utility of fear conditioning has
31 not been trans-situationally validated in real-life contexts. We thus investigated whether fear conditioning
32 readily occurs and guides the animal's future behavior in an ecologically-relevant environment. To do so,
33 Long-Evans rats foraging for food in an open arena were presented with a tone CS paired with electric
34 shock US to their dorsal neck/body that instinctively elicited escape UR to the safe nest. On subsequent
35 test days, the tone-shock paired animals failed to exhibit fear CR to the CS. In contrast, animals that
36 encountered a realistic agent of danger (a looming artificial owl) paired with a shock, simulating a realistic
37 predatory strike, instantly fled to the nest when presented with a tone for the first time. These results
38 illustrate the survival function and precedence of a nonassociative process, rather than associative
39 conditioning, in life-threatening situations that animals are likely to encounter in nature.

40
41
42

43 **Main Text**

44 **Introduction**

45 Since the time of Watson and Morgan's (1) conception that emotions, such as fear, should be studied
46 as conditioned (acquired) reactions and Watson and Rayner's (2) demonstration that fear can be rapidly
47 learned in 9-month-old "Little Albert," Pavlovian (or classical) fear conditioning has been the paradigm par
48 excellence for studying both normal and abnormal fear behaviors (3-7). Briefly, fear conditioning focuses
49 on how an initially innocuous conditioned stimulus (CS; e.g., auditory, visual, contextual cues), upon
50 pairing with a noxious unconditioned stimulus (US; usually electric shock) that reflexively elicits
51 unconditioned response (UR; namely defensive reactions), becomes capable of eliciting conditioned
52 response (CR; e.g., freezing in rodents, increased skin conductance in humans). A century of fear
53 conditioning research has led to wide-ranging discoveries. In particular, fear conditioning experiments
54 have fundamentally transformed learning theories from the archaic contiguity (or temporal) relationship
55 (8-10) to the modern contingency (or informational) relationship between the CS and US (11-14),
56 revealed detailed neurobiological mechanisms of learning and memory (15-17) and influenced
57 contemporary cognitive behavioral therapy for various anxiety and traumatic-stressor related disorders,
58 such as panic, phobic and posttraumatic stress disorders (18-22).

59 Despite its utility and appeal, fear conditioning paradigms nonetheless simplify behavioral analyses of
60 fear, ignoring the multitude of actions and decisions that animals and humans utilize to survive the
61 breadth of risky situations in the real world (23-28). Moreover, the prevalent notion that fear conditioning
62 produces adaptive associative fear memory has yet to be ecologically validated. In fact, some
63 researchers have questioned the evolutionary logic underlying fear conditioning; "No owl hoots or whistles
64 5 seconds before pouncing on a mouse...Nor will the owl give the mouse enough trials for the necessary
65 learning to occur...What keeps animals alive in the wild is that they have very effective innate defensive
66 reactions which occur when they encounter any kind of new or sudden stimulus" (29). Indeed, laboratory
67 rodents exhibit unlearned, instinctive fear responses to advancing artificial terrestrial and aerial predators
68 (30, 31), overhead looming stimuli (32), and predator odors (33).

69 Here, we investigated for the first time whether fear conditioning readily transpires and modifies
70 subsequent behavior of animals in a naturalistic environment. To achieve this, hunger-motivated rats

71 searching for a food pellet in a large arena—a purposive behavior (34)—were presented with a discrete
72 tone CS followed by a painful US to their dorsal neck/body region by means of chronically implanted
73 subcutaneous wires (Fig. 1A). A dorsal neck/body shock better simulates real predatory strike compared
74 to footshock used in standard fear conditioning studies, as it is unlikely that predators direct their attacks
75 on small prey animal’s paws. Additionally, in nature, bodily injuries are normally inflicted by external
76 agents (namely, predators in animals and perpetrators in humans). Thus, other groups of rats were
77 presented with a looming aerial predator (i.e., a lifelike great horned owl) preceded with and without a
78 tone CS and followed by the same US (Fig. 1B-D). A single trial tone-shock, tone-owl, tone/owl-shock and
79 owl-shock training was employed because multiple bodily harm encounters would prove fatal in nature,
80 antithetical to the natural selection of fear conditioning (29). Later, all animals’ reactions to the tone cue
81 were examined while foraging for food in the open arena.

82 **Results**

84 Baseline foraging

85 Female and male rats were pseudo-randomly assigned to tone-shock (8 females, 8 males), owl-
86 shock (8 females, 8 males), tone/owl-shock (6 females, 8 males), and tone-owl (4 females, 4 males)
87 groups and implanted with subcutaneous wires in their dorsal neck/body (Fig. 1A-C). After recovery
88 from the surgery, the rats were trained to exit a nest compartment upon gate opening to procure a
89 sizable 0.5 g food pellet placed at variable distances in a large, expanding open arena (Fig. 1D, top
90 panel). On the first baseline day, female rats took a significantly longer amount of time to procure the
91 food pellet compared to male rats (*Supplementary materials*, Fig S1, Baseline day 1). This initial
92 difference in foraging behavior likely represents heightened spatial neophobia (risk-averse to novel
93 environments) in female rats. As rats became familiar with the foraging arena, the latency and
94 duration measures declined across 5 baseline days comparably in both sexes, with no further
95 statistical differences in latencies for pellet procurement. Because there were no reliable sex
96 differences in subsequent fear conditioning dependent variables (*Supplementary materials*, Fig. S2),
97 the four groups were collapsed across sexes.

98

99 Fear conditioning

100 On the training day, all rats first underwent three foraging trials with pellets fixed at the longest
101 distance (125 cm) to confirm comparable pre-fear conditioning foraging behavior between groups (Fig.
102 2A, Baseline). Afterwards, animals were exposed to a tone-shock, an owl-shock, a tone/owl-shock or a
103 tone-owl pairing in the manner shown in Fig. 1 (*Supplementary materials*, Movie S1). Those rats
104 presented with the tone CS 5-sec prior to the gate opening (i.e., tone-shock, tone-owl, tone/owl-shock
105 groups) took more time to enter the foraging arena in comparisons to owl-shock animals unexposed to
106 the tone (Fig. 2B, Leave nest latency); this indicates that the tone was a salient cue that animals were
107 attentive to and thus conditionable. Once in the foraging arena, all animals readily advanced toward the
108 pellet and breached the trigger zone (25 cm from the pellet) to activate the shock, owl, or owl-shock
109 stimuli (Fig. 2B, Trigger zone latency). In response to the shock, owl, or owl-shock, all rats promptly fled
110 from the foraging arena to the nest (Fig. 2B, Escape latency; Fig. 2D,E, Escape speed). Figure 2C shows
111 representative track plot examples of tone-shock, owl-shock, tone/owl-shock and tone-owl animals
112 successfully procuring the pellet during pre-tone baseline but not during tone conditioning. The fact that
113 the escape latency and running speed were not significantly different between the tone-owl and other
114 groups indicates that the looming owl-induced innate fear sans pain was just as effective in eliciting the
115 flight UR as the painful shock or shock-owl combination. However, inspections of the escape trajectories
116 revealed that the tone-shock and tone-owl groups tended to flee linearly to the nest, whereas the owl-
117 shock and tone/owl-shock groups that experienced a dorsal neck/body shock 100 ms after the looming
118 owl (mimicking realistic predatory attack) and begun their flight to the nest inclined to escape circuitously
119 (Fig. 2F,H). This was supported by significant group differences in the escape distances (Fig. 2G) and
120 trajectory angles (Fig. 2I), where owl-shock and tone/owl-shock groups traveled longer distances and had
121 higher angle variances, respectively, during their escape routes than tone-shock and tone-owl groups.

