Survival Decision Making

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Abstract

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All animals must make decisions in order to survive. Prey animals especially must not only forage for food, but avoid predators and if fortunate enough to survive a predator encounter, learn how to survive in the future. Over time, the rat has evolved to maximize the exploitation of resources in its environment and quickly adapt to its surroundings, making it the most successful mammal on the planet. In doing so, they have acquired many innate behavioral responses to different stimuli in their environment and possess exceptional spatial memory. Translational fear and avoidance research for clinical disorders in humans owes much to rodent research on the topic, but restricts the full behavioral repertoire of the rat through limited behavioral paradigms like fear conditioning. This dissertation explores the natural foraging behaviors of the rat in the laboratory, brain areas implicated in naturalistic predator avoidance, and the ability of rats to use information about a predator attack to survive in the future. The first study compared the fear responses to various types of 2D and 3D looming aerial stimulus threats. This study also includes the inactivation of the basolateral amygdala and superior colliculus and how they are required for eliciting innate fear responses to aerial threat. The results show a drastic increase in fear to a 3D aerial predator threat than a 2D threat, and also found significant sex differences with females taking longer to habituate to overhead fear-inducing stimuli. The second study focuses on the utility of Pavlovian fear conditioning in a naturalistic environment and what cues or information rats will use after surviving a predator attack. Here rats that have undergone a pairing of shock and tone while foraging, continue to forage unimpeded when that tone is played again. Alternatively, pairing a shock with a predator attack lead to an enhanced fear of the foraging context and to a novel tone played within this context. This more adaptive sensitization process is better utilized in a natural setting than traditional cue-based fear conditioning because it would not require the learning of discrete tones to avoid a predator attack.

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Chapter I. Background and Introduction

"Given enough time for the generations to evolve, the predator produces particular survival adaptations in its prey which, through the circular operation of feedback, produce changes in the predator which again change the prey". Frank Herbert

The Wild Rat

Despite their small stature and near constant prey status, the rat has become the most successful mammal on the planet by population (World Wildlife Foundation). Multiple factors are responsible for the rat's success such as rapid sexual development, multiple mating periods each year, and strong immune systems (Paccola, Resende et al. 2013), leading to over 64 species developing across the globe. However, the rat does not live in isolation. In their dynamic environments that allow them to thrive there exists the almost constant threat of attack from terrestrial (foxes, cats, and snakes) and aerial (owls, hawks) predators and they are forced to make decisions to survive.

Decisions can be influenced by instincts or innate responses and learned through experience, but together they result in a robust and flexible behavioral repertoire that allows the rat to subsist in almost any environment. Many of these survival tactics take the form of fear behaviors and are produced by the rat at the moment of predator perception, while other avoidance responses may be learned after a dangerous predator encounter. The immediate defensive responses elicited by a predator are also known as species-specific defense responses (SSDR's) (Bolles 1970), and it is another tool through which the rat can rapidly detect and respond to a threat instead of the costly lesson of learning a predator is a threat. Although SSDRs provide the opportunity for a rat to escape, there are many times when it would be more beneficial to avoid a certain predator location altogether. The learning and recalling of fear memories representing locations where predators may be is nearly as important for survival as the avoidance of a predator attack in the first place. While foraging, it is not enough to navigate and learn where certain food locations are, the rat needs to learn where the predators are as well.

Across multiple sub-species, rats tend to live in groups and must leave their own nest area to forage for food (Barnett 1975). While foraging they usually move about 20-30 meters from their nests, highlighting their preference to live near abundant food sources (Barnett 1976). Although this may seem like a limited foraging area, rats will fully exploit their foraging zone until food is not readily available, and then find a new nest location. During exploration of a dynamic environment, whether that be a forest, desert, or city, the rat must learn to integrate its surroundings and navigate optimally to collect and utilize resources. The environment in which the rat species has evolved in can play a large role in the behaviors exhibited when foraging. For instance, the kangaroo rat (*dipodomys spectabilis)* lives in underground burrows in deserts and dry grasslands on a diet of seeds and where water can be scarce (Schroder 1979). Open desert offers little cover to an animal easily preyed upon from above, so to maximize time within their burrows to save water and provide protection from predators, they hoard seeds within their nests to feed on for future use (Shaw 1934). This seed hoarding system allows the kangaroo rat to spend most of its time within the burrow, especially during periods of seed scarcity when increased foraging time outside the burrow would not only waste energy but drastically increase the risk of predation.

Another species of rat that lives in many different habitats around the world is the black rat (*rattus rattus)*, which exhibits nonspecialist foraging behaviors (Clark 1982). As the black rat lives in environments usually abundant in food, especially when living amongst humans, it does not need to store food for later use. This has led to a foraging strategy where the rats consume food as they move through their environment and sample many other potential food sources (Clark 1982). One key factor driving continuous foraging in the black rat is the population density of rats in that area where the more rats that are present is positively correlated with the distance traveled from the nest area, and negatively correlated with the amount of food brought back to the nest to hoard. So, while this more temperate environment provides more potential resources, it also means higher competition among conspecifics and an increased risk of predation through longer foraging times.

Regardless of the environment a species of rat lives in, rats leave their nest areas to forage at night, which provides an extra layer of protection from predators (Barnett 1976). Navigation, whether to a site with abundant food, or foraging as a rat moves through their environment, requires multiple strategies to not only find the location of food but to successfully navigate back to the nest area, especially when under attack from a predator. Rats lack foveal vision and cannot see intricate details that would be required to visually navigate through a dynamic environment (Burn 2008). They do however, possess considerable spatial memory of specific locations and will consistently return to food patches once they are known (Bowers 1982). While male rats are territorial, food patches are shared by all that live within the same nest area and navigation to these patches can rely on trails frequented by all rats living together. When a rat has found a food patch and is returning to the nest area, it will deposit droplets of urine only along the path of its return, not towards the food path itself (Galef and Buckley 1996). The trails to and from the food sites rats create most often share the quality of being under cover and against barriers, created through the process of thigmotaxis or wall-running (Crozier 1928). Interestingly, while thigmotaxis is equally displayed in wild and laboratory rats, rats raised in environments without any opaque boundaries do not display thigmotaxis in adulthood (Patrick and Laughlin 1934). Therefore, thigmotaxis may develop through habit formation from the rat's innate avoidance of open spaces. When raised in an artificial environment with unseen boundaries, the fear of open spaces might habituate over time and the thigmotaxis habit will never develop. Future research into this now presumed innate behavior would be interesting to see the impact of the avoidance motivation of open spaces on a habitual behavior that appears to affect all rats' navigation tendencies in the lab and in their natural habitats.

Both male and female rats leave their nests to forage for food and explore the immediate surroundings, but only female rats feed and care for rat pups up until about post-natal day 21, when they are weaned and begin exploring outside the dam themselves (Sengupta 2011). Sex differences in foraging emerge early on with female rats taking more frequent and shorter trips to food areas than males (Inglis, Shepherd et al. 1996, Spinks, Branch et al. 1999). Females continue making short, frequent return trips after giving birth, presumably to monitor pups more frequently as maternal separation is a source of anxiety for both the pups and the mother (Lehmann, Stohr et al. 2000, Aguggia, Suarez et al. 2013). Female rats also explore novel contexts at a slower rate than males and exhibit less risk-taking behaviors (Jolles, Boogert et al. 2015, Zambetti, Schuessler et al. 2019). Other than food motivation, sexual motivation may be driving a riskier and more exploratory phenotype seen in male rats. Although female rats can go into heat quite frequently when in the presence of males (about every five days), male rats exploring further outside the nest will allow for a greater chance of encountering a willing female (Song, Kalyani et al. 2018).

Fear is Survival

Despite the facts that rats have maintained population success all over the world 95% of wild rats die within their first year (Davis 1948), with many of these deaths occurring from predators. Because of their small stature and lack of physical defensive capabilities, rats are preyed upon by nearly any meat-eating animal in their environment including, but not limited to, snakes, birds of prey, foxes, and coyotes (Lim 1974, Duckett 1976, Windberg and Mitchell 1990, Banks 1997). Therefore, in order to avoid capture and certain death by these much larger animals, rats have evolved to innately avoid certain situations and react instantaneously to a predator strike. For example, as mentioned above rats will naturally avoid open areas when foraging in the wild, which is also observed in the lab in captured wild rats placed into an open-field arena (Price and Huck 1976).

For terrestrial predators like the fox, rats' olfaction has evolved to not only avoid an area where compounds found in fox feces are present (Trimethylthiazoline), but elicit fear responses like freezing as well (Endres, Apfelbach et al. 2005). In addition to fox odor, multiple odor sources from the cat will also induce fear behaviors in the rat. A collar worn by a cat, cat urine and feces, and cotton left in cat-housing all elicit innate avoidance behaviors from the rat (Blanchard, Griebel et al. 2001, Dielenberg and McGregor 2001, Staples, McGregor et al. 2008). Rats also possess sensitive hearing, and can detect sounds from the wide range of 0.25-80 kHz (Burn 2008). Unlike olfaction however, rats do not display any innate to fear to owl or other birds of prey calls and while these calls can be used as a conditional stimulus (CS) during fear conditioning, they do not condition any faster when compared with pure tones (Kindermann, Siemers et al. 2009). When looking at how rats are exposed to predators it becomes clearer why there is a discrepancy between innate avoidance behaviors in audition and olfaction. There are few discrete cues in nature that can alert a rat to a predator attack, scent being one of them. The presence of fox or cat odors is a clear indicator of those predators being nearby, especially when considering the fox utilizes urine and feces to mark their territories (Iossa, Soulsbury et al. 2008). Alternatively, birds of prey, especially owls are known for their silent hunting and have evolved distinct patterns of plumage to fly completely silently (Jaworski and Peake 2020). Over time rats have developed innate olfactory fear responses to specific predator cues, but as Robert Bolles famously said, “No owl hoots or whistles 5 seconds before pouncing on a mouse…” (Bolles 1970), never allowing for any associative processes to develop into an innate fear response.

With this silent advantage in mind, the lack of any predictive scent, and their nocturnal foraging habits, it may appear the rat would have no innate defensive response to any predatory attack from above. The only sensory system left for detection of aerial predators is vision, which rats greatly lack acuity in (Burn 2008). However, as rats travel through their environment, they will reflexively keep one eye focused above them that allows for a near 360° view of their surroundings (Wallace, Greenberg et al. 2013). When an object of interest like prey comes into view, or depth-perception is required, the eyes will realign onto said object allowing for a more flexible visual perception depending on the circumstances. In the repeated presence of aerial predators during foraging, rats will not only flee from flying owls, but avoid sequestered locations that have a high owl presence even though they have not been individually attacked (Brown, Kotler et al. 1988). This suggests the visual stimulus of a flying predator will not only drive a fleeing response, but can be contextually tied to certain foraging locations.

Foraging in the Laboratory

The ethological studies and experiments described above have laid the foundation for further manipulation and control of foraging behaviors and its neural correlates in a laboratory setting.

Of course, there are obvious limitations when attempting to mimic foraging in a limited laboratory space. First, the subjects themselves are not natural foragers, these tests will most likely be performed using lab rats that have been bred for generations in sterile and undynamic environments. A more extreme critique would be that most laboratory foraging paradigms have almost nothing to do with foraging at all, and instead are just rediscoveries of operant reinforcement schedules (Shettleworth 1989). That being said, the level of control, specificity of task performance, and the ability to focus in on specific questions of foraging theories not possible to manipulate in the field all are great benefits to studying foraging in the lab

For example, according to optimal foraging theory, after visiting two separate food patches, rats should exploit the one more abundant in resources and switch to the more abundant patch quickly to get as many resources as possible (Krebs and McCleery 1984). To test the timescale of patch exploitation, Timberlake et al (1987) created a simple lever pressing task where one side immediately provided the rat with food, but required lever presses increased as the rat consumed more. The other side would provide more food per lever press (a richer patch), but had a time delay for when the rat would be able to utilize the lever. The most optimal foraging strategy for the rat would be to lever press on the first open side, until the richer patch was available then quickly get the remainder of their food from that location. Surprisingly, the rats only switched to the richer foot patch if the lever-activation occurred within 16 minutes of entering the first patch. Otherwise, the rats continued putting higher effort into lever pressing and fully exploited the less-optimal patch. This effect is also known as ‘sunk cost’ and it appears humans are also sensitive to time spent in a patch after making that decision (Sweis, Abram et al. 2018). Multiple brain areas such as the amygdala, hippocampus, orbitofrontal cortex, and ventral tegmental area are also involved in not only the evaluation of reward, but encoding for time spent in reward locations (Pessiglione 2014, Papale, Zielinski et al. 2016, Solomon, Conover et al. 2017). The amygdala and hippocampus have also been shown to be involved in fear and avoidance behaviors (LeDoux 2003, Sanders, Wiltgen et al. 2003), most likely representing the locus of the approach-avoid conflict within the brain.

After demonstrating with field studies the sensitivity of rodents to overhead threat, laboratory research on the topic has shown that specific stimuli characteristics are necessary for looming defense responses (LDR; freezing and/or fleeing) to occur (Yilmaz and Meister 2013). Unfortunately, as is the case with many behavioral studies, as more labs have utilized looming stimuli as an aversive stimulus, the variability in responses have increased with a similar amount of theories accompanying them. For instance, De Franceschi et al. (2016) claims the stimulus itself will determine the behavioral response, with a sweeping disc representing a distal predator leading to freezing to avoid detection, and an expanding disc representing an immediate strike leading to fleeing. Yilmaz & Meister (2013) have shown using the same overhead stimulus (expanding disc) that the further an animal is away from a shelter, the more likely it is to freeze, but will flee to the shelter when close Wei et al. (2015) have reported only freezing in response to an expanding disc, with rapid habituation after only a few trials. Although variable, all of these studies show a reliable fear response in the lab to overhead stimuli and each provide explanations that are evolutionarily viable.

The most advantageous use of laboratory foraging is the study of foraging under the risk of predation where fear and risk assessment systems can be analyzed without the risk of death or injury to the animal. The fear systems of the brain have evolved over time to avoid and react to predators in dynamic environments, not in small, sterile chambers that limit an animal’s behavior. As the rat moves through its environment, it forms a cellular spatial representation within the interconnected circuits of the hippocampus and entorhinal cortex (Moser, Kropff et al. 2008). When encountering a predatory threat, after a spatial memory has been formed there is a shift in place cell firing presumably from fear-inputs from the amygdala to the hippocampus (Kim, Park et al. 2015). The general nature of the context has now shifted from a safe-foraging zone that provided food to a risky, possibly life-threatening location. In the wild, there are few places a rat will go that has such defined boundaries as foraging arenas used in the lab, so how far does a rat need to travel for a new place-field or context to develop? Instead of only focusing on location, the perception of the context and how it will affect the rat is more likely to determine hippocampal place fields (Smith and Mizumori 2006). These results and conclusions are only possible through the use of laboratory paradigms.

Chapter II. Fear Responses to Naturalistic Aerial Predator Stimuli and the Effects of Basolateral Amygdala and Superior Colliculus Inactivations

Introduction

The study of fear in laboratory rats and mice has traditionally utilized paradigms concerned with basic associative fear learning, or how fear of specific stimuli are acquired (Kim and Jung 2018). For example, in Pavlovian (classical) fear conditioning—the most widely used associative fear learning paradigm—an initially neutral conditioned stimulus (CS; e.g., a tone, light, or context) is temporally paired with the presentation of an aversive unconditioned stimulus (US; e.g., an electric shock to the animal's paws), typically over several trials. Through repeated CS-US pairings, the CS becomes a predictor of the US, and its presentation alone elicits fear-related behavior (such as freezing) in the animal. Similarly, in instrumental (operant) fear conditioning paradigms, the focus is on how the animal learns to avoid or terminate an aversive stimulus by responding appropriately to predictive stimuli over the course of several trials. While these associative paradigms have been useful in studying principles of learned fear and its underlying neural substrates, they address only a facet of fear as a functional behavior intended to keep animals alive in nature (Pellman and Kim 2016).