122
123 Context (pre-tone) testing

124 On the following day, animals were placed back in the nest and underwent three pre-tone baseline
125 trials (maximum 300 sec to retrieve the food pellet placed at 125 cm) to assess whether previous
126 encounters with tone-shock, owl-shock, tone/owl-shock and tone-owl stimuli combinations produced fear

127 of the arena. As can be seen in Figure 3A, the owl-shock and tone/owl-shock groups took significantly
128 longer latencies to procure the pellet (i.e., the time from gate opening-to-return to nest with the pellet)
129 than the tone-shock and tone-owl groups on the first day of testing. The lengthened times to enter the
130 foraging arena exhibited by owl-shock and tone/owl-shock rats likely reflect inhibitory avoidance resulting
131 from the previous predatory attack experience in the arena (35). In contrast, the fact that the pre-tone test
132 baseline latencies of tone-shock and tone-owl rats (*Supplementary materials*, Fig. S3) were not reliably
133 different from their baseline latencies from the fear conditioning day (prior to experiencing tone-shock or
134 tone-owl) suggests that contextual fear conditioning failed to transpire in these animals despite their
135 robust escape behavior to tone-shock and tone-owl experiences. Similar patterns of group differences,
136 albeit lesser magnitudes, were observed on the second day of pre-tone baseline trials (Fig. 3C).

137
138 Tone testing

139 Immediately after the pre-tone baseline, all groups were subjected to three successive tone test trials
140 (one minute apart). The owl-shock and tone/owl-shock animals continued to take longer latencies to exit
141 the nest compared to tone-shock and tone-owl animals (Fig. 3B, Leave nest latency). Once in the
142 foraging arena, the tone/owl-shock group's latency to approach 25 cm from the pellet to trigger the tone
143 were marginally but reliably longer than those of tone-shock and tone-owl groups, but not owl-shock
144 group (Fig. 3B, Trigger zone latency). Upon the activation of tone (60 s continuous), the majority of owl-
145 shock and tone/owl-shock animals promptly fled to the nest, thereby significantly increasing the latency to
146 procure the pellet (60 s = unsuccessful), whereas the tone-shock and tone-owl animals were largely
147 unaffected by the tone and readily procured the pellet (Fig. 3B, Procure pellet latency). The second day of
148 tone testing yielded similar patterns of group differences (Fig. 3D). Figure 3E shows individual track plots
149 from all animals with the initial number of trial(s) necessitated for successful foraging. Further analyses
150 across tone testing days (3 trials/day) showed that the overall success rates of procuring the pellet were
151 significantly lower in owl-shock and tone/owl-shock groups compared to tone-shock and tone-owl groups
152 (Fig. 3F), and that owl-shock and tone/owl-shock animals required extended trials to reliably obtain the
153 pellet (Fig. 3G). Because the temporal interval between the CS and US is well known to be crucial in
154 various types of Pavlovian conditioning, including fear conditioning (36), we examined whether tone fear

155 conditioning transpired in a specific (optimal) range of interstimulus intervals (ISI) but was masked by
156 non-optimal ISIs. We found no significant correlation between the ISIs and the magnitudes of tone-
157 induced suppression of pellet procurement in tone-shock animals, indicating that tone fear conditioning
158 failed to materialize across varying ISIs of delay conditioning (Fig. 3H). Conversely, in the tone/owl-shock
159 animals, the tone-induced suppression of pellet procurement was uniformly observed across different
160 ISIs, suggesting that the observed fear in these animals may not necessarily reflect Pavlovian
161 conditioning (Fig 3H). These results of delayed tone-shock paired animals failing to show conditioned
162 tone fear and contextual fear suggest that standard fear conditioning does not readily occur in naturalistic
163 environment. Instead, the finding of owl-shock animals displaying robust fear to a novel tone, which the
164 animals never heard before, suggests that non-associative sensitization-like processes play a crucial role
165 in protecting animals in the real world.

166 **Discussion**

168 It is generally believed (though never validated) that there is behavioral continuity of Pavlovian fear
169 conditioning from the laboratory to real-life situations, and thus understanding the mechanisms of fear
170 conditioning will have clinical relevance. The present study directly investigated whether fear conditioning
171 readily occurs in naturalistic situations that animals are likely to encounter in their habitats. Standard fear
172 conditioning in rodents takes place in small experimental chambers, and several studies have shown that
173 a single tone CS-footshock US pairing (i.e., delay fear conditioning) reliably produces conditioned
174 freezing in rats and conditioned tachycardia/freezing in mice (37). One-trial delay tone fear conditioning
175 has also been demonstrated in human subjects using a loud white noise US and assessing conditioned
176 skin conductance response (38). However, in the present study, where rats are exhibiting a purposive
177 foraging behavior (34) in a large arena, a delayed pairing of tone CS and dorsal neck/body shock US
178 (tone-shock group) produced virtually no evidence of auditory (and contextual) fear conditioning across a
179 range of CS durations (i.e., ISIs). A similar pairing of tone CS and looming owl (tone-owl group) also
180 failed to produce auditory fear conditioning despite the owl US evoking robust fleeing UR. In contrast,
181 foraging rats that experienced a looming owl and shock pairing (owl-shock group) later exhibited robust
182 fear (escape) behavior to a novel tone presentation. In the tone/owl-shock animals, the escape behavior

183 was uniformly observed across different ISIs, suggesting that the observed fear to the tone stimulus
184 in this group may not be a Pavlovian response. These findings then point to a nonassociative
185 sensitization (or sensitization-like) process, rather than associative fear conditioning, as playing a vital
186 function in risky (i.e., predatory attack) situations that animals encounter in nature.