In contrast, studies investigating innate (unconditioned) fear employ stimuli that are instinctively threatening to the species being tested. Historically used by ethologists (Kavaliers and Choleris 2001, Schleidt, Shalter et al. 2011), innate fear paradigms often use predator cues (Takahashi, Nakashima et al. 2005), or in some cases, actual or simulated predators (Blanchard and Blanchard 1989, Choi and Kim 2010) to elicit fear responses. Responding to such predatory stimuli does not require previous experience or learning about the stimuli over multiple trials, and therefore fear toward these stimuli is considered to be genetically “pre-wired” (Öhman and Mineka 2001). These ethologically-relevant fears offer an evolutionary advantage over trial-and-error learning typical of associative fear learning, which can be both costly and time consuming for the organism (Pellman and Kim 2016). Indeed, despite the prominence of associative fear learning models, it is debatable whether associative fear learning is the primary mechanism by which fear operates in nature (Bolles 1970). Accordingly, the use of innate fear paradigms in fear research has been steadily increasing (Kim and Jung 2018).

One of the most common predatory threats in rodents comes in the form of large aerial predators, such as owls (Andersson and Erlinge 1977). Upon detection of overhead predators, rodents will display characteristic looming defense responses (LDR’s), such as fleeing or freezing (Bolles 1970), with the probability of either behavior prevailing being modulated by the perception of successful escape (Fanselow 1994, Edut and Eilam 2003, Yilmaz and Meister 2013). Recently, the overhead, two dimensional “looming stimuli” developed by Yilmaz and Meister (2013) have been used to study LDR’s in the laboratory setting (Wallace, Greenberg et al. 2013, Yilmaz and Meister 2013, Wei, Liu et al. 2015, De Franceschi, Vivattanasarn et al. 2016, Shang, Chen et al. 2018). In these studies, rodents (usually mice) are placed in chambers commonly fitted with overhead monitors capable of producing visual stimuli. These stimuli are intended to simulate a rapidly approaching or distant aerial predator and come in the form of a rapidly expanding black disk or a sweeping black bar, respectively. Presentation of the looming stimuli transiently evokes freezing or fleeing, depending on whether the animal is distant from or nearby, respectively, an enclosed shelter providing a refuge (Yilmaz and Meister 2013). The advantages of looming-induced fear paradigms are that the visual stimuli are simple to generate, precise, and highly controllable.

While previous studies have demonstrated that overhead 2D looming stimuli can evoke innate fear responding, the types of LDR's (freezing or fleeing to an enclosed nest) vary despite using similar stimuli and testing apparatus (Yilmaz and Meister 2013, De Franceschi, Vivattanasarn et al. 2016). Moreover, no looming stimuli study to date has utilized female rats; thus any potential sex differences in rats are largely unknown. To further characterize and facilitate an understanding of innate fear responding to looming stimuli, the present experiment employed a more naturalistic, goal-directed risky-foraging task in male and female rats. Briefly, hunger-motivated animals are allowed to forage for food pellets in a large arena partitioned into two distinct zones; a safe, enclosed "nest area" and a risky, open "foraging area." After a period of baseline foraging assessment, rats are challenged with presentations of the looming stimuli in the foraging area. Furthermore, in addition to using standard 2D looming stimuli projected above the animal (Yilmaz and Meister 2013), there were projections of the stimuli onto the floor of the arena to examine whether animals display fear behavior toward shadows casted on the ground. We also employed a discrete, localizable 3D looming stimulus; i.e., a life-like owl programmed to plunge toward the animal during the foraging task (Figure 2.1).

In previous risky foraging tasks with a terrestrial pseudo-predator the basolateral amygdala (BLA) was shown to be necessary for the fleeing response, and when inactivated, rats successfully foraged under predatory threat (Choi and Kim 2010). The amygdala is also crucial in the fear circuit involved with response to the 2D looming stimuli, through projections from the superior colliculus (SC), which when inactivated eliminates the fear response to 2D stimuli (Wei, Liu et al. 2015). While the amygdala has long been known to play a large role in both learned and innate fear, the SC is a large layered structure that until more recently was primarily known as an physical orientating structure as mainly part of the visual system (White and Munoz 2011). Current studies of the SC have revealed its part in the unconditional fear response during Pavlovian fear conditioning (Muthuraju, Talbot et al. 2016), as well as the enhanced startle response in a fear-potentiated startle task (Zhao and Davis 2004). In order to begin to understand the brain structures involved with the innate fear response to a 3D aerial predator, the basolateral amygdala and superior colliculus (SC) were temporarily inactivated or lesioned respectively, while rats engaged in the risky foraging task.

It was hypothesized that the 3D owl stimulus would be most effective in eliciting LDRs, as it more closely resembles an ethologically relevant threat compared to the 2D looming stimuli. To comprehensively test this hypothesis, separate groups of animals were challenged with the two stimuli types over multiple days of testing. Finally, we hypothesized that female rats would display greater innate fear to all looming stimuli, and consequently, be less successful in procuring pellets during stimuli testing. This is predicated on previous literature showing that female rats react more defensively and engage in more risk-assessment behaviors following predator exposure (Blanchard, Shepherd et al. 1991, Pellman, Schuessler et al. 2017).

Materials and Methods

Subjects

A total of 56 rats (39 males and 17 females; nulliparous; 3-4 months old) were ordered from Charles-Rivers Laboratories in small cohorts (n=4 for each sex) and tested independently. Once transported, animals were individually housed in a climate-controlled vivarium (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care) and maintained on a reverse 12-h light/dark cycle (lights on at 7:00 PM). After 5-7 days, animals were placed on a standard food deprivation schedule (with *ad libitum* access to water) to gradually reach and maintain 85% of their normal weight. All experiments were conducted during the dark phase of the cycle and in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Amygdala Surgery

Under anesthesia (80 mg/kg ketamine and 5 mg/kg xylazine, i.p.), male rats (n = 6) were mounted in a stereotaxic instrument (Kopf) and were chronically implanted with guide cannulae (Plastics One Inc.) bilaterally into the amygdalae. Stereotaxic coordinates for 26-gauge guide cannulae were (referenced from bregma) anteroposterior (AP) – 2.5; mediolateral (ML) +- 5.0 and, dorsoventral (DV) +- 7.4 mm. Implanted cannulae were cemented to the skull with 6 anchoring screws. All rats were given 5-7 days of surgical recovery and daily handling and weighing before experimental procedures began.

Drug Infusion

Cannulated animals underwent habituation and baseline foraging training the same as non-cannulated animals. Muscimol-free base (Sigma-Aldrich) dissolved (10mM) in artificial cerebrospinal fluid (ACSF) were microinfused into the amygdala bilaterally by backloading the drugs up a 33-gauge infusion cannula into polyethylene (PE 20) tubing connected to 10-µl Hamilton micro-syringes (Hamilton Company). The infusion cannula protruded 1 mm beyond the guide cannula. 30 minutes prior to the Owl testing infusion cannulae were placed in the guide cannulae to deliver 0.3 µl of muscimol or ACSF at a rate of 0.1 µl/min using a Harvard PHD2000 syringe pump (Harvard Apparatus). The infusion cannulas remained in place for 1 minute after the infusion. Over the next 2 testing days, the infused drug was alternated for each animal.

Superior Colliculus Electrolytic Lesion

Male rats were assigned to one of two surgical groups: sham lesion control (n = 3) and electrolytic lesion (n = 7). Under anesthesia (80 mg/kg ketamine and 5 mg/kg xylazine, i.p.), rats were mounted onto a stereotaxic unit (Kopf) and stainless-steel insect pins (#00) coated with epoxy except for 0.5 mm at the tip were bilaterally lowered into the superior colliculi. The target coordinates were as follows relative to bregma, AP -6.0; ML +1.5; DV -4.4. Once the insect pins were lowered into place, lesions were created by passing DC current through the electrodes (1.5mA, 20s). The sham animals had insect pins lowered into place for 20 seconds, with no running current. Afterwards, the rats' scalps were sutured using wound-clips and placed into surgery recovery until the wound-clips were removed (5-7 days).

Histology

At the completion of behavioral testing, animals were given a lethal dose of Buthanesia (pentobarbital sodium) and perfused intracardially with 0.9 % saline followed by 10% buffered formalin. The brains were removed and stored in 10% formalin for 24 hours and then kept in 30% sucrose solution until they sank (~4 days). Transverse 50-µm sections were taken through the locations of the cannulae or lesion sites, mounted on gelatin-coated slides with a gelatin mounting solution, and stained with a cresyl violet dye in addition to a Prussian blue stain for the lesioned animals (Figures 2.8 D and 2.9 D).

Foraging Apparatus

A custom-built foraging arena consisted of a nest (69 cm L x 58-66 cm W x 61 cm H) that opened via a sliding gate to reveal a large foraging area (208 cm L x 66-120 cm W x 61 cm H) where food pellets were placed and where rats encountered 2-D and 3-D looming stimuli. The testing room was kept under red light (11 lux foraging area, 2 lux nest area) with constant white noise (72 dB) playing in the background. The ANY-maze software and AMi interface system (Stoelting) connected to a PC tracked the animal’s position in the arena, via a ceiling mounted camera, and triggered the looming stimuli.

Floor looming. A drop-ceiling projector mount (Amer) was used to position the projector (Epson EX7240) above and perpendicular to the foraging zone so that nearly the entire arena (except the nest) was covered by the projected image (85 lux).

Ceiling looming. The projector was mounted at the end of the foraging arena and directed upwards to a white insulation foam (244 x 122 cm) screen suspended 2.19 m above the foraging arena (52 lux).

Owl. A life-like model owl (84 cm wingspan) was mounted to a pneumatic air cylinder (Bimba, 92 cm) and positioned at a 45° angle above the arena (76 cm) at the opposite end from the nest. A bidirectional double-solenoid air-valve (IMI Norgren) placed outside of the arena was used to activate the air cylinder, which generated an increase to 75 dB (from 72 dB) in the foraging arena when the valve switched position. Exhaust dampeners (McMaster-Carr) were attached to the solenoid valve ports to lessen noise. While in the retracted position, the owl was hidden from the view of the animal by a black curtain spanning the width of the apparatus. When fully extended (at the rate of 46 cm/s), the bottom of the owl was 43 cm above the arena floor at the long pellet location (100 cm from nest). The owl was triggered when the animal entered predetermined distances of 75, 50, and 25 cm from the nest for long, medium, and short pellet location tests, respectively.

Behavioral Procedures

All animals underwent habituation and baseline foraging sessions prior to floor looming, ceiling looming or owl encounter tests on consecutive days. The ANY-maze tracking data (sampled at 60 Hz) were used offline to quantify (i) freezing, defined as no movement apart from respiration (Blanchard and Blanchard, 1969) for at least 2 sec, (ii) outbound foraging speed (the time from exiting the nest to either reaching the pellet or entering the looming stimuli trigger zone), (iii) inbound speed (the time from either procuring the pellet or triggering the looming stimuli to returning to nest) and (iv) latency to procure food pellet (time it takes for rat to retrieve pellet and begin consumption). Fleeing/escape behavior was defined as the animal running back to the nest without securing the pellet.

Habituation. Rats were placed in the nest area for 30 min/day for two days with 20 food pellets (0.5 g, Bio-Serv dustless precision pellets, #F0171) to acclimate to the experimental setting and food source, and to also associate the nest area with feeding.

Baseline sessions. Each day rats were placed in the nest area with 2 food pellets. Once the pellets were eaten, the nest door to the foraging area opened with a pellet placed 25 cm away. The latency to retrieve the pellet and return to the nest area for consumption was measured, after which the door was closed. This process repeated with the pellet placed 50 and 75 cm away from the nest opening. Across the subsequent 4 days, the pellet gradually moved further away from the nest until it reached 100 cm from the nest opening. From the 3rd day of baseline through the end of testing, the projector was on to habituate rats to projector generated background light and sound. The owl encountering rats remained in red light throughout the entire experiment.

Floor looming testing. On the test day, animals initially underwent 3 pre-looming baseline trials with 100 cm pellet placement. Afterwards, the pellet was moved 100-175 cm from the nest for looming tests. When the rat was within ~25 cm of the pellet, the experimenter remotely activated black expanding disk, sweeping bar and sweeping block stimuli (counterbalanced order). Specifically, the expanding disk started at the center of the floor with a diameter of 0.635 cm and extended to 66 cm (at a rate of 59.43 cm/s) encompassing nearly the entire foraging arena; the sweeping bar was a narrow rectangle that moved at 61.21 cm/s to the nest; and the large sweeping block started at the edge and filled the entire foraging area at 61.21 cm/s. During each stimulus test (3 minutes), as the animal entered the trigger zone, the looming stimulus was triggered five times in rapid succession. The visual stimuli were created and converted into executable files using Python packages TKinter/Turtle/PyInstaller, and inserted into an ANY-maze procedural file.

Ceiling looming testing. Animals underwent the same procedure as described above, except the looming stimuli projected on the ceiling.

Owl encounter testing. Each testing day began with rats being placed into the nest with 2 food pellets and then undergoing 3 pre-owl baseline trials with the pellet placed 100 cm away from the nest. Afterwards, each time the rat was within ~25 cm of the pellet, the owl automatically plunged towards the rat, and after one second retracted to its original position behind the blackout curtain. If the animal was unable to procure the pellet within 3 minutes, on subsequent trials, the pellet was moved closer to the nest (75 and 50 cm away) and the animals were again given 3 minutes to retrieve the pellet. Daily testing continued until all rats successfully procured the pellet while facing the owl. Four of the 8 female rats previously underwent 2D floor looming stimuli; however, there were no differences in behavior between 2D pre-exposed and naïve rats to the owl.

Data Analyses

Statistical analyses were performed using SPSS (IBM, version 19), and graphs were created using GraphPad (Prism, version 8). Because the Levene’s test for normality showed significance, nonparametric tests were used to analyze data.

Baseline sessions. To compare initial baseline latencies to procure the pellet, Mann-Whitney U tests were used to compare male and female rats.

2-D looming testing. Chi-square tests for independence were applied to both floor and ceiling looming data to compare the escape response frequency between males and females. A Mann-Whitney U test was also used to compare the latency to procure the food pellet between sexes for each 2D looming type.

Owl testing. A Mann-Whitney U test was used to determine sex differences in the foraging latencies and speed, and the number of attempts to procure the pellet across the baseline and owl encounter trials.

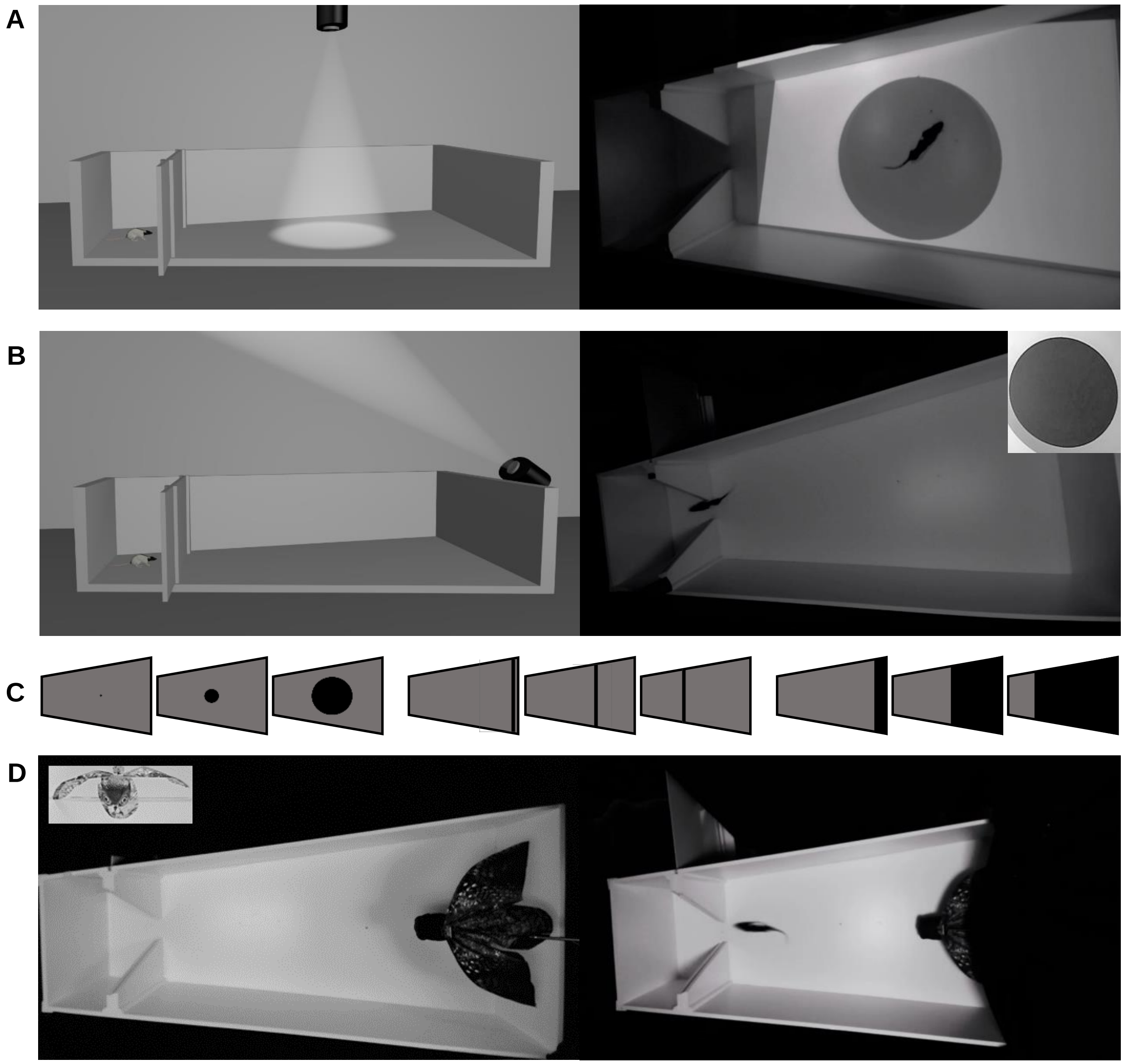

Figure 2.1. Experimental apparatus and looming stimuli. (A, left) 3-D representation of nest (69 cm length x 58-66 cm width x 61 cm height) and foraging arena (208 cm length x 66-120 cm expanding width x 61 cm height) with floor looming projector orientation alongside (**right**) a still image of rat encountering expanding disc on the floor of the arena. (**B, left**) 3-D representation of foraging arena with ceiling looming projector orientation alongside (**right**) a still image of a rat fleeing the overhead expanding disc stimuli. (**C)** Representations of each looming stimuli (expanding disc, sweeping bar, and large sweeping block) from beginning to end of the presentation and how they appear within the foraging arena. (**D, left**) Foraging arena owl in the prone position and no black-out curtain. Insert displays front-facing image of an owl. (**right**) Rat fleeing from activated owl (completely extended) back to the nest area. The arena was piped with masking white noise (72 dB at the aerial stimuli trigger zone). The sound associated with the activation of the owl briefly raised the total sound level to 75 dB.

Results

Female rats initially have increased foraging times than male rats.

Projector and owl encounter rats' data were pooled together as baseline foraging session methods were identical across experiments and no increase in latency was found from the projector being turned on, leading to baseline sample sizes of 20 male and 17 female rats. Rats were required to forage for food pellets at increasingly greater distances across the 5 baseline session days. Briefly, each baseline session day consisted of 3 trials in which a pellet was placed 25, 50, or 75 cm away from the nest (eventually increasing to 100 cm). As expected, the latencies to procure the pellet were longer on the first day since rats are venturing into a novel environment (Figure 2.2 A). Additionally, the foraging apparatus expands in width as the rats move further away from the nest, adding a continuum of increasing danger to the task. A Mann-Whitney U test revealed female rats had significantly longer foraging times than male rats on all baseline session days, except for baseline session day 2 However, once the animals emerged from the nest, there were no significant differences between males and females in the outbound speed (distance/time) to the pellet location across any baseline days (Figure 2.2 B). Females on average had significantly higher inbound speeds (food pellet to the nest area) on the second day of baseline, but there were no significant differences on the following days (Figure 2.2 C). Although females took a significantly longer time foraging for pellets on the last baseline day, both sexes were quickly able to retrieve the pellet in under 25 seconds.

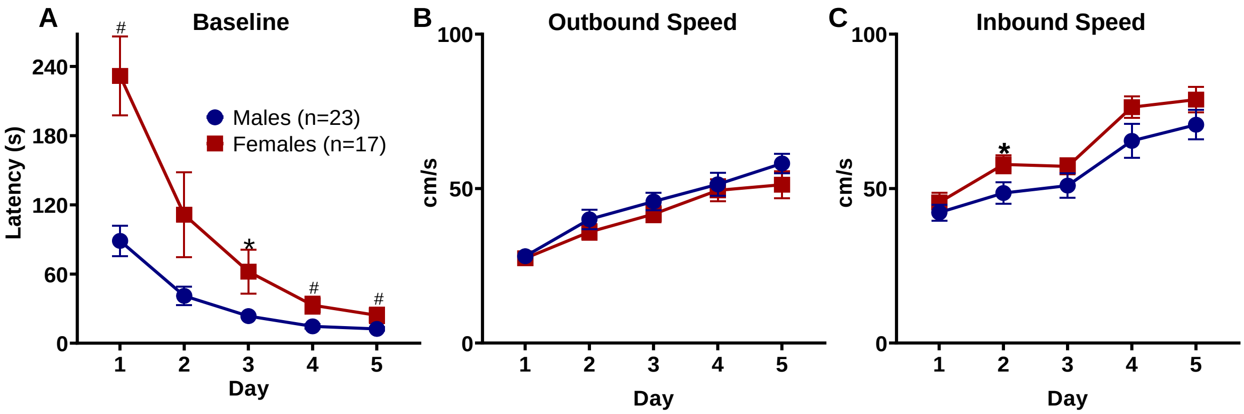

Figure 2.2. Average latencies to retrieve pellet during initial baseline session days between sexes. Rats first engaged in a foraging task where food pellets were placed at increasing distances from the nest area, and no looming stimuli were presented. (**A**) Females took a significantly longer time foraging for food pellets than males on baseline session day 1, 3, 4, and 5 (Baseline 1: z=3.535, p<0.01; Baseline 2: z=1.798, p>0.05; Baseline 3: z=2.424, p<0.05; Baseline 4: z=2.82, p<0.01; Baseline 5: z=2.851, p<0.01). (**B**) There were no significant differences between sexes for outbound speed during baseline foraging days (Mann-Whitney U, Baseline 1: z=-0.274, p>0.05; Baseline 2: z=-0.958, p>0.05; Baseline 3: z=-0.821, p>0.05; Baseline 4: z=-0.123, p>0.05; Baseline 5: z=-1.245, p>0.05). (**C**) Inbound speed during baseline foraging where females were significantly faster on the second day (Mann-Whitney U, Baseline 1: z=-0.821, p>0.05; Baseline 2: z=-2.496, p<0.05; Baseline 3: z=-1.724, p>0.05; Baseline 4: z=-1.594, p>0.05; Baseline 5: z=-1.246, p>0.05). Data are represented as mean ± SEM. *p<0.05, #p<0.01.

2D floor looming elicited weak fear responses

Foraging rats were exposed to a 2D stimulus, either an expanding disc, a large sweeping block or a sweeping bar, projected onto the floor of the arena (Figure 2.1A). None of the male rats exhibited LDRs to floor looming stimuli, whereas 5 out of 8 females fled from the block stimulus, but not to the other visual stimuli (Figure 2.3 A). A chi-square test for independence found females had a significantly higher fear response than males to the floor looming block. This fleeing response quickly habituated following repeated presentations. The outbound and inbound speeds were compared between males and females with no significance found (Figure 2.3 C, E). For the inbound speed during Block stimulus trials, only the speeds of females that fled from the stimulus were included in the analysis (n=5). Freezing behavior (i.e., no movement apart from respiration for ≥2 s automatically scored by Anymaze) was never observed in males and females during the floor looming test.

With respect to looming stimuli presentation, the first cohort of rats tested was presented with the large block stimulus first, the expanding disc second, and the sweeping bar third, whereas the second cohort was exposed to the expanding disc first, the sweeping bar second, and the large block third. The pellet was also placed further from the nest area (175 cm). Amongst the females, there was no order-effect found for stimuli presentation, with females in either cohort responding to the large block regardless of its presentation order. None of the animals underwent both the floor looming and ceiling looming conditions. It is possible, however, that an animal that did not show a fear response to the floor looming stimuli would exhibit fear toward the ceiling looming stimuli if tested consecutively. No significant differences were found between sexes for latencies to procure the pellet for each stimulus using a Mann-Whitney U test (Figure 2.4 A).

2D ceiling looming elicited moderate fear responses

While the floor looming condition produced very weak fear in few females and none in males, the visual stimuli projected onto the ceiling above the foraging arena (“Ceiling looming” condition; Figure 2.3 B) were moderately effective in evoking LDRs. Specifically, a chi-square test for independence revealed that females (8/8) responded significantly more than males (4/7) to the ceiling looming disc. The sweeping block stimulus elicited fewer fleeing responses in both sexes (4/8 females; 2/7 males), whereas almost none of the animals (1/7 females; 0/8 males) fled in response to the overhead, sweeping bar stimulus. Once again, none of the male and female rats displayed freezing in the arena when looming stimuli were presented and quickly habituated to the stimuli (Figure 2.4 B). Hence, our sweeping stimuli results in rats contrast with a previous study conducted in mice that reported freezing responses to a similar stimulus (De Franceschi, Vivattanasarn et al. 2016).

Latencies to procure the pellet mirrored the fleeing response data, such that the highest latencies occurred with the expanding disc, followed by the block and bar stimuli, respectively (Figure 2.4 A). Each trial lasted a maximum of 180 s. Half of the female rats and 1 male rat reached this cutoff before procuring the pellet (Figure 2.3 B). There were no significant differences in outbound speed in either testing day, except for females having significantly higher speeds during the Bar trial on testing day 2 (Figure 2.3 D). There were no significant changes in inbound speed following stimulus exposure (Figure 2.3 F), and only the rats that fled from the stimulus (as mentioned above) were included in the analysis. On the second day of projector testing most males (n=6) consumed the food pellets in the foraging area so no inbound speed to the nest was able to be calculated, resulting in a low n-size across all trials (Figure 2.3 F). Interestingly, females exhibited a large increase in inbound speed during this day of testing.

In general, there were trends of female rats exhibiting higher latencies to procure the pellet than male rats during the stimulus trials (Figure 2.4 A).

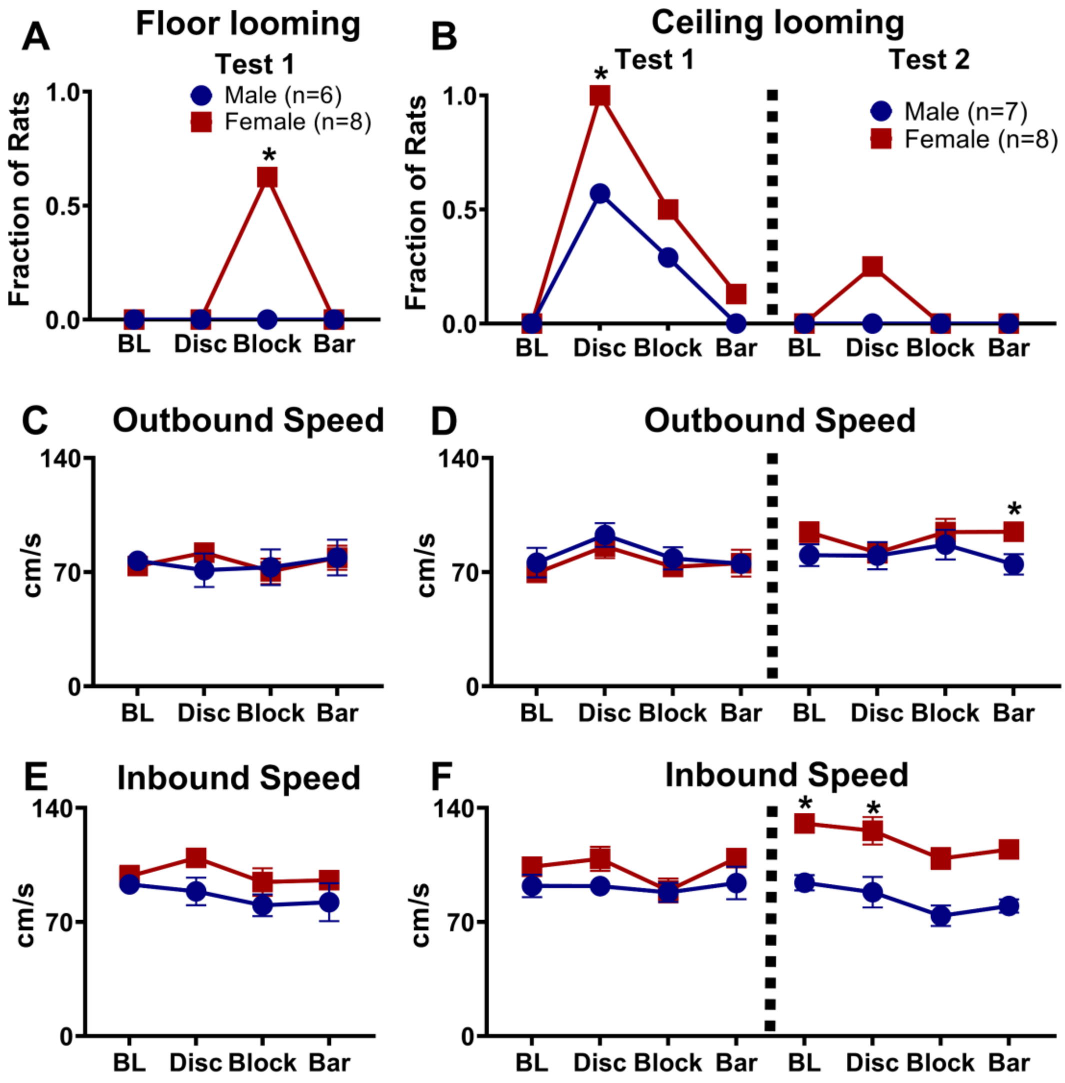

Figure 2.3. Higher female sensitivity to 2D looming stimuli. (**A**) The fraction of male and female rats that fled from 2D floor-looming stimuli ($\chi^2(1, n=14) = 5.833$, $p = 0.016$). (**B**) The fraction of animals that fled from 2D ceiling-looming stimuli ($\chi^2(1, n=15) = 4.29$, $p = 0.038$). (**C**) Outbound speed during baseline and each projected stimuli. (**D**) There were no significant differences in outbound speed on the first day of testing. On the second day, females were on average significantly faster than males for the outbound speed during the Bar trial (Outbound: Mann-Whitney U, Baseline, $z = -0.645$, $p > 0.05$; Disc, $z = -0.387$, $p > 0.05$; Block, $z = 0$, $p > 0.05$; Bar, $z = -0.214$, $p > 0.05$. Inbound: Baseline, $z=-0.665$, $p>0.05$; Disc, $z=-1.717$, $p>0.05$; Block, $z = -1.278$, $p > 0.05$; Bar, $z = -1.393$, $p > 0.05$). (**E**) Inbound speed from the pellet location to the nest, the Block stimulus only includes females that fled to the nest during stimulus presentation (n=5). Both sexes fled from the Expanding Disc in this condition (Mann-Whitney U, Test 1: Baseline: $z = -0.579$, $p > 0.05$ expanding disc: $z = -0.340$, $p > 0.05$; large block: $z = -0.694$, $p > 0.05$; sweeping bar: $z = -0.463$, $p > 0.05$. Test 2: Baseline: $z = -1.62$, $p > 0.05$ expanding disc: $z = -0.463$, $p > 0.05$; large block: $z = -0.688$, $p > 0.05$; sweeping bar: $z = -2.199$, $p < 0.05$). (**F**) There were no significant differences in inbound speed on the first day of

projected stimuli presentations. Only the inbound speed data of males that fled from the Expanding Disc were used for analysis (n=4; (Mann-Whitney U, Test 1: Baseline: $z = -1.678$, $p > 0.05$ expanding disc: $z = -1.189$, $p > 0.05$; large block: $z = -0.387$, $p > 0.05$; sweeping bar: $z = -1.254$, $p > 0.05$. Females had significantly faster inbound speeds on the 2nd day of projector testing during the Baseline and Disc trials. On the second testing day of Ceiling Looming most males consumed the food pellet outside the nest area, so inbound speed for only 2 males could be calculated (Mann-Whitney U, Test 2: Baseline: $z = -2.646$, $p < 0.05$ expanding disc: $z = -2.089$, $p < 0.05$; large block: $z = -2.049$, $p > 0.05$; sweeping bar: $z = -2.049$, $p > 0.05$). Data are represented as mean ± SEM. * $p < 0.05$.