187 The tone CS (3 kHz, 80 dB, ranging 9-86.6 s) and shock US (2.5 mA, 1 s) employed in the present
188 study were effective in eliciting orienting and fleeing responses, respectively, and were presented to
189 animals in the manner (i.e., a delay conditioning) that satisfied the stimuli saliency, intensity, surprising,
190 and temporal contiguity requirements for conditioning (39-41). Then, what can account for one-trial
191 auditory fear conditioning, demonstrated in standard Pavlovian paradigms (35, 37, 38, 42), not emerging
192 in animals that left the safe nest to forage for food in an open arena? It may well be that rats are not
193 biologically predisposed to associate discrete CS and US in natural (complex) environments where
194 competing hunger-driven and fear-driven motivated behaviors are freely expressed. Indeed, in real-life,
195 only a small minority of people experiencing trauma develop posttraumatic stress disorder (PTSD) and
196 even with re-exposure to the same trauma there is low incidence PTSD (43, 44). In contrast, standard
197 experimental chambers may be conducive to fear conditioning because they are simple and limit the
198 repertoire of behaviors. The absence of one-trial fear conditioning in a naturalistic setting may be
199 analogous to “The Rat Park Experiment,” where rats housed in an enriched environment with plants,
200 trees and social interaction resist drug addiction behavior evident in standard cage-housed rats (45, 46).
201 Animals tested in naturalistic paradigms are given choices that do not force their behaviors into
202 dichotomies (i.e., freezing or no freezing; drug craving or no drug craving). Allowing for an expanded
203 behavioral repertoire, while more difficult to study, may thus yield a greater understanding of behaviors
204 and their underlying brain mechanisms.

205 It should also be noted that fear encounters in real life generally occur in the presence of external
206 agents or forms (i.e., predators/conspicuous in animals and assailants/combatants in humans), which is
207 virtually nonexistent in standard Pavlovian fear conditioning paradigms. Thus, the effects of a discernable
208 entity in associative fear learning have never been investigated. By simulating a realistic life-threatening
209 situation, i.e., a looming aerial predator that instinctively elicited flight behavior followed by somatic pain,
210 we found that rats engaged in purposive behavior utilize nonassociative sensitization as their primary

211 defensive mechanism. The fact that the owl-shock and tone/owl-shock animals exhibited relatively
212 nonlinear, erratic escape trajectories to the nest compared to linear escape trajectories in tone-shock
213 animals (Fig. 2F-I) suggests the intriguing possibility that the same dorsal neck/body shock US may be
214 interpreted as a life-or-death (panic) situation in the presence of an external threat agent versus a mere
215 startling (nociceptive) situation in the absence of an external threat agent. The erratic flight behavior in the
216 presence of a looming owl may represent the penultimate stage of circa-strike, or “life-or-death,” behavior
217 within the “predatory imminence continuum” theory (47). Functionally, a sensitized fear system may
218 intensify avoidance behavior, which in turn effectively transposes novel, neutral cues into “false positives”
219 to prioritize survival in natural environment (29). In other words, nonspecific sensitization-based
220 overestimation of danger may be a more prudent course for survival than relatively more specific
221 association-based prediction of danger.

222 Clark Hull (48) has posited that Pavlovian fear conditioning offers biological utility by circumventing a
223 “bad biological economy” of defense reaction always necessitating injury. This prevailing view that
224 ascribes preeminent importance of fear conditioning as the primary defensive mechanism is likely to be a
225 theoretical simplification and provides an incomplete picture of fear, as its function in a natural
226 environment may be rather limited (i.e., lacks face validity). It may well be possible to produce fear
227 conditioning in naturalistic settings with further CS-US trials but then this too would be a bad biological
228 economy as such learning will dramatically reduce biological fitness. It is also important to recognize
229 inconsistencies in the literatures, such as clinical studies that have reported that patients with anxiety
230 disorders, such as phobias, have trouble recalling the particular pairing of the fear event with its aversive
231 consequences (49, 50). The increased utilization of naturalistic fear paradigms that simulate dangers that
232 animals and humans encounter in real life will enable us to clarify, update, and revise fear concepts
233 derived largely from fear conditioning studies and in doing so facilitate future progress in the treatment of
234 fear disorders.

235

236

237

238 **Materials and Methods**

239

240 Subjects

241 Sixty-two Long-Evans rats (3-4 months old; 32 females and 30 males), purchased from Charles-
242 Rivers Laboratories, were initially pair-housed by sex for 5-7 days of acclimatization in a climate-
243 controlled vivarium (accredited by the Association for Assessment and Accreditation of Laboratory
244 Animal Care), with a reversed 12-h light/dark cycle (lights on at 7 PM). After undergoing
245 subcutaneous wire implant surgery (described below), animals were individually housed and placed
246 on a standard food-deprivation schedule with *ad lib* access to water to gradually reach and maintain
247 ~85% normal body weight. All experiments were performed during the dark phase of the cycle in strict
248 compliance with the University of Washington Institutional Animal Care and Use Committee
249 guidelines.

250

251 Surgery

252 Under isoflurane anesthesia, rats were mounted on a stereotaxic instrument (Kopf), and two Teflon-
253 coated stainless-steel wires (0.0003 inch bare, 0.0045 inch coated; A-M Systems, Everett, WA) were
254 inserted in the dorsal neck/back region of body. The wire tips were exposed (~1 cm), bent to a V-shape,
255 and hooked to subcutaneous tissue (36). The other ends of the wires were affixed to a headstage
256 (Plastics One, MS303-120), which was then cemented to the animal's skull embedded with 6 anchoring
257 screws. While still under anesthesia, animals were connected to a shock-apparatus and given a mild
258 shock to observe muscle twitching; 6 rats that showed no reaction to shock were removed from the
259 experiment. Animals were given 4 days of postoperative recovery and were adapted to handling for 5
260 days before nest habituation.