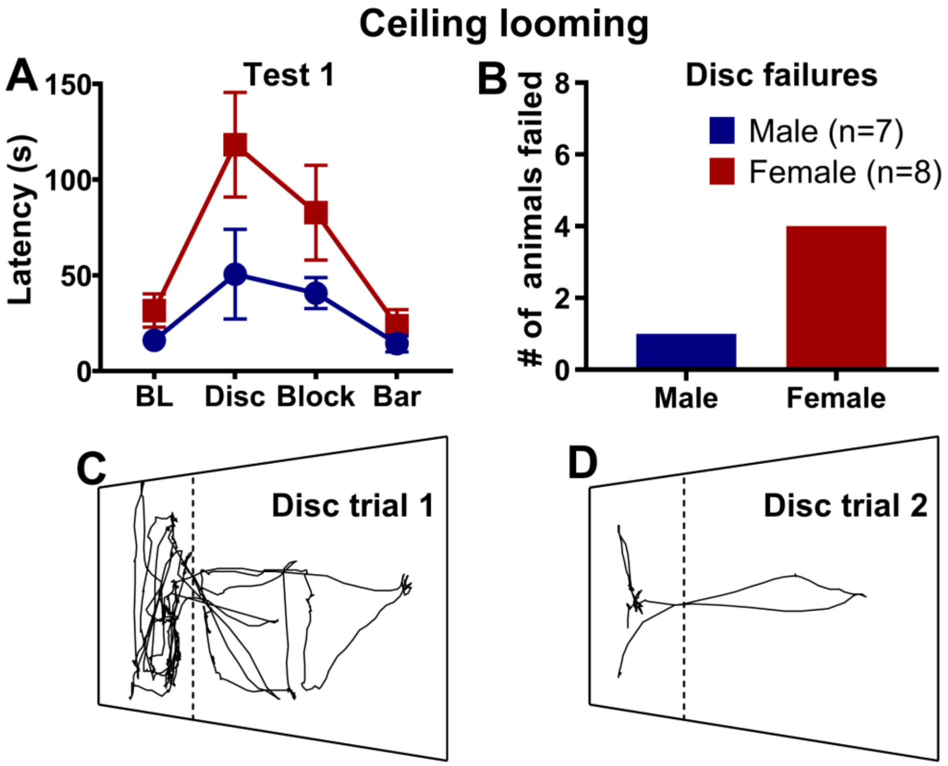

Figure 2.4. Foraging latencies to ceiling looming stimuli. (**A**) Group average latencies + SEM to retrieve food pellet (Mann-Whitney U Test, expanding disc: $z = 1.68$, $p > 0.05$; large block: $z = 0.389$, $p > 0.05$; sweeping bar: $z = 0.969$, $p > 0.05$). (**B**) A number of rats who failed to retrieve the pellet on Expanding Disc trial (did not retrieve pellet within 180 seconds). (**C**) Representative position plots of a female rat that failed to retrieve a pellet on the first day of Expanding Disc exposure. (**D**) Position plot from the same rat demonstrating rapid habituation to Expanding Disc during the second day of expanding disc exposure.

3D predatory threat evokes a stronger fear response in female than male rats

Rats foraging for pellets placed 100 cm away from the nest encountered a model owl that emerged from behind a blackout curtain. Two subsequent trials each testing day had the pellet placed at 75 and 50 cm away from the nest, totaling to three (180 s) trials per day. The latency to procure the pellet and number of attempts to acquire the pellet (i.e., the number of owl activations) were averaged each testing day. Initially, both male and female rats failed to procure the pellet under threat of the owl (Figure 2.5 A). Across days, however, male rats habituated to the owl faster than female rats, with a significant difference in latency on day 5. Notably, it took 19 days for all female rats to successfully procure the pellet during the 3 baseline and 3 owl exposure trials given per test day (Figure 2.7). During the first two days of owl exposure, male rats also triggered the owl significantly more (Figure 2.5 B), signifying more attempts were made to retrieve the food pellet. There is a noticeable decrease in outbound speed in male rats on day 2 following owl exposure (Figure 2.5 C), and on the same testing day females had significantly higher outbound speeds. There were no other significant differences in outbound or inbound speed (Figure 2.5 D).

Contextual fear from 3D predatory threat encounters

Each owl testing day began with 3 baseline trials (pellet placed at 100 cm) to measure any contextual fear memory that may transpired from encounters with the owl the previous day. Both male and female rats' baseline trial latencies significantly increased following the first day of owl exposure (Figure 2.6 A). Females, however, exhibited a stronger contextual fear response than males during owl testing days 1, 2, 4, and 5 baseline trials. There were no significant differences in outbound or inbound speed during baseline trials on owl testing days (Figure 2.6 B-C).

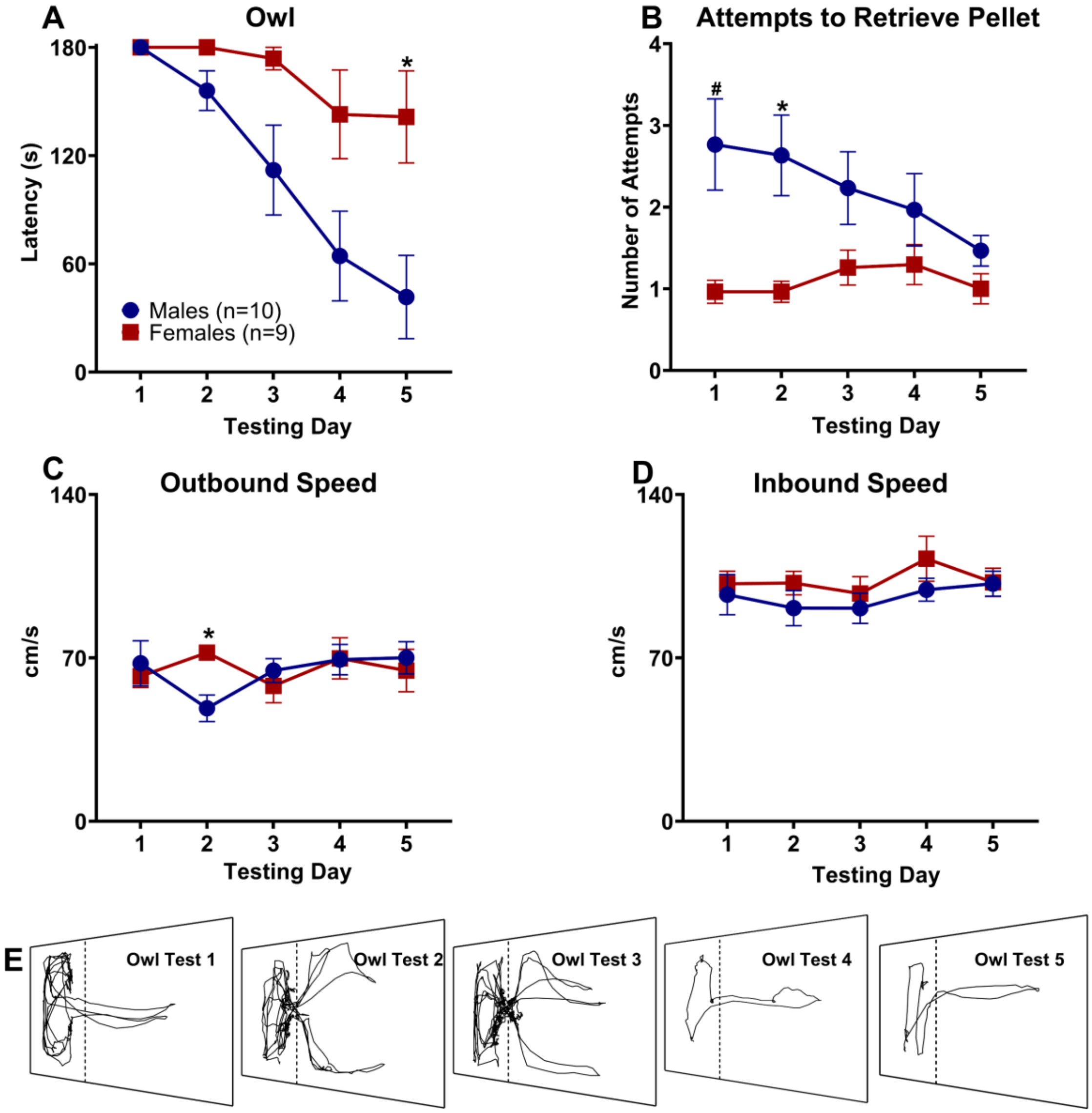

Figure 2.5. Female rats exhibited stronger fear to the owl than male rats across the testing days. (**A**) Male and female rats average group latencies ± SEM to retrieve the pellet under aerial predator threat. Male rats habituated to the owl faster than females (Mann-Whitney U test, $z = 2.331$, $p < 0.05$). (**B**) Average attempts made to procure the food pellet across owl exposure days. Male rats made significantly more attempts than females to reach the food pellet, leading to more exposure to the predator early on in testing Mann-Whitney U test, Owl day 1: $z = 2.684$, $p < 0.01$; Owl day 2: $z = 2.199$, $p < 0.05$). (**C**) Outbound speed during owl exposure testing days. Females were significantly faster outbound on day 2 (Mann-Whitney U test, Day 2 Outbound Speed: $z = -3.022$, $p < 0.05$). (**D**) There are no significant differences between males and females speed during inbound responses to the owl (Mann-Whitney U test, Outbound, Day 1: $z = -0.408$, $p > 0.05$, Day 3: $z = -0.653$, $p > 0.05$, Day 4: $z = -0.041$, $p > 0.05$, Day 5: $z = -0.449$, $p > 0.05$. Inbound, Day 1: $z = -0.082$, $p > 0.05$, Day 2: $z = -0.735$, $p > 0.05$, Day 3: $z = -0.123$, $p > 0.05$, Day 4: $z = -0.776$, $p > 0.05$, Day 5: $z = -0.204$, $p > 0.05$). (**E**) Representative position plots of male rat across 5 days of Owl exposure. *$p < 0.05$, #$p < 0.01$

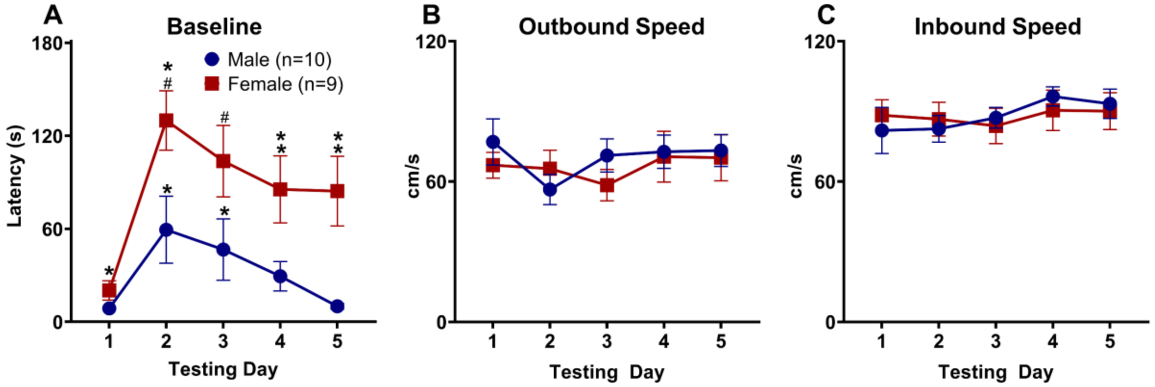

Figure 2.6. Sex differences in contextual fear from owl encounters. (**A**) Group average latencies ± SEM to procure the food pellet during baseline trials performed on each testing day before the Owl encounter trials. Male and female rats both exhibited significantly longer baseline foraging times the day following the predator encounter when compared to the first day of predator exposure (related samples Wilcoxon signed ranks test, Males: BL1-BL2, $z = 2.395$, $p < 0.05$; BL1-BL3, $z = 2.296$, $p < 0.05$. Females: BL1-BL2, $z = 2.666$, $p < 0.01$; BL1-BL3, $z = 2.666$, $p < 0.01$; BL1-BL4, $z = 2.521$, $p < 0.05$; BL1-BL5, $z = 2.134$, $p < 0.05$). Female rats took a significantly longer time than males to procure the pellet on days 1, 2, 4, and 5 (Mann-Whitney U test, BL1: $z = 3.535$, $p < 0.01$; BL2: $z = 1.798$, $p > 0.05$; BL3: $z = 2.424$, $p < 0.05$; BL4: $z = 2.82$, $p < 0.01$; BL5: $z = 2.851$, $p < 0.01$). (**B**) There are no significant differences between males and females during outbound responses to the owl (Mann-Whitney U test, Outbound, Day 1: $z = -0.899$, $p > 0.05$, Day 2: $z = -0.613$, $p > 0.05$, Day 3: $z = -1.551$, $p > 0.05$, Day 4: $z = -0.683$, $p > 0.05$, Day 5: $z = -0.898$, $p > 0.05$). (**C**) There are no significant differences between males and females during inbound responses to the owl (Inbound, Day 1: $z = -0.613$, $p > 0.05$, Day 2: $z = -0$, $p > 0.05$, Day 3: $z = -0.41$, $p > 0.05$, Day 4: $z = -1.103$, $p > 0.05$, Day 5: $z = -0.082$, $p > 0.05$). Data are represented as mean +/- SEM. *$p<0.05$, #$p<0.01$.

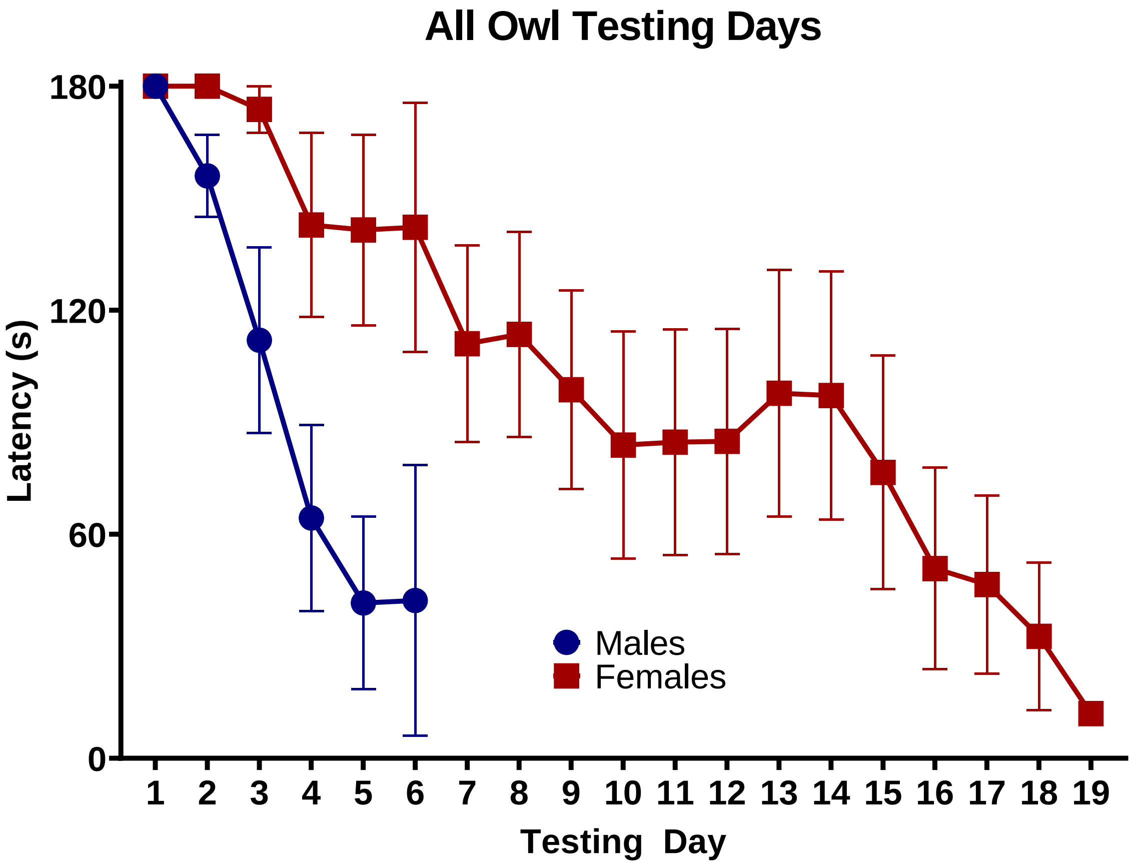

Figure 2.7. Average latencies across owl testing days. Mean group latencies ± SEM for male and female rats. For females, n=9, n=7 and n= 6 rats for testing days 1-12, 13-17 and 18-19, respectively. For males, n=10 and n=4 for testing days 1-5 and day 6, respectively.

Reversible inactivation of the BLA eliminates Owl fear response

To determine whether the expression of fear in response to an overhead predator required the BLA as has been shown with terrestrial predators (Choi and Kim 2010), the GABA agonist muscimol was infused into the BLA during Owl testing. After baseline foraging training had been completed (4-6 days), on the first day of testing 30 minutes before the trial began, the animal was infused with 0.3µl of either muscimol or ACSF (0.1µl/min). The muscimol infused animals successfully retrieved the pellet at all distances when confronted with the Owl, while the ACSF infused animals failed to retrieve the pellet from any distance (Figure 2.8 A). The next day the infused drug from the first day of testing was alternated for each animal. Once again, the muscimol infused animals did not show any fear responses, while the ACSF infused animals failed to retrieve the pellet. On the third and final day of testing the rats were infused with the original drug they received during the first Owl exposure test. The ACSF infused animals once again were not able to retrieve the pellet and the muscimol infused animals showed no fear response to the Owl.

In addition to the lack of fear responses displayed by the muscimol infused rats, they also showed an increase in novelty seeking behaviors when exposed to the owl. For all rats on their first owl trial after the muscimol infusion, they spent a significantly longer amount of time in the far-end of the foraging arena where the owl returns to its prone position (Figure 2.8 B). For the subsequent owl exposure trials, the rats do not approach the owl and quickly procure the food pellet.

Lesioning of the superior colliculus eliminates fear responses to the Owl

After recovery from surgery, lesioned rats underwent habituation and baseline foraging training sessions for 5 days. 24 hours after the last day of baseline they were exposed to the owl in the same manner as described above. All of the lesioned animals were able to retrieve the food pellet and had significantly shorter latencies when retrieving the food pellet (Figure 2.9 A). The sham rats failed to retrieve the food pellet across the 3 testing days and had significantly increased baseline latencies following each owl exposure (Figure 2.9 B). Unlike the BLA inactivated rats however, the SC lesioned rats never approached the owl after triggering it and quickly procured the pellet to eat in the nest during every trial (Figure 2.9 C).

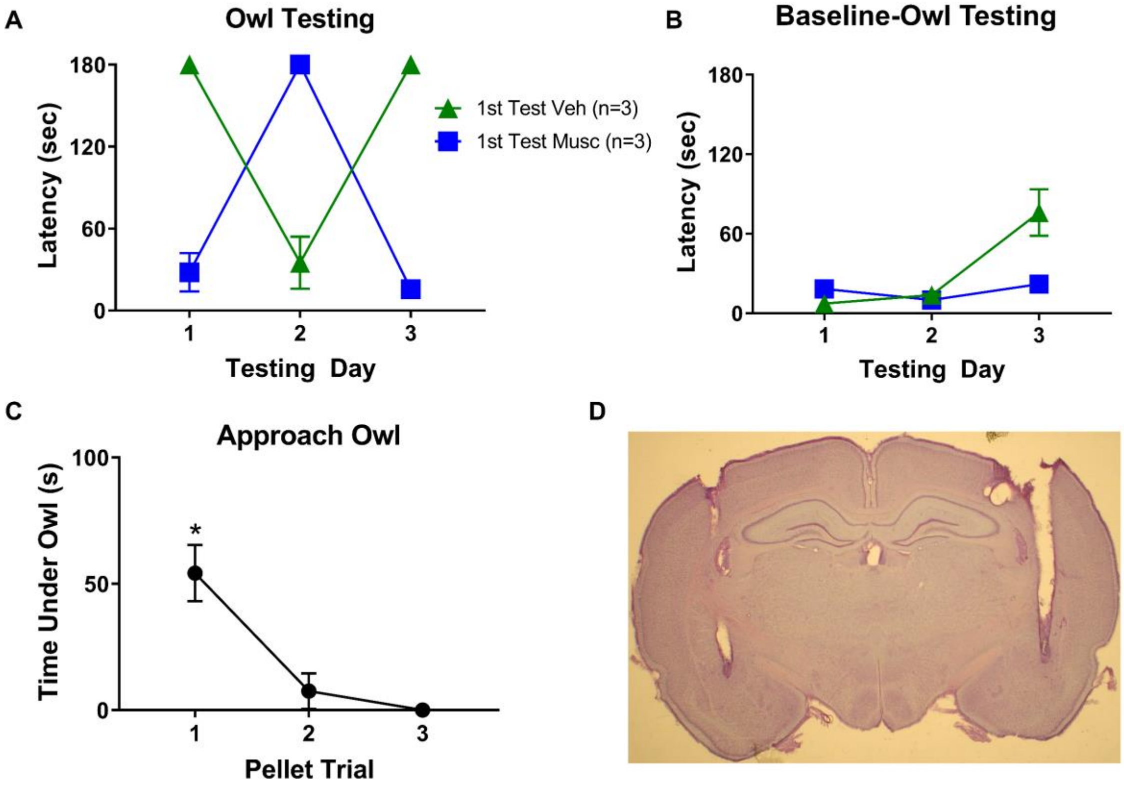

Figure 2.8. Inactivation of the basolateral amygdala eliminates fear response to owl. (A) Latency to retrieve the food pellet after intracranial infusion into the BLA of either ACSF or muscimol. Rats that received muscimol showed no fear response to the owl and rats that received ACSF failed to retrieve the pellet, regardless of treatment order. **(B)** Baseline latencies for each testing day before owl exposure trials. Rats that received ACSF on the first exposure day showed an enhanced latency to retrieve the food pellet their second time after ACSF infusion. **(C)** Time spent under the prone owl during the first exposure after infusion of muscimol. All rats, regardless of drug treatment order, spent a significant amount of time underneath the owl but only during their first exposure after muscimol infusion (related samples Wilcoxon signed ranks test; 1-2, z = -2.041, *p* = 0.031; 1-3, z = -2.201, *p* = 0.028). **(D)** Representative cresyl-violet stained section showing bilateral cannulae placement in the BLA.

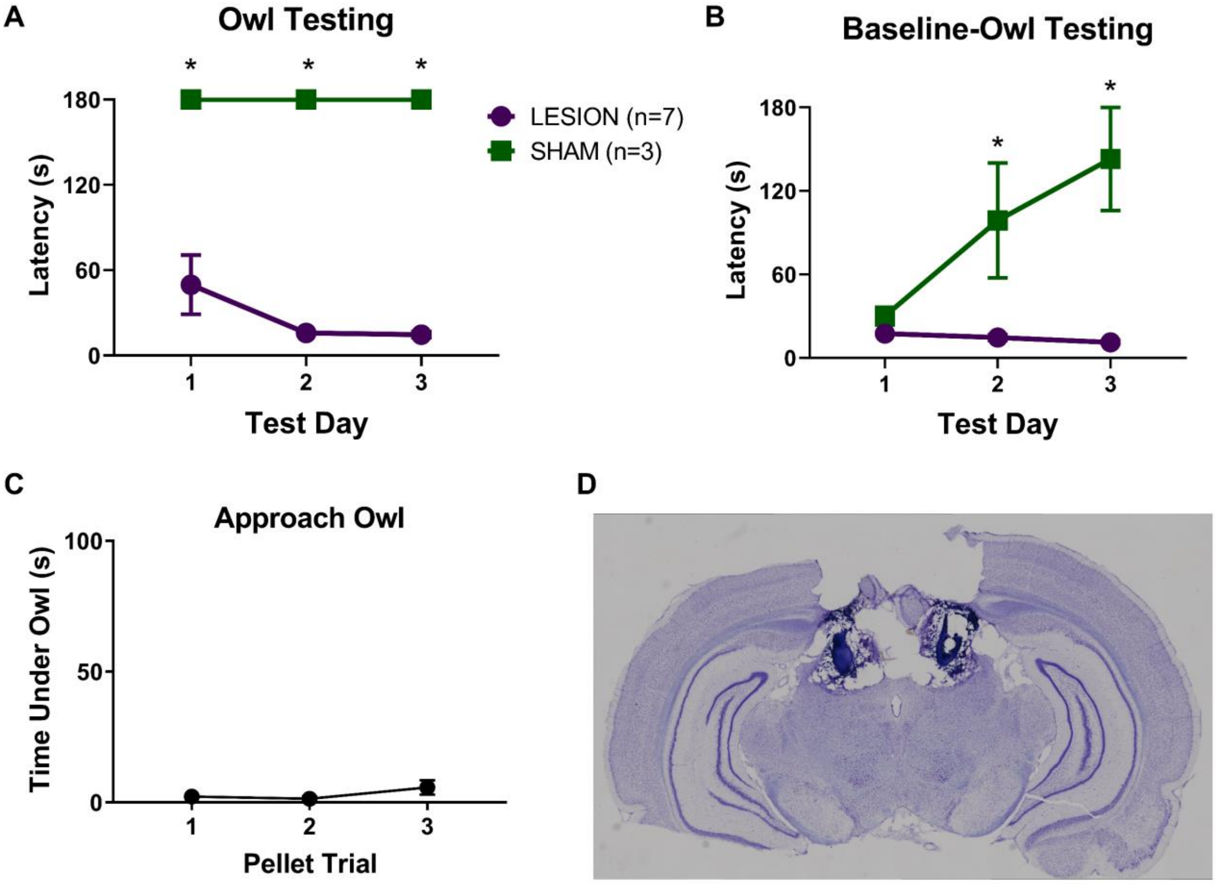

Figure 2.9. Lesioning of the superior colliculus eliminates fear response to the owl. (A) Average latencies (± SEM) to procure the food pellet during owl exposure testing days. Superior colliculus lesioned animals did not flee from the owl and had significantly shorter latencies to retrieve the pellet compared to sham animals (Mann-Whitney U Test Day 1: $z = -2.423$, $p = 0.017$; Day 2: $z = -2.433$, $p = 0.015$; Day 3: $z = -2.455$, $p = 0.017$). **(B)** Baseline latencies during testing days before owl exposure. Sham animals had significant increases in fear of the foraging context compared to lesioned animals (Mann-Whitney U Test Day 1: $z = -1.257$, $p = 0.267$; Day 2: $z = -2.393$, $p = 0.017$; Day 3: $z = -2.400$, $p = 0.016$). **(C)** Lesioned animal's time spent under the owl in its prone position during testing. There was no trial or novelty effect when lesioned animals encountered the owl for the first time. **(D)** Representative cresyl violet and Prussian blue stained section showing the extant of the electrolytic lesion.

Discussion

Prior to looming stimuli testing, evidence of sex differences was apparent during the baseline foraging sessions. Female rats were more reluctant to enter the larger, open foraging area to obtain the food pellet, preferring to remain in the safe, enclosed nest area. This is especially evident on the first baseline day. These baseline data are in accordance with reports that female rats spend less time out of cover and are more hesitant to consume food in a novel environment (Carrier and Kabbaj 2013, Turner and Burne 2014, Jolles, Boogert et al. 2015).

More female than male rats fled from the ceiling looming expanding disc on the first day of testing. Whereas the ceiling looming expanding disc most accurately simulates a rapidly descending predator among the 2D stimuli, the ceiling bar stimulus is intended to resemble an approaching "cruising" predator (Yilmaz and Meister 2013, De Franceschi, Vivattanasarn et al. 2016). That more females than males displayed LDRs specifically to the ceiling looming disc suggests that female rats show exaggerated fear toward imminent predation threat, and not just more defensive/risk-assessment behavior following predator exposure as previously reported (Blanchard, Shepherd et al. 1991). Notwithstanding, female rats showed greater hesitancy to leave the safe nest area following stimulus presentation during testing, reflected in their increased latencies and failed attempts to procure the pellet.

Previous research has shown that Long-Evans female rats perform better than males in an object recognition task in which visual acuity was measured (Seymoure and Juraska 1997). Thus, it is possible that the observed sex differences in the 2D looming experiments may be explained by a poorer vision in male rats. However, differential fear responding was evident when stimuli were projected downward onto the arena, which minimizes visual acuity differences. Specifically, more female than males fled from the floor looming block stimulus.

The block stimulus encompassed a greater area when projected onto the arena than when projected upwards onto the smaller projector screen. Thus, during the floor looming condition, when the shapes of the stimuli are largely indecipherable, the block stimulus may be perceived as higher in intensity and therefore in aversiveness compared to the floor disc and bar stimuli, which do not cover the entirety of the arena. One way to possibly eliminate this difference is by lowering the ceiling projector screen so that the stimuli appear more similar in size between conditions. The floor looming block stimulus also produces a stronger change in arena brightness. Insofar as male mice are concerned, rapid changes in brightness alone do not elicit an innate fear response (Yilmaz and Meister 2013), which is consistent with our male rat results. Nonetheless, such a large and fast change in illumination may be perceived as aversive in female rats. Finally, the absence of LDRs in males and females to the floor projected expanding disc may also be indicative of the visual system's need to perceive a rapidly approaching predator from above to initiate an LDR and not merely the shadow cast by the predator on the floor beneath the rat. Yilmaz and Meister (2013) have also shown in mice that only when presented above does the expanding disc trigger a flight response.

When animals were confronted with the 3D looming stimulus, a plastic owl "predator," there was no difference in LDRs on the first day of testing, and all animals failed to obtain the pellet. However, differences emerged over subsequent test days. Starting with the second test day, male rats' latencies began to rapidly decrease with each subsequent test. Female rats' latencies only began to decrease starting the third day of testing, but ultimately remained high for the remainder of the testing schedule. Also pronounced is the difference in baseline trial latencies following the first test day. Female rats' baseline trial latencies remained significantly higher than male rats consistently across the remaining test days, evidence of a stronger contextual fear memory formed after initial owl exposure and greater resistance to extinction. Such findings conflict with data generated from context fear conditioning studies, which show that male rats display greater levels of fear when freezing is used as the index of fear (Maren, De Oca et al. 1994, Graham, Yoon et al. 2009, Colon, Odynocki et al. 2018), and that females are quicker to return to areas previously associated with footshock (Van Oyen, Van De Poll et al. 1979, Heinsbroek, van Haaren et al. 1988, Beatty, Gregoire et al. 2013). Notably, none of the animals displayed freezing behavior to either the 2D or 3D looming stimuli in the foraging area.

Few significant changes in outbound or inbound speed were observed throughout baseline and testing sessions. Those that were significant did not correlate with a fear response or a rapid return to the nest area and might be an effect of the animals having completed many trials. As stated above, most male rats began consuming the food pellets in the foraging area during the second day of projector testing, indicating low fear to that space even though it is a bright-light condition. Female rats having significantly higher outbound speeds than male rats on the second day of owl exposure is in agreement with past research showing female rats will explore an area previously associated with predator odors faster than male rats, but show heightened fear to the odor itself (Jolles, Boogert et al. 2015). Female rats have an increase in outbound speed over male rats immediately before the owl is triggered, but male rats habituate to the direct predator encounter at a substantially faster rate. As outbound and inbound speeds were not reliably different across test days, sex differences in latency to procure the pellet were due to increased hesitancy to approach the pellet as well as number of attempts during owl encounters.

Factors relating to our foraging paradigm, chamber design, and species tested may explain the discrepant findings discussed above. In our paradigm, the animals are engaged in goal-oriented, purposive behavior (Tolman 1932) in a large arena, which effectively expands the animal’s behavioral repertoire. This paradigm was also designed to simulate an ethologically relevant "approach food-avoid predator” conflict situation, contrasting with the paradigms in which animals are arbitrarily placed into small chambers that restrict movement and behavioral capacity. However, the animals in the present study also had extensive experience in the arena before testing, and undoubtedly had thoroughly mapped the locations of the safe (nest area) versus dangerous (foraging area) zones (Kim, Park et al. 2015). These factors perhaps explain why we observed only fleeing LDRs in both 2D and 3D looming experiments. In regards specifically to our 2D looming stimuli results, the fact that we used rats instead of mice may explain why none of the animals froze to the expanding disc, despite being a considerable distance from the nest area during stimulus presentation. Though physically similar, rats and mice show considerable evolutionary divergence (Gibbs, Weinstock et al. 2004) and are thus not entirely interchangeable in behavioral experiments.