261

262 Foraging Apparatus and Stimuli

263 A custom-built foraging arena consisted of a nest (69 cm length x 58-66 cm width x 61 cm height)
264 that opened via an automated sliding gate to reveal a large, expanded foraging area (208 cm length x
265 66-120 cm width x 61 cm height) where 0.5 g food pellets (grain-based; F0171, Bio-Serv) were
266 placed at variable locations (Fig. 1A). The testing room was kept under red light (11 lux foraging area,
267 2 lux nest area) with constant white noise (72 dB) playing in the background. Prior to placing each

268 animal, the arena was wiped with 70% ethanol. The ANY-maze software and Ami interface system
269 (Stoelting) connected to a PC automatically tracked the animal's position in the arena, via a ceiling
270 mounted camera, and triggered the tone, shock and aerial predator stimuli: (i) 3 kHz, 80 dB tone CS was
271 produced using Anymaze (Stoelting) and presented through two speakers mounted on the nest-foraging
272 border; (ii) 1 s, 2.5 mA shock US was delivered to the animal's dorsal neck/back region via a headstage
273 tethered to a stimulus-isolator (Bak); (iii) A life-like model owl (31), mounted onto a 92 cm pneumatic air
274 cylinder (Bimba) at the opposite end of the foraging arena and hidden behind a black curtain, plunged
275 downward towards the rat (46 cm/s), then retracted back to its starting position.

276
277 Behavioral Procedure

278 Upon reaching and maintaining 85% normal body weight, animals were transported to the
279 experimental room and underwent series of habituation, baseline, fear conditioning, and testing sessions.

280 (*Habituation days*) Animals were placed in the nest scattered with 20 food pellets (0.5 g, grain-based,
281 Bio-Serv) for 30 min/day for 2 consecutive days to acclimatize and associate the nest with food
282 consumption.

283 (*Baseline days*) After 1 minute in the nest sans food pellets, the gate opened, and the animal was
284 allowed to explore the large foraging arena and find a pellet placed 25 cm away from the nest (first trial).
285 As soon as the animal took the sizeable 0.5 g pellet back to the nest, the gate closed. Once the animal
286 finished eating, the second trial with the pellet placed 50 cm and then the third trial with the pellet placed
287 75 cm commenced in the same manner. Animals underwent 3-5 consecutive baseline days, with the
288 pellet distances gradually extending to 75, 100 and 125 cm, and they were also accustomed to tethering
289 beginning on baseline day 3 onward.

290 (*Fear conditioning day*) Rats, pseudo-randomly assigned into tone-shock, tone-owl, tone/owl-shock
291 and owl-shock groups (Fig. 1), underwent 3 baseline trials with the pellet placed at 125 cm from the nest.
292 On the 4th trial, the tone-shock, tone-owl and tone/owl-shock animals were exposed to a tone CS that
293 came on 5 seconds before the gate opened and remained on until they reached the trigger zone (25 cm
294 to the pellet). For tone-shock and tone-owl animals, the tone co-terminated with the shock US and the owl
295 looming, respectively. For tone/owl-shock animals, the shock occurred 0.1s sec after the owl looming and

296 co-terminated with the tone. Two animals in the tone/owl-shock group were excluded because they
297 failed to leave the nest within 2 min. The owl-shock animals were subjected to the same owl looming-
298 shock pairing (as the tone/owl-shock animals) but in the absence of tone. All rats fled to the nest in
299 reaction to the shock and/or looming owl, at which time the gate was closed. After 1 minute in the
300 nest, the animals were placed back into their homecage.

301 (*Testing days*) All rats underwent 3 baseline trials (a maximum of 300 sec to retrieve the pellet) to
302 assess whether shock and/or looming owl encounter the previous day resulted in the fear of the
303 arena (i.e., contextual fear). Afterwards, animals were presented with the tone cue when they
304 approached the trigger zone (25 cm to the pellet). The tone played continuously for 60 sec, after
305 which the tone test trial ended. Animals underwent 3 tone tests daily until they successfully attained
306 the pellet (i.e., fear extinction).

307 Data Analyses

309 Statistical analyses were performed using SPSS (IBM, version 19) and R (The R Foundation,
310 version 3.5.3). Body tracking positions were obtained using Deep Lab Cut (51) and analyzed using a
311 self-written script in Python (Python Software Foundation). Animal sample sizes were determined
312 using a power analysis performed by G*Power (G*Power, version 3.0.1, Franz Faul; power=0.95,
313 alpha=0.05, effect size=0.5, two-tailed). A Levene's test for normality showed significance for the
314 data, thus nonparametric tests were used for analysis. Because there were no significant sex
315 differences in any stages of the experiment after the first day of baseline (*Supplementary materials*,
316 Fig. S1), data from females and males were pooled together for all analyses (*Supplementary*
317 *materials*, Fig. S2). Statistical significance was set at $P < 0.05$. Graphs were made using GraphPad
318 Prism (version 8).

319 Data Availability

320 The data that support the findings of this study and the relevant analysis code are available from the
321 Dryad data repository. <https://doi.org/10.5061/dryad.76hdr7sxx> Reviewer Link:
322 https://datadryad.org/stash/share/00_D25HmXortJJoB9bMz5YMUvKOM09RLtEv-TOR2sRc

323

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328 **Disclosures**

329 All authors report no biomedical financial interests or potential conflicts of interests.

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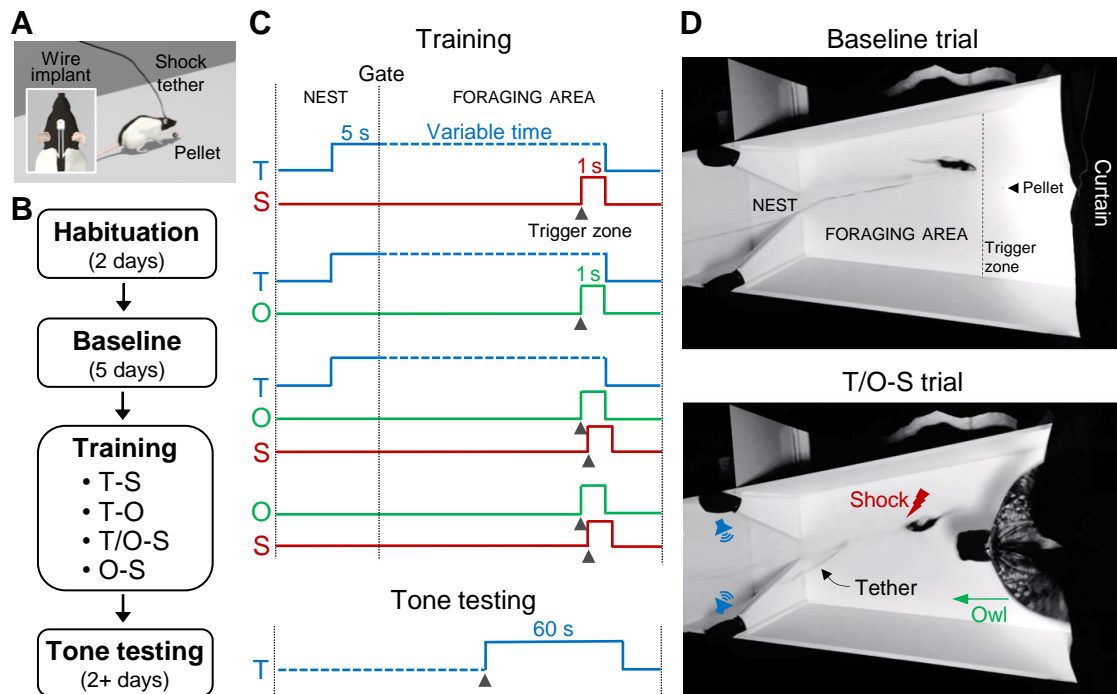
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431 **Figures and Tables**