Lastly, the predator stimuli used were also made more realistic in our design, such that 2D stimuli were presented above the animal at more plausible heights (2.19 m) instead of being presented 30 cm above the animal, as in contemporary looming studies (Wallace, Greenberg et al. 2013, Yilmaz and Meister 2013, Wei, Liu et al. 2015, De Franceschi, Vivattanasarn et al. 2016). The 3D looming stimulus also realistically resembled an owl. While the 3D looming stimulus uniquely produced sound (a 3 dB increase in the background of 72 dB white noise) and potentially caused an air breeze upon trigger—potential confounds—the owl likely represents a more intense predator stimulus than the 2D stimuli, which can support contextual fear conditioning (Blanchard, Griebel et al. 2003). In fact, most animals fully habituate to the 2D stimuli by test day 2, suggesting that they are relatively weak danger stimuli. Indeed, one other study performed two days of testing with an overhead expanding disc and likewise found a dramatic reduction in fear responses by the second day of testing (Wei, Liu et al. 2015).

An evolutionary-developmental theory may explain in a more general sense the observed sex differences in risky-foraging behavior. As male and female mammals develop, divergent behaviors are predicted to emerge as a result of differential reproductive and survival goals (Kodric-Brown and Brown 1987, Ellis, Figueredo et al. 2009, West-Eberhard 2014). Males typically devote more effort toward reproduction, whereas females typically devote more effort toward offspring development (i.e., “parenting”); thus, risk-taking may be more beneficial for males in that it can increase the chances of mate access, whereas risk-aversion in female rats helps ensure stable parenting of offspring (Kodric-Brown and Brown 1987, Steinberg 2008, Jolles, Boogert et al. 2015). However, the possibility that food restriction, implemented in our study to motivate the animals to engage in the foraging task, in part accounted for the observed sex differences cannot be excluded.

Not surprisingly, rats with inactivated BLA’s showed no fear response when encountering the owl, as has been demonstrated in other foraging-predator encounter tasks (Choi and Kim 2010), and it has been shown to play a crucial role in the expression of most fear behaviors (Fanselow and Gale 2006). An interesting result of the BLA inactivation was the approach towards novelty that was observed only during the first muscimol trial of the cannulated rats. Even for the group of rats that had two days of muscimol testing, on the 1st trial on the 2nd day of muscimol infusion, there was no approach into the foraging area under the owl. This suggests that the memory of the owl from the previous days of testing has remained even with muscimol infusion, but only as another object in the foraging arena. In both human and rat studies the amygdala has been implicated in novelty detection and avoidance responses like marble burying tasks (Burns, Annett et al. 1996, Blackford, Buckholtz et al. 2010). For prey animals like the rat, it is in their best interest to react to most novel objects in a fearful manner, especially when it is plunging down at them from above.

There is also evidence to support that this innate fear to looming stimuli has been evolutionarily conserved through to humans. When faced with looming and threatening animate pictures (spiders and snakes), humans have a significant increase in activity within the amygdala (Coker-Appiah, White et al. 2013). No responses in fear or threat detection brain areas are observed when the same participants are presented with looming images of inanimate threatening objects (knives and guns). However, there were no fear behaviors or self-reported feelings of fear from the participants. It could be that the initial recognition of an evolutionarily relevant and dangerous animal primes the defense circuits of the brain in case a survival response is necessary.

The electrolytic lesioning of the SC also ablated any fear response to the owl, while having no other effects on foraging behaviors. Previous studies have shown the necessity of the SC in the innate fear response to looming stimuli (Wei, Liu et al. 2015), but this was only performed using 2D stimuli that rats normally habituate to rapidly. The current study has shown that even with the remaining fear network of the brain intact, without the SC processing the 3D owl stimulus, no LDR’s will occur. It would be interesting for future studies to use eye-tracking techniques in freely roaming rats, like that used in Wallace et al. (2013), to determine if constant monitoring of the area above the rat is still performed with lesioned or inactivated SC's.

In conclusion, both 2D and 3D looming stimuli can elicit LDRs in male and female rats, albeit differentially. 2D looming stimuli are most effective in female rats, who reliably respond to the ceiling looming expanding disc on the first day of testing. Males, on the other hand, responded to the expanding disc at lower rates. Both sexes exhibit robust fear responding to a 3D owl stimulus, which can support contextual fear conditioning. However, female rats consistently show greater, longer-lasting fear across the owl test sessions. Ultimately, these data reflect the notion that female rats are more sensitive to aerial predation threat and respond more defensively following predator exposure. Notably, in contrast to fear conditioning studies that reported stronger contextual freezing in male rats than females rats (Maren, De Oca et al. 1994, Graham, Yoon et al. 2009, Colon, Odynocki et al. 2018), our ecologically-relevant foraging with looming threat paradigm, where female rats show significantly greater and enduring fear behavior than male rats, may have translational relevance as the observed sex differences parallel human conditions where women have a higher incidence of anxiety and posttraumatic stress disorders than men (Olff 2017). Future experiments utilizing our naturalistic looming paradigm may seek to identify the neural circuits involved in generating the general LDR seen in both sexes following 2D and 3D stimuli, as well elucidate whether potential differences in anatomy and/or function of those circuits contributes to the observed sex differences.

Chapter III: Pavlovian Fear Conditioning Does Not Readily Occur in a Naturalistic Setting

Introduction

Since the time of Watson and Morgan's (Watson and Morgan 1917) conception that emotions, such as fear, should be studied as conditional (acquired) reactions and Watson and Rayner's (Watson and Rayner 1920) demonstration that fear can be rapidly learned in 9-month-old "Little Albert," Pavlovian (or classical) fear conditioning has been the paradigm par excellence for studying both normal and abnormal fear behaviors (LeDoux 1998, Fendt and Fanselow 1999, Bouton, Mineka et al. 2001, Maren and Quirk 2004, Kim and Jung 2006). Briefly, fear conditioning focuses on how an initially innocuous conditional stimulus (CS; e.g., auditory, visual, contextual cues), upon pairing with a noxious unconditional stimulus (US; usually electric shock) that reflexively elicits unconditional responses (UR; namely defensive reactions), becomes capable of eliciting conditional responses (CR; e.g., freezing in rodents, increased skin conductance in humans). A century of fear conditioning research has led to wide-ranging discoveries. In particular, fear conditioning experiments have fundamentally transformed learning theories from the archaic contiguity (or temporal) relationship (Watson 1913, Pavlov 1927, Guthrie 1930) to the modern contingency (or informational) relationship between the CS and US (Kamin 1968, Rescorla 1968, Wagner, Logan et al. 1968, rescorla and Wagner 1972), revealed detailed neurobiological mechanisms of learning and memory (Haubensak, Kunwar et al. 2010, Tovote, Fadok et al. 2015, Josselyn and Tonegawa 2020) and influenced contemporary cognitive behavioral therapy for various anxiety and traumatic-stressor related disorders, such as panic, phobic and posttraumatic stress disorders (Foa and Rothbaum 1998, Butler, Chapman et al. 2006, Delgado, Olsson et al. 2006, Craske, Rauch et al. 2011, Mahan and Ressler 2012).

Despite the utility and appeal of fear conditioning paradigms, in particular the fact that conditioned fear memory can transpire after *a single* CS-US pairing and be retained across the adult lifespan (LeDoux 1996, Fanselow 2010), they nonetheless simplify behavioral analyses of fear, ignoring the multitude of actions and decisions that animals and humans utilize to survive the breadth of risky situations in the real world (Lima and Dill 1990, Bednekoff 2007, Stephens 2008, Beckers, Krypotos et al. 2013, Mobbs and Kim 2015, Pellman and Kim 2016). Indeed, standard rodent fear conditioning studies performed in small experimental chambers encapsulate Thorndike's notion of studying unadulterated learning by placing animals in artificial situations that inhibit "instinctive activities (e.g., instinctive fears)," as instinctive behaviors may be opposite to learned behaviors in complex environments (Thorndike 1900). Hence, the prevalent notion that fear conditioning produces biologically functional associative fear memory needs to be ecologically validated. In fact, some researchers have questioned the evolutionary logic underlying fear conditioning; "No owl hoots or whistles 5 seconds before pouncing on a mouse…Nor will the owl give the mouse enough trials for the necessary learning to occur…What keeps animals alive in the wild is that they have very effective innate defensive reactions which occur when they encounter any kind of new or sudden stimulus" (Bolles 1970). Consistent with this contrarian view are findings that laboratory rodents exhibit unlearned, instinctive fear responses to advancing artificial terrestrial and aerial predators (Choi and Kim 2010, Zambetti, Schuessler et al. 2019), overhead looming stimuli (Yilmaz and Meister 2013), and predator odors (Papes, Logan et al. 2010).

Here, we investigated for the first time whether fear conditioning readily transpires and modifies subsequent behavior of animals in a naturalistic environment. To achieve this, hunger-motivated rats searching for a food pellet in a large arena—that is, engaging in a purposive behavior as they would in nature (Tolman 1948)—were presented with a discrete tone CS followed by a painful US to their dorsal neck/body region by means of chronically implanted subcutaneous wires (Figure 3.1 A). A dorsal neck/body shock better simulates real predatory strike compared to footshock used in standard fear conditioning studies, as it is unlikely that predators direct their attacks on small prey animal's paws. Additionally, in nature, bodily injuries are normally inflicted by external agents (namely, predators in animals and perpetrators in humans). Thus, other groups of rats were presented with a looming aerial predator (i.e., a lifelike great horned owl) preceded with and without a tone CS and followed by the same US (Figure 3.1 B-D). A single trial tone-shock, tone-owl, tone/owl-shock and owl-shock training was employed because multiple CS-US trial-and-error (rehearsal) learning, endangering the animal to repeated bodily harm would prove fatal in nature, antithetical to the natural selection of fear conditioning (Bolles 1970, LeDoux 1996, Fanselow 2010). Later, all animals' reactions to the tone cue were examined while foraging for food in the open arena.

Methods

Subjects

Seventy Long-Evans rats (3-4 months old; 36 females and 34 males, RRID:RGD_2308852), purchased from Charles-Rivers Laboratories, were initially pair-housed by sex for 5-7 days of acclimatization in a climate-controlled vivarium (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), with a reversed 12-h light/dark cycle (lights on at 7 PM). After undergoing subcutaneous wire implant surgery (described below), animals were individually housed and placed on a standard food-deprivation schedule with *ad lib* access to water to gradually reach and maintain ~85% normal body weight. All experiments were performed during the dark phase of the cycle in strict compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Surgery

Under isoflurane anesthesia, rats were mounted on a stereotaxic instrument (Kopf), and two Teflon-coated stainless-steel wires (0.0003 inch bare, 0.0045 inch coated; A-M Systems, Everett, WA) were inserted in the dorsal neck/back region of body. The wire tips were exposed (~1 cm), bent to a V-shape, and hooked to subcutaneous tissue (Lee and Kim 2004). The other ends of the wires were affixed to a headstage (Plastics One, MS303-120), which was then cemented to the animal's skull embedded with 6 anchoring screws. While still under anesthesia, animals were connected to a shock-apparatus and given a mild shock to observe muscle twitching; 6 rats that showed no reaction to shock were removed from the experiment. Animals were given 4 days of postoperative recovery and were adapted to handling for 5 days before nest habituation.

Foraging Apparatus and Stimuli

A custom-built foraging arena consisted of a nest (69 cm length x 58-66 cm width x 61 cm height) that opened via an automated sliding gate to reveal a large, expanded foraging area (208 cm length x 66-120 cm width x 61 cm height) where 0.5 g food pellets (grain-based; F0171, Bio-Serv) were placed at variable locations (Figure 3.1 A). The testing room was kept under red light (11 lux foraging area, 2 lux nest area) with constant white noise (72 dB) playing in the background. Prior to placing each animal, the arena was wiped with 70% ethanol. The ANY-maze software and Ami interface system (Stoelting) connected to a PC automatically tracked the animal's position in the arena, via a ceiling mounted camera, and triggered the tone, shock and aerial predator stimuli: (i) 3 kHz, 80 dB tone CS was produced using Anymaze (Stoelting) and presented through two speakers mounted on the nest-foraging border; (ii) 1 s, 2.5 mA shock US was delivered to the animal's dorsal neck/back region via a headstage tethered to a stimulus-isolator (Bak); (iii) A life-like model owl (Zambetti, Schuessler et al. 2019), mounted onto a 92 cm pneumatic air cylinder (Bimba) at the opposite end of the foraging arena and hidden behind a black curtain, plunged downward towards the rat (46 cm/s), then retracted back to it starting position.

Behavioral Procedure for Naturalistic Fear Conditioning

A total of 62 rats (32 females and 30 males) were used to investigate fear conditioning in an ecologically-relevant environment. Upon reaching and maintaining 85% normal body weight, animals were transported to the experimental room and underwent series of habituation, baseline, fear conditioning, and testing sessions.

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

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

Fear conditioning day Rats, pseudo-randomly assigned into tone-shock, tone-owl, tone/owl-shock and owl-shock groups (Figure 3.1), underwent 3 baseline trials with the pellet placed at 125 cm from the nest. On the 4th trial, the tone-shock, tone-owl and tone/owl-shock animals were exposed to a tone CS that came on 5 seconds before the gate opened and remained on until they reached the trigger zone (25 cm to the pellet). For tone-shock and tone-owl animals, the tone co-terminated with the shock US and the owl looming, respectively. For tone/owl-shock animals, the shock occurred 0.1s sec after the owl looming and co-terminated with the tone. Two animals in the tone/owl-shock group were excluded because they failed to leave the nest within 2 min. The owl-shock animals were subjected to the same owl looming-shock pairing (as the tone/owl-shock animals) but in the absence of tone. All rats fled to the nest in reaction to the shock and/or looming owl, at which time the gate was closed. After 1 minute in the nest, the animals were placed back into their homecage.

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

Behavioral Procedure for Standard Fear Conditioning

A total of eight rats (four females and four males) with *ad libitum* access to food and water underwent one trial tone fear conditioning (Wilensky, Schafe et al. 2000). A day prior to fear conditioning (day 0), animals were tethered and placed in an experimental chamber for 10 minutes of pre-exposure. Fear conditioning (day 1) commenced after 3 minutes of baseline in the chamber by exposing animals to 24.1 s tone CS (3 kHz, 80 dB) that co-terminated with 1 sec dorsal neck/body shock (2.5 mA). The 24.1 s tone was based on the mean tone CS duration from the tone-shock group of the naturalistic fear conditioning experiment. Postshock freezing was assessed for 1 min before animals were removed from the conditioning chamber. For tone test (day 2), animals were placed in a novel chamber that differed in terms of the wall pattern, floor texture, background light, and smell (Lee and Kim 1998). After 1 minute of baseline, the tone was presented for 3 minutes to assess CS-evoked freezing response, and the animals were left in the chamber for an additional minute before being placed back in their homecage. Freezing was quantified using Anymaze (Stoelting) tracking software with the freezing threshold set to 2 seconds.

Data Analyses

Statistical analyses were performed using SPSS (IBM, version 19) and R (The R Foundation, version 3.5.3). Body tracking positions were obtained using Deep Lab Cut (Mathis, Mamidanna et al. 2018) and analyzed using a self-written script in Python (Python Software Foundation). Animal sample sizes were determined using a power analysis performed by G*Power (G*Power, version 3.0.1, Franz Faul; power=0.95, alpha=0.05, effect size=0.5, two-tailed). A Levene's test for normality showed significance for the data, thus nonparametric tests were used for analyses. Because there were no significant sex differences in any stages of the experiment after the first day of baseline (Figure 3.2 and Table 3.1), data from females and males were pooled together for all analyses. Statistical significance was set at $P < 0.05$. Graphs were made using GraphPad Prism (version 8).

For the analyses of escape trajectories (Figure 3.3 H and I), the coordinate data of each rat in the foraging arena taken at a frequency of 10 Hz was used to obtain the change in position vectors between each time point (black) and an overall change in position vector (red). To obtain the individual change in position vectors, we used Python and the Numpy, Pandas, and Matplotlib packages to calculate the changes in x and y position between coordinates. With each change in x and y positions, we were able to calculate the magnitude of the distance traveled and the angle of travel using an inverse tangent function. The resultant vector representing the average change in position vector was determined by taking the average change in x position and average change in y position to calculate an overall magnitude and angle. The (population) variance and standard deviation of the angles of the change in position vectors were obtained using Numpy.