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Fig. 1 Experimental design of fear conditioning in a naturalistic setting. (A) An illustration of a

435 tethered rat foraging for a food pellet in the open arena (inset shows a headstage and placement of

436 subcutaneous shock wires). **(B)** Timeline of experiment. *Habituation*: Rats were placed in a closed nest

437 with dispersed food pellets for 30 min/day. *Baseline*: Rats were allowed to leave the nest to discover food

438 pellets placed 25-125 cm (in 25 cm increments from the nest) in the foraging arena. *Training*: Animals

439 approaching the pellet location experienced a delayed pairing of tone-shock (T-S), tone-owl (T-O),

440 tone/owl-shock (T/O-S), or owl-shock (O-S). *Tone Test*: On subsequent days, all rats were placed back in

441 the foraging arena and upon nearing the food pellet, the tone was activated. **(C)** Schemas of delayed

442 pairings of stimuli. The T-S, T-O and T/O-S (but not O-S) groups were presented with a tone 5 s before

443 the gate opening that stayed on until the animals were within 25 cm of the food pellet, at which the tone

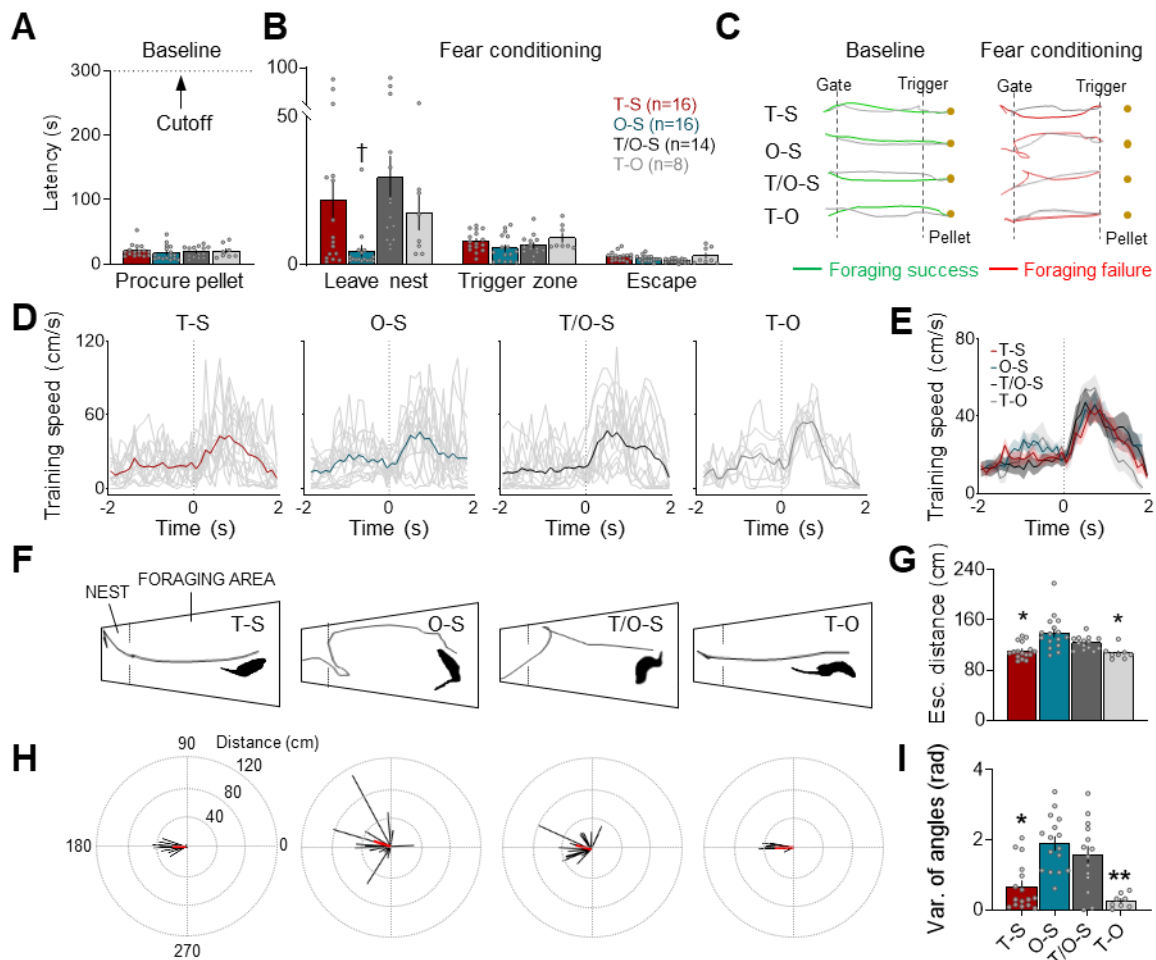
444 co-terminated with the triggered shock (1 s), owl (1 s) or owl-shock (100 ms interstimulus interval, ISI)

445 stimuli. **(D)** A representative rat in the foraging arena (208 cm length x 66-120 cm expanding width x 61

446 cm height) during a *baseline trial*, where the animal successfully acquires the pellet, and during a *T/O-S*

447 trial, where the animal flees from looming owl and shock into the nest (69 cm length x 58-66 cm width x
448 61 cm height).

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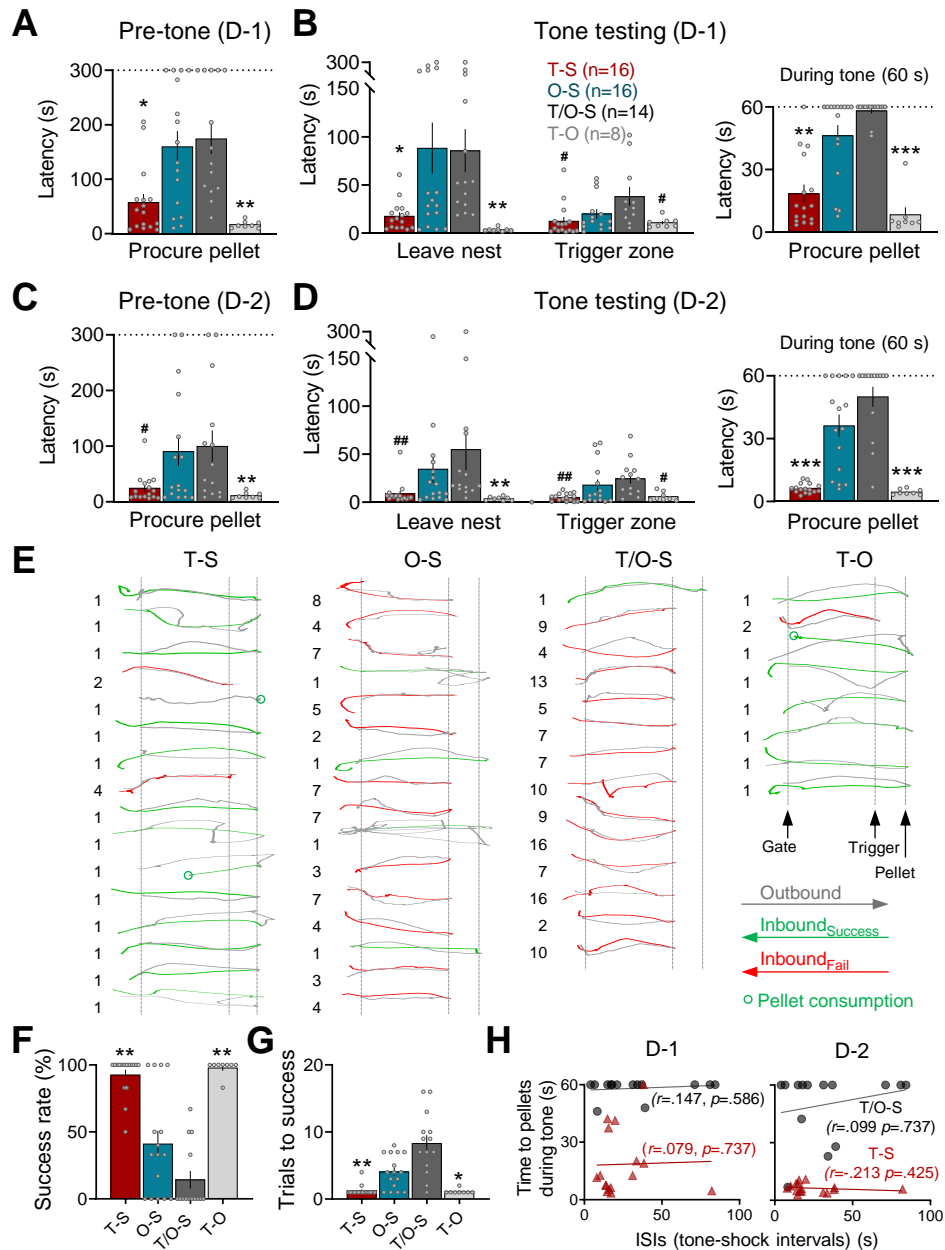
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Fig. 2 Foraging and escape behaviors during fear conditioning. (A) Pre-conditioning baseline

468 latencies (mean \pm SEM) to procure food pellets in the foraging arena were equivalent between T-S (red),
469 O-S (blue), T/O-S (dark gray) and T-O (light gray) groups (Kruskal-Wallis, $H = 2.694$, $p = 0.441$). **(B)**
470 During fear conditioning, the T-S, T/O-S and T-O groups exposed to the tone 5 s before the gate opening
471 had significantly longer latencies to leave the nest than the O-S group (left panel, Kruskal-Wallis, $H =$
472 18.6 , $p < 0.001$; pairwise comparisons, $p = 0.008$ for T-S vs. O-S, $p = 0.011$ for O-S vs. T-O, $p < 0.001$ for
473 O-S vs. T/O-S, $p = 0.69$ for T-S vs. T-O, $p = 0.631$ for T-S vs. T/O-S, $p = 0.343$ for T/O-S vs. T-O). Once
474 outside the nest, however, the latency to breach the trigger zone, enroute to the pellet, was not reliably
475 different among the groups (Kruskal-Wallis, $H = 7.453$, $p = 0.059$). In response to the triggered shock, owl
476 or owl-shock, all groups showed similar escape-to-nest latencies (Kruskal-Wallis, $H = 6.141$, $p = 0.105$).
477 **(C)** Representative track plot examples from T-S, O-S, T/O-S and T-O animals during the baseline, when

478 animals successfully procured the pellet, and during the fear conditioning, when the same animals fled
479 from shock, owl or owl-shock stimuli and thus unable to attain the pellet. **(D)** Mean instantaneous speed
480 (\pm SEM) of each group 2 sec before and after the shock, owl or owl-shock onset ($t = 0$). Thin, grey lines
481 represent individual animal data. **(E)** All groups showed comparable escape speed to the shock, owl, and
482 owl-shock stimuli (Kruskal-Wallis, $H = 0.901$, $p = 0.825$). **(F)** Representative track plots showing escape
483 paths of T-S, O-S, T/O-S and T-O animals. The inset silhouette images show that the T-S and T-O
484 animals were facing forward at the time of the shock or owl stimulus whereas the O-S and T/O-S animals
485 were turning back at the time of the shock stimulus because of the 100 ms owl-shock interstimulus
486 interval. **(G)** Mean escape distance (\pm SEM) from the trigger zone to the nest. The O-S and T/O-S groups
487 travelled longer distances to escape compared to the T-S and T-O groups (Kruskal-Wallis, $H = 21.98$, $p <$
488 0.001 ; pairwise comparisons, $p = 0.014$ for T-S vs. T/O-S, $p = 0.008$ for T/O-S vs T-O, $p = 0.001$ for T-S
489 vs. O-S, $p = 0.001$ for O-S vs T-O). **(H)** Representative vector plots of each group showing variabilities in
490 their escape paths. **(I)** Mean variance (\pm SEM) of escape trajectory angles (radian) from the trigger zone
491 to the nest. The O-S and T/O-S groups had greater variance in their escape trajectories when fleeing
492 back to the nest (Kruskal-Wallis, $H = 22.37$, $p < 0.001$; pairwise comparisons, $p = 0.022$ for T-S vs. T/O-S,
493 $p = 0.003$ for T/O-S vs T-O, $p = 0.002$ for T-S vs. O-S, $p < 0.001$ for O-S vs T-O). (\dagger compared to T-S,
494 T/O-S, and T-O; * compared to O-S and T/O-S, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # compared to T/O-S,
495 $p < 0.05$, ## $p < 0.01$).