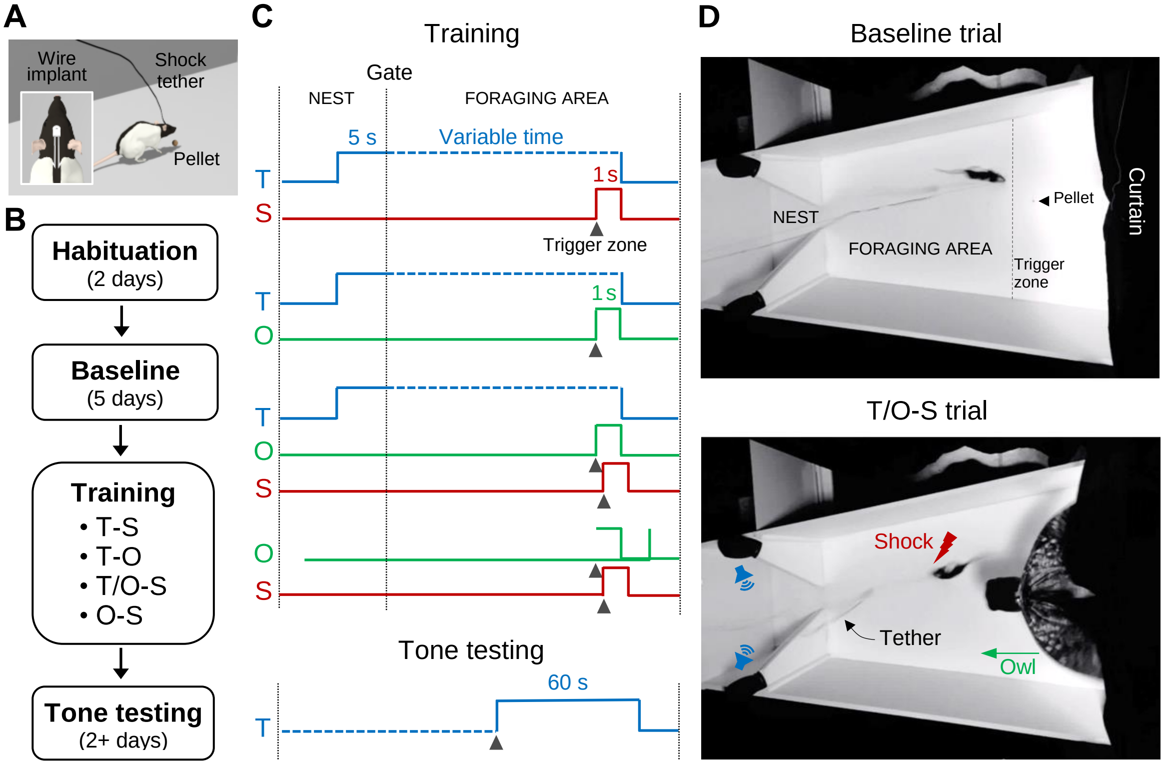

Figure 3.1. Experimental design of fear conditioning in a naturalistic setting. (A) An illustration of a tethered rat foraging for a food pellet in the open arena (inset shows a headstage and placement of subcutaneous shock wires). (**B**) Timeline of experiment. *Habituation*: Rats were placed in a closed nest with dispersed food pellets for 30 min/day. *Baseline*: Rats were allowed to leave the nest to discover food pellets placed 25-125 cm (in 25 cm increments from the nest) in the foraging arena. *Training*: Animals approaching the pellet location experienced a delayed pairing of tone-shock (T-S), tone-owl (T-O), tone/owl-shock (T/O-S), or owl-shock (O-S). *Tone Test*: On subsequent days, all rats were placed back in the foraging arena and upon nearing the food pellet, the tone was activated. (**C**) Schemas of delayed 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 the gate opening that stayed on until the animals were within 25 cm of the food pellet, at which the tone co-terminated with the triggered shock (1 s), owl (1 s) or owl-shock (100 ms interstimulus interval, ISI) stimuli. (**D**) A representative rat in the foraging arena (208 cm length x 66-120 cm expanding width x 61 cm height) during a baseline trial, where the animal successfully acquires the pellet, and during a T/O-S trial, where the animal flees from looming owl and shock into the nest (69 cm length x 58-66 cm width x 61 cm height).

Results

Baseline foraging in an ethologically-relevant environment

Female and male rats were pseudo-randomly assigned to tone-shock (8 females, 8 males), owl-shock (8 females, 8 males), tone/owl-shock (6 females, 8 males), and tone-owl (4 females, 4 males) groups and implanted with subcutaneous wires in their dorsal neck/body (Fig. 1a-c). After recovery from surgery and habituation to the nest compartment of the arena, the rats were trained to exit the nest via a computer-controlled automated gateway to procure a sizable 0.5 g food pellet placed at variable distances in the large, expanding open area of the arena (Figure 3.1 D, top panel). Once the animals returned to the nest for pellet consumption, the gateway closed until the next trial (3 trials/day). On the first baseline day, female rats took a significantly longer amount of time to procure the food pellet compared to male rats (Figure 3.2, Baseline day 1). This initial difference in foraging behavior likely represents heightened spatial neophobia (risk-averse to novel environments) in female rats. As rats became familiar with the foraging arena, the latency and duration measures declined across 5 baseline days comparably in both sexes, with no further statistical differences in latencies for pellet procurement. Because there were no reliable sex differences in subsequent fear conditioning dependent variables (Table 3.1), the four groups were collapsed across sexes.

Fear conditioning in an ethologically-relevant environment

On the training day, all rats first underwent three foraging trials with pellets fixed at the longest distance (125 cm) to confirm comparable pre-fear conditioning foraging behavior between groups (Figure 3.3 A, Baseline). Afterwards, animals were exposed to a tone-shock, an owl-shock, a tone/owl-shock or a tone-owl pairing in the manner shown in Figure 3.1. Those rats presented with the tone CS 5-sec prior to the gate opening (i.e., tone-shock, tone-owl, tone/owl-shock groups) took more time to enter the foraging arena in comparisons to owl-shock animals unexposed to the tone (Figure 3.3 B, Leave nest latency); this indicates that the tone was a salient cue that animals were attentive to and thus conditionable. Once in the foraging arena, all animals readily advanced toward the pellet and breached the trigger zone (25 cm from the pellet) to activate the shock, owl, or owl-shock stimuli (Figure 3.3 B, Trigger zone latency). In response to the shock, owl, or owl-shock, all rats promptly fled from the foraging arena to the nest (Figure 3.3 B, Escape latency; Figure 3.3 D, E, Escape speed). Figure 3.3 C shows representative track plot examples of tone-shock, owl-shock, tone/owl-shock and tone-owl animals successfully procuring the pellet during pre-tone baseline but not during tone conditioning. The fact that the escape latency and running speed were not significantly different between the tone-owl and other groups indicates that the looming owl-induced innate fear sans pain was just as effective in eliciting the flight UR as the painful shock or owl-shock combination. However, inspections of the escape trajectories revealed that the tone-shock and tone-owl groups tended to flee linearly to the nest, whereas the owl-shock and tone/owl-shock groups that experienced a dorsal neck/body shock 100 ms after the looming owl (mimicking realistic predatory attack) and begun their flight to the nest inclined to escape circuitously (Figure 3.3 F, H). This was supported by significant group differences in the escape distances (Figure 3.3 G) and variance of trajectory angles (Figure 3.3 I), where owl-shock and tone/owl-shock groups traveled longer distances and had higher angle variances, respectively, during their escape routes than tone-shock and tone-owl groups.

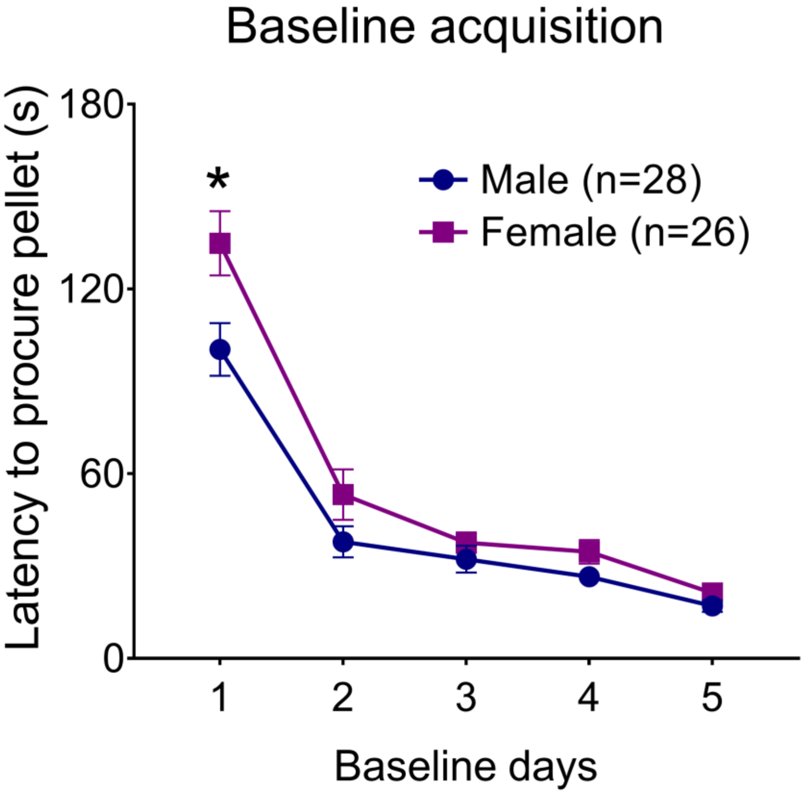

Figure 3.2. Initial sex differences in the baseline latency to procure pellets. Average latencies (±SEM) to procure food pellets in foraging area. Females had longer latencies to procure pellets than males during the first baseline session day 1 (Mann-Whitney U, $z = 2.476$, $p = 0.013$) but not subsequent 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: $z = 1.904$, $p = 0.057$). * $p < 0.05$.

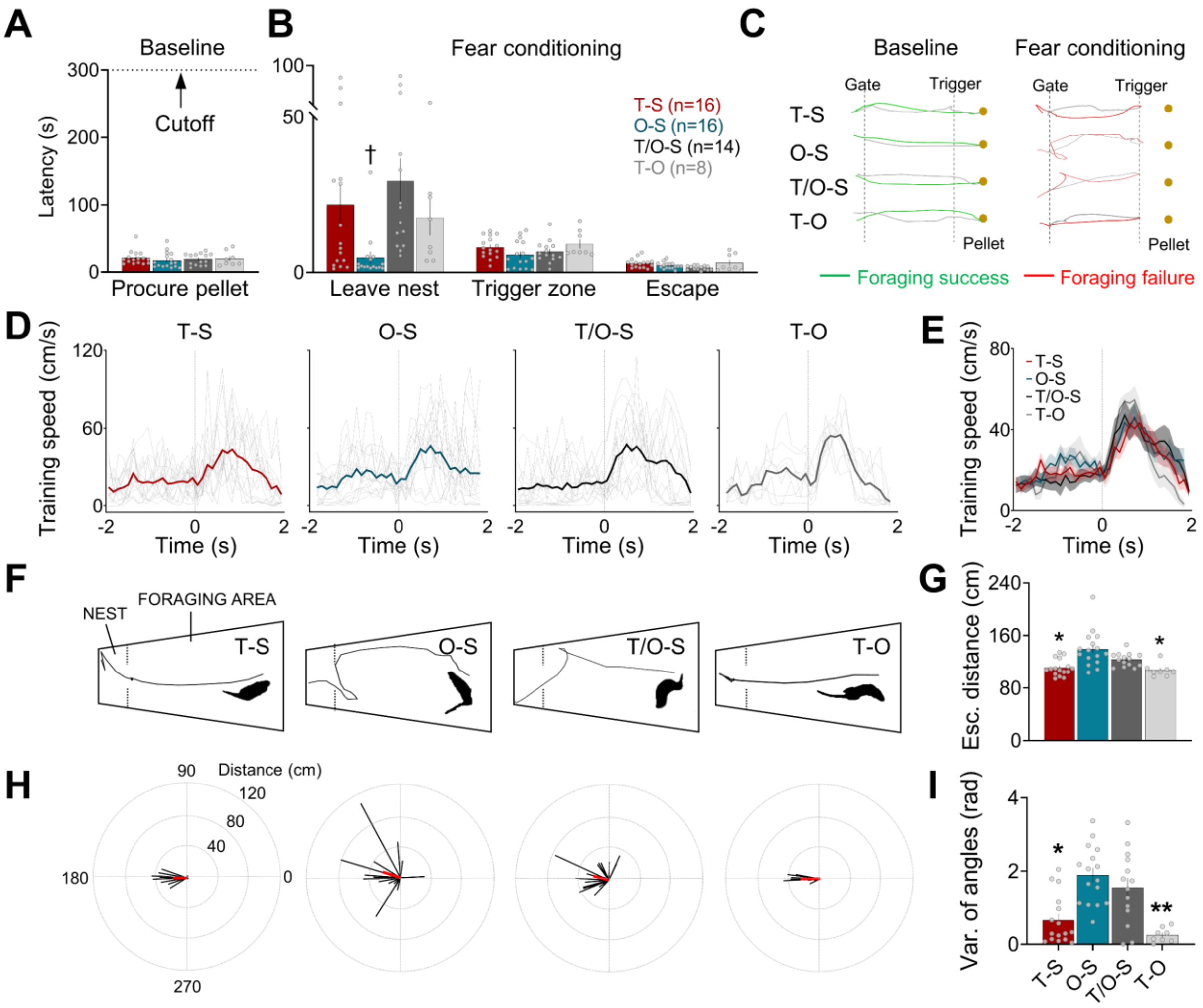

Figure 3.3. Foraging and escape behaviors during fear conditioning. (A) Pre-conditioning baseline latencies (mean + SEM) to procure food pellets in the foraging arena were equivalent between T-S (red), O-S (blue), T/O-S (dark gray) and T-O (light gray) groups (Kruskal-Wallis, H = 2.694, *p* = 0.441). (**B**) During fear conditioning, the T-S, T/O-S and T-O groups exposed to the tone 5 s before the gate opening had significantly longer latencies to leave the nest than the O-S group (left panel, Kruskal-Wallis, H = 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 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 outside the nest, however, the latency to breach the trigger zone, en route to the pellet, was not reliably different among the groups (Kruskal-Wallis, H = 7.453, *p* = 0.059). In response to the triggered shock, owl or owl-shock, all groups showed similar escape-to-nest latencies (Kruskal-Wallis, H = 6.141, *p* = 0.105). (**C**) Representative track plot examples from T-S, O-S, T/O-S and T-O animals during the baseline, when animals successfully procured the pellet, and during the fear conditioning, when the same animals fled from shock, owl or owl-shock stimuli and thus unable to attain the pellet. (**D**) Mean instantaneous speed (+ SEM) of each group 2 sec before and after the shock, owl or owl-shock onset (t = 0). Thin, grey lines represent individual animal data. (**E**) All groups showed comparable escape speed to the shock, owl, and owl-shock stimuli (Kruskal-Wallis, H = 0.901, *p* = 0.825). (**F**) Representative track plots showing escape 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 animals were facing forward at the time of the shock or owl stimulus whereas the O-S and T/O-S animals were turning back at the time of the shock stimulus because of the 100 ms owl-shock interstimulus interval. (**G**) Mean escape distance (± SEM) from the trigger zone to the nest. The O-S and T/O-S groups travelled longer distances to escape compared to the T-S and T-O groups (Kruskal-Wallis, H = 21.98, $p < 0.001$; 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 vs. O-S, $p = 0.001$ for O-S vs T-O). (**H**) Representative vector plots of each group showing variabilities in their escape paths. (**I**) Mean variance (± SEM) of escape trajectory angles (radian) from the trigger zone to the nest. The O-S and T/O-S groups had greater variance in their escape trajectories when fleeing back to the nest (Kruskal-Wallis, H = 22.37, $p < 0.001$; pairwise comparisons, $p = 0.022$ for T-S vs. T/O-S, $p = 0.003$ 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). († compared to T-S, 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, $p < 0.05$, ## $p < 0.01$).