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501 **Fig. 3 Foraging and escape behaviors during tone testing.** (A) The mean latency (\pm SEM) to procure
502 the pellet during the pre-tone baseline trials on testing day 1 (D-1). Both O-S and T/O-S groups took
503 significantly longer times to exit (gate opening, $t=0$) and return to the nest with the pellet than T-S and T-O
504 groups (Kruskal-Wallis, $H = 20.518$, $p < 0.001$; pairwise comparisons, $P = 0.003$ for T-S vs. T/O-S, $p <$
505 0.001 for T/O-S vs. T-O, $p = 0.013$ for T-S vs. O-S, $p < 0.001$ for O-S vs. T-O). (B) The times (mean \pm
506 SEM) to leave nest and reach trigger zone on day 1 tone test trials. Both O-S and T/O-S groups had

507 longer latencies to leave nest (Kruskal-Wallis, $H = 27.071$, $p < 0.001$; pairwise comparisons, $p = 0.003$ for
508 T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.044$ for T-S vs. O-S, $p < 0.001$ for O-S vs. T-O. Once
509 outside the nest, the T/O-S group took longer time to reach the trigger zone than the T-S and T-O
510 (Kruskal-Wallis, $H = 9.153$, $p = 0.027$; pairwise comparisons, $p = 0.019$ for T-S vs. T/O-S, $p = 0.042$ for
511 T/O-S vs. T-O). During the tone test, the latencies to procure the pellet within the 60 s allotted time were
512 significantly longer in O-S and T/O-S animals compared to T-S and T-O animals (Kruskal-Wallis, $H =$
513 34.428 , $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p =$
514 0.002 for T-S vs. O-S, $p < 0.001$ for O-S vs. T-O). **(C)** The mean latency (\pm SEM) to procure the pellet
515 during the pre-tone baseline trials on testing day 2 (D-2). O-S and T/O-S groups continued to have longer
516 latencies to exit (gate opening, $t=0$) and return to the nest with the pellet than T-S and T-O groups
517 (Kruskal-Wallis, $H = 12.47$, $p = 0.006$; pairwise comparisons, $p = 0.022$ for T-S vs. T/O-S, $p = 0.002$ for
518 T/O-S vs. T-O, $P = 0.009$ for O-S vs. T-O). **(D)** The times (mean \pm SEM) to leave nest and reach trigger
519 zone on day 2 tone test trials. There were group differences in the latencies to leave nest (Kruskal-Wallis,
520 $H = 21.505$, $p < 0.001$; pairwise comparisons, $p = 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, p
521 $= 0.002$ for O-S vs. T-O). Once outside the nest, there were group differences in the latencies to reach
522 the trigger zone (Kruskal-Wallis, $H = 21.531$, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-
523 S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.037$ for O-S vs. T-O). During the tone test, the latencies to procure
524 the pellet within the 60 s allotted time were significantly longer in O-S and T/O-S animals compared to T-S
525 and T-O animals (Kruskal-Wallis, $H = 37.223$, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-
526 S, $p < 0.001$ for T/O-S vs. T-O, $p < 0.001$ for T-S vs. O-S, $p < 0.001$ for O-S vs. T-O). **(E)** Individual track
527 plots during the first tone exposure from all animals from each group. The parenthesized numbers next to
528 plots represent the trial(s) needed for successful foraging. **(F)** The overall success rates of procuring the
529 pellet on the first testing day were significantly lower in the O-S and T/O-S groups compared to the T-S
530 and T-O groups (Kruskal-Wallis, $H = 32.299$, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-
531 S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.001$ for T-S vs. O-S, $p = 0.003$ for O-S vs. T-O). **(G)** The O-S and
532 T/O-S animals required extended trials to obtain the pellet (Kruskal-Wallis, $H = 32.004$, $p < 0.001$;
533 pairwise comparisons, $p < 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.002$ for T-S vs. O-
534 S, $p = 0.011$ for O-S vs. T-O). **(H)** In T-S and T/O-S animals, there were no reliable correlations

535 (Spearman's correlation coefficient) between the tone-induced suppression of pellet procurement (an
536 index of fear) and the temporal intervals (i.e., ISIs) between tone CS onset and shock US onset in neither
537 testing day 1 nor 2. (* compared to both O-S and T/O-S, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # compared
538 to T/O-S, $p < 0.05$, $p < 0.01$).

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555 **Supplementary Information for**
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557 Pavlovian fear conditioning does not readily occur in rats in naturalistic
558 environments

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571 **This PDF file includes:**

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Figures S1 to S3

574 Legends for Movies S1 to S2
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577 **Other supplementary materials for this manuscript include the following:**

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Movies S1 to S2

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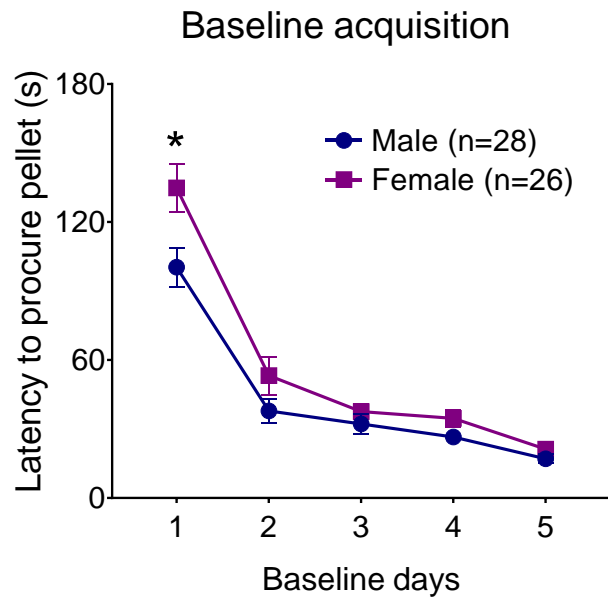


Fig. S1. Initial sex differences in the baseline latency to procure pellets. Average latencies (\pm SEM)

to procure food pellets in foraging area. Females had longer latencies to procure pellets than males during the first baseline session day 1 (Mann-Whitney U, $z = 2.476$, $p = 0.013$) but not subsequent baseline session days 2-5 (Mann-Whitney U, Baseline 2: $z = 1.039$, $p = 0.299$; Baseline 3: $z = 1.922$, $p = 0.055$; Baseline 4: $z = 1.112$, $p = 0.266$; Baseline 5: $z = 1.904$, $p = 0.057$). * $p < 0.05$.

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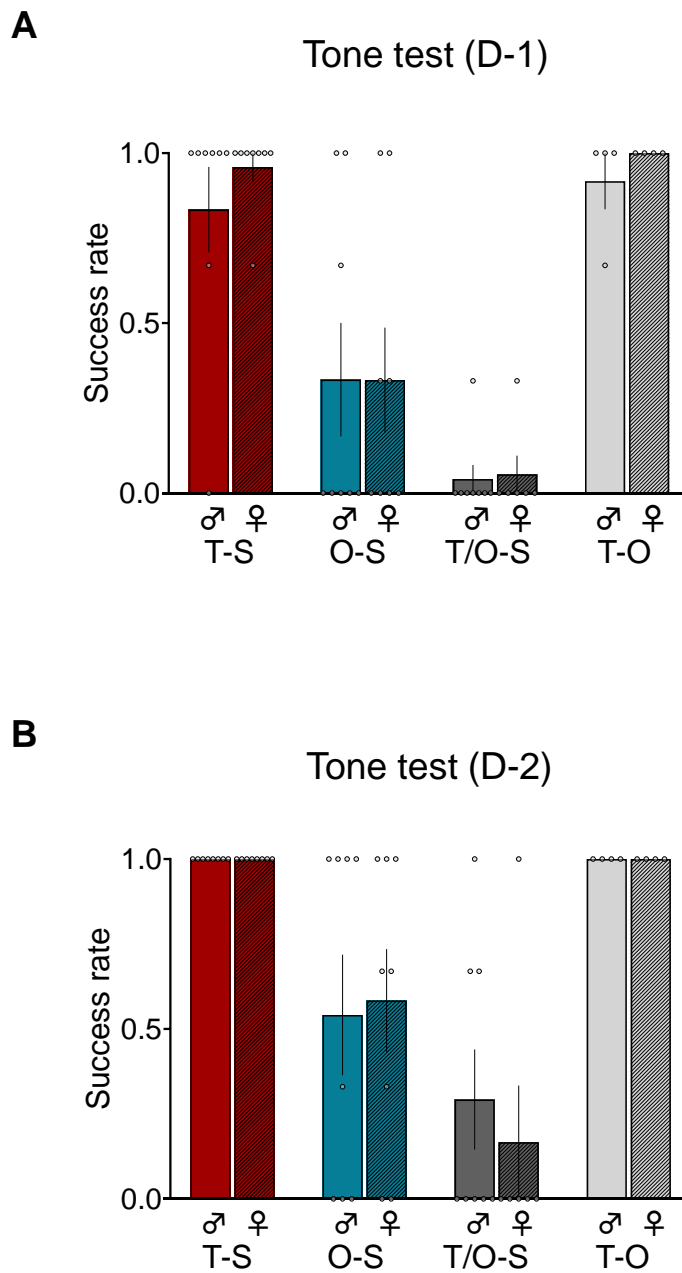


Fig. S2. No reliable sex differences in the procurement of pellets during tone tests. (A) Mean (\pm SEM) success rate for procuring food pellets during the first day of tone testing. No significant differences were found between males and females in tone-shock (T-S), owl-shock (O-S), tone/owl-shock (T/O-S) and tone-owl (T-O) groups (Mann-Whitney U; $z = 0.694$, $p = 0.645$ for T-S; $z = 1.0$, $p = 0.317$ for O-S; $z = 0.212$, $p = 1.0$ for T/O-S; $z = 0.234$, $p = 0.815$ for T-O). **(B)** Mean (\pm SEM) success rate for procuring food

690 pellets during the second day of tone testing. No sex differences were observed in all groups (Mann-
691 Whitney; $z = 0$, $p = 1.0$ for T-S; $z = 0.056$, $p = 0.955$ for O-S; $z = -0.649$, $p = 0.662$ for T/O-S; $z = 0$, $p =$
692 1.0 for T-O).

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Pre-tone baselines

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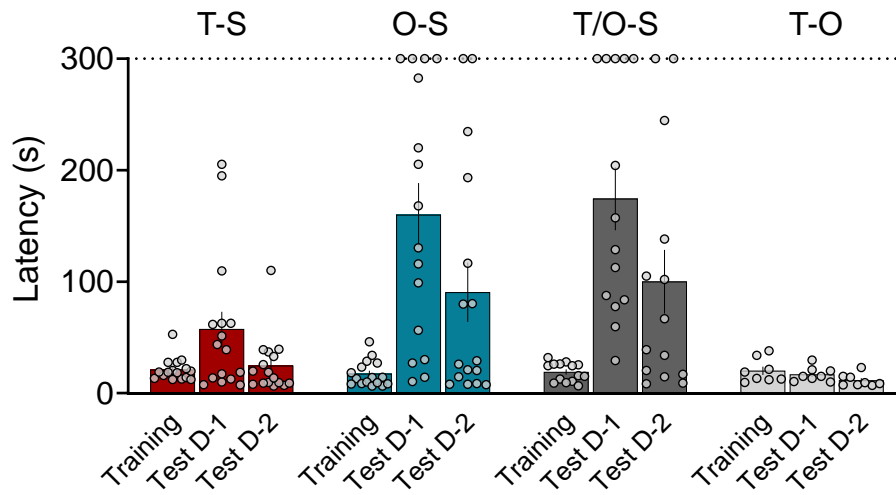
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Fig. S3. Comparisons of latencies to procure pellets during pre-fear conditioning baseline and

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pre-tone testing baseline days 1 and 2. The baseline latencies to procure pellets prior to the fear

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conditioning session (Fig. 2A) were not statistically different from the day 1 (Fig. 3A) and day 2 (Fig. 3C)

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pre-tone test baseline latencies after the fear conditioning session in both tone-shock (T-S) and tone-owl

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(T-O) paired animals (Related-samples Wilcoxon signed rank test; Baseline vs. D-1: $z = 1.293$, $p = 0.196$

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for T-S; $z = -0.560$, $p = 0.575$ for T-O; Baseline vs. D-2: $z = -0.155$, $p = 0.877$ for T-S; $z = -1.82$, $p = 0.069$

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for T-O). This indicates that neither the tone-shock group nor the tone-owl group showed evidence of

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contextual fear conditioning.

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722 **Legends for supplementary movies**

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724 **Movie S1.**

725 Representative foraging and escape behaviors of a rat presented with an owl-shock pairing. As the
726 animal come near a pellet, it encounters a swooping owl (from behind a black curtain) followed by a
727 dorsal neck/body shock pain. The rat flees to the nest without procuring the pellet.

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729 **Movie S2.**

730 The next day, as the same O-S rat advances towards a pellet, a novel tone is presented for the first time.
731 In response to the tone, the rat promptly flees to the nest without procuring the pellet.

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