Context (pre-tone) testing in an ethologically-relevant environment

On the following day, animals were placed back in the nest and underwent three pre-tone baseline trials (maximum 300 sec to retrieve the food pellet placed at 125 cm) to assess whether previous encounters with tone-shock, owl-shock, tone/owl-shock and tone-owl stimuli combinations produced fear of the arena. As can be seen in Figure 3.4 A, the owl-shock and tone/owl-shock groups took significantly longer latencies to procure the pellet (i.e., the time from gate opening-to-return to nest with the pellet) than the tone-shock and tone-owl groups on the first day of testing. The lengthened times to enter the foraging arena exhibited by owl-shock and tone/owl-shock rats likely reflect inhibitory avoidance resulting from the previous predatory attack experience in the arena (Wilensky, Schafe et al. 2000). In contrast, the fact that the pre-tone test baseline latencies of tone-shock and tone-owl rats (Figure 3.5) were not reliably different from their baseline latencies from the fear conditioning day (prior to experiencing tone-shock or tone-owl) suggests that contextual fear conditioning failed to transpire in these animals despite their robust escape behavior to tone-shock and tone-owl experiences. Similar patterns of group differences, albeit lesser magnitudes, were observed on the second day of pre-tone baseline trials (Figure 3.4 C).

Tone testing in an ethologically-relevant environment

Immediately after the pre-tone baseline, all groups were subjected to three successive tone test trials (one minute apart). The owl-shock and tone/owl-shock animals continued to take longer latencies to exit the nest compared to tone-shock and tone-owl animals (Figure 3.4 B, Leave nest latency). Once in the foraging arena, the tone/owl-shock group’s latency to approach 25 cm from the pellet to trigger the tone were marginally but reliably longer than those of tone-shock and tone-owl groups, but not the owl-shock group (Figure 3.4 B, Trigger zone latency).

Upon the activation of tone (60 s continuous), the majority of owl-shock and tone/owl-shock animals promptly fled to the nest, thereby significantly increasing the latency to procure the pellet (60 s = unsuccessful), whereas the tone-shock and tone-owl animals were largely unaffected by the tone and readily procured the pellet (Figure 3.4 B, Procure pellet latency). The second day of tone testing yielded similar patterns of group differences (Figure 3.4 D). Figure 3.4 E shows individual track plots from all animals with the initial number of trial(s) necessitated for successful foraging. Further analyses across tone testing days (3 trials/day) showed that the overall success rates of procuring the pellet were significantly lower in owl-shock and tone/owl-shock groups compared to tone-shock and tone-owl groups (Figure 3.4 F), and that owl-shock and tone/owl-shock animals required extended trials to reliably obtain the pellet (Figure 3.4 G). Because the temporal interval between the CS and US is well known to be crucial in various types of Pavlovian conditioning, including fear conditioning (Lee and Kim 2004), we examined whether tone fear conditioning transpired in a specific (optimal) range of interstimulus intervals (ISI) but was masked by non-optimal ISIs. We found no significant correlation between the ISIs and the magnitudes of tone-induced suppression of pellet procurement in tone-shock animals, indicating that tone fear conditioning failed to materialize across varying ISIs of delay conditioning (Figure 3.4 H). Conversely, in the tone/owl-shock animals, the tone-induced suppression of pellet procurement was uniformly observed across different ISIs, suggesting that the observed fear in these animals may not necessarily reflect Pavlovian conditioning (Figure 3.4 H). These results of delayed tone-shock paired animals failing to show conditioned tone fear and contextual fear suggest that standard fear conditioning does not readily occur in naturalistic environment. Instead, the finding of owl-shock animals displaying robust fear to a novel tone, which the animals never heard before, suggests that non-associative sensitization-like processes play a crucial role in protecting animals in the real world.

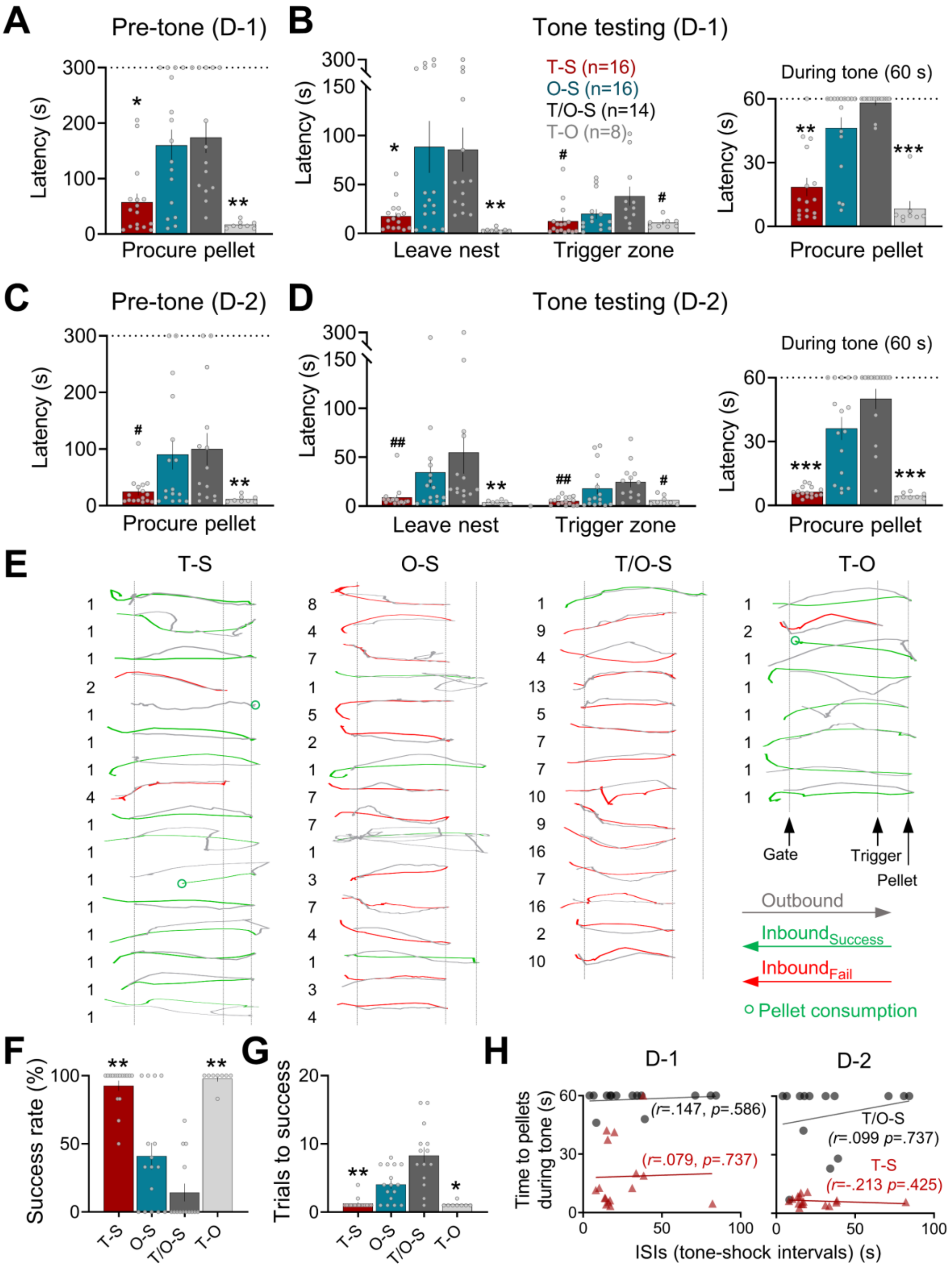

Figure 3.4. Foraging and escape behaviors during tone testing. (**A**) The mean latency (± SEM) to procure the pellet during the pre-tone baseline trials on testing day 1 (D-1). Both O-S and T/O-S groups took significantly longer times to exit (gate opening, t=0) and return to the nest with the pellet than T-S and T-O groups (Kruskal-Wallis, H = 20.518, *p* < 0.001; pairwise

comparisons, $P = 0.003$ for T-S vs. T/O-S, $p < 0.001$ 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 ± SEM) to leave nest and reach trigger zone on day 1 tone test trials. Both O-S and T/O-S groups had longer latencies to leave nest (Kruskal-Wallis, H = 27.071, $p < 0.001$; pairwise comparisons, $p = 0.003$ for 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 outside the nest, the T/O-S group took longer time to reach the trigger zone than the T-S and T-O (Kruskal-Wallis, H = 9.153, $p = 0.027$; pairwise comparisons, $p = 0.019$ for T-S vs. T/O-S, $p = 0.042$ for T/O-S vs. T-O). During the tone test, the latencies to procure the pellet within the 60 s allotted time were significantly longer in O-S and T/O-S animals compared to T-S and T-O animals (Kruskal-Wallis, H = 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, $p = 0.002$ for T-S vs. O-S, $p < 0.001$ for O-S vs. T-O). (**C**) The mean latency (± SEM) to procure the pellet during the pre-tone baseline trials on testing day 2 (D-2). O-S and T/O-S groups continued to have longer latencies to exit (gate opening, t=0) and return to the nest with the pellet than T-S and T-O groups (Kruskal-Wallis, H = 12.47, $p = 0.006$; pairwise comparisons, $p = 0.022$ for T-S vs. T/O-S, $p = 0.002$ for T/O-S vs. T-O, $P = 0.009$ for O-S vs. T-O). (**D**) The times (mean ± SEM) to leave nest and reach trigger zone on day 2 tone test trials. There were group differences in the latencies to leave nest (Kruskal-Wallis, 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, $p = 0.002$ for O-S vs. T-O). Once outside the nest, there were group differences in the latencies to reach the trigger zone (Kruskal-Wallis, H = 21.531, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.037$ for O-S vs. T-O). During the tone test, the latencies to procure the pellet within the 60 s allotted time were significantly longer in O-S and T/O-S animals compared to T-S and T-O animals (Kruskal-Wallis, H = 37.223, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p < 0.001$ for T-S vs. O-S, $p < 0.001$ for O-S vs. T-O). (**E**) Individual track plots from all animals from each group displaying the XY trajectory coordinates each rat took during the first tone exposure. The parenthesized numbers next to plots represent the trial(s) needed for successful foraging. (**F**) The overall success rates of procuring the pellet on the first testing day were significantly lower in the O-S and T/O-S groups compared to the T-S and T-O groups (Kruskal-Wallis, H = 32.299, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.001$ for T-S vs. O-S, $p = 0.003$ for O-S vs. T-O). (**G**) The O-S and T/O-S animals required extended trials to obtain the pellet (Kruskal-Wallis, H = 32.004, $p < 0.001$; pairwise comparisons, $p < 0.001$ for T-S vs. T/O-S, $p < 0.001$ for T/O-S vs. T-O, $p = 0.002$ for T-S vs. O-S, $p = 0.011$ for O-S vs. T-O). (**H**) In T-S and T/O-S animals, there were no reliable correlations (Spearman’s correlation coefficient) between the tone-induced suppression of pellet procurement (an index of fear) and the temporal intervals (i.e., ISIs) between tone CS onset and shock US onset in neither testing day 1 nor 2. (* compared to both O-S and T/O-S, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # compared to T/O-S, $p < 0.05$, $p < 0.01$).

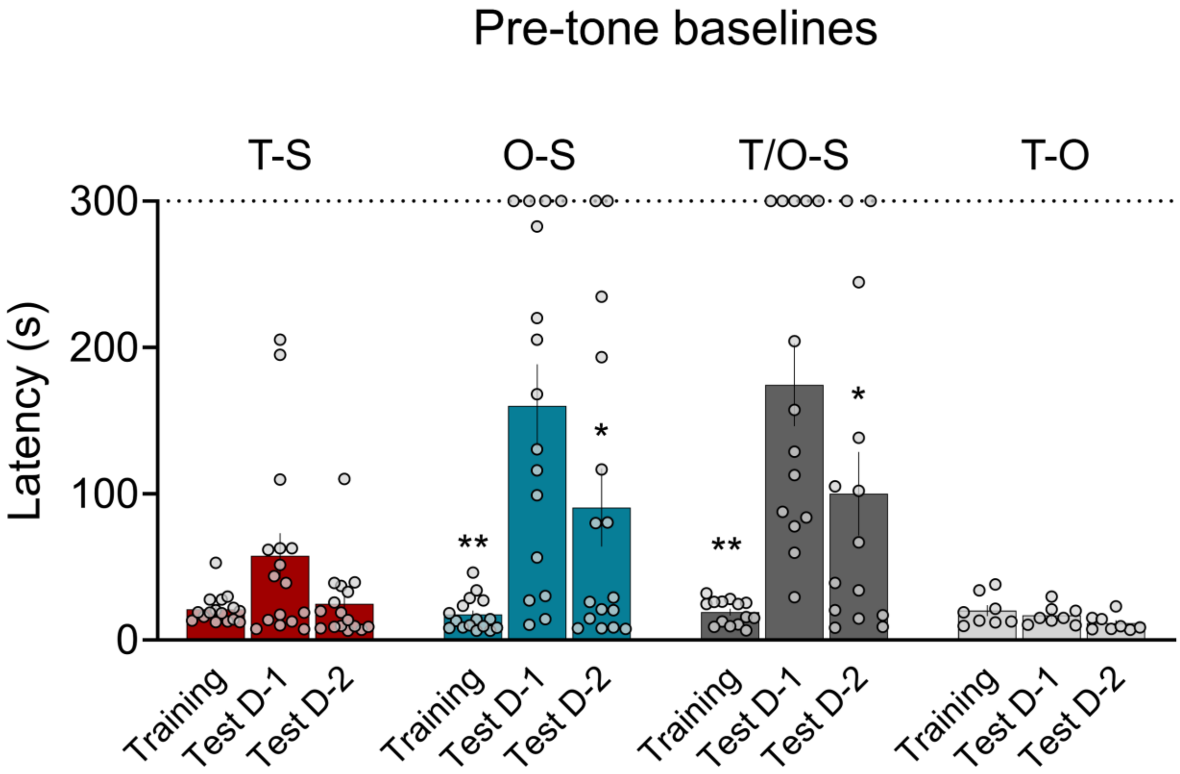

Figure 3.5. Comparisons of latencies to procure pellets during pre-fear conditioning baseline and pre-tone testing baseline days 1 and 2. The baseline latencies to procure pellets prior to the fear conditioning session (Figure 3.3 A) were not statistically different from the day 1 (Figure 3.4 A) and day 2 (Figure 3.4 C) pre-tone test baseline latencies after the fear conditioning session in both tone-shock (T-S) and tone-owl (T-O) paired animals (Related-samples Wilcoxon signed rank test; Baseline vs. D-1: $z = 1.293$, $p = 0.196$ 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; $z = -1.82$, $p = 0.069$ for T-O). This indicates that neither the tone-shock group nor the tone-owl group showed evidence of contextual fear conditioning. However, the O-S and T/O-S groups pre-tone test baseline latencies were significantly longer than the training day baseline latencies (Related-samples Wilcoxon signed rank test; Baseline vs D-1: $z = 3.517$, $p < 0.001$ for O-S; $z = 3.296$, $p = 0.001$ for T/O-S; Baseline vs D-2: $z = 2.095$, $p = 0.036$ for O-S; $z = 2.542$, $p = 0.011$ for T/O-S), suggesting the O-S and T/O-S groups did form a contextual fear memory of the foraging arena. * $p < 0.05$, ** $p < 0.01$

Fear conditioning in a standard chamber

To determine whether the absence of tone fear conditioning in a naturalistic environment (Figure 3.4) was due to rats receiving subdermal pain to their dorsal neck/body region, opposed to dermal pain to their paws in standard fear conditioning, eight experimentally naïve rats (four females and four males) that underwent the same subcutaneous wire implant surgery were presented with a tone CS and dorsal neck/body shock US pairing in an experimental chamber (Figure 3.6 A). The fixed CS duration (24.1 s) employed was based on the mean CS duration of tone-shock animals in the naturalistic fear conditioning experiment (Figure 3.3 H). Following the CS-US pairing, animals exhibited reliable postshock freezing (fear conditioning day 1; Figure 3.6 B) and tone CS-elicited freezing in a contextually-altered chamber (tone testing day 2; Figure 3.6 C, D). The fact that fear conditioning transpired with a single tone-shock pairing in a standard chamber suggests that the absence of fear conditioning in a naturalistic environment is unlikely due to attributes of tone CS and dorsal neck/body shock US (as opposed to a footshock).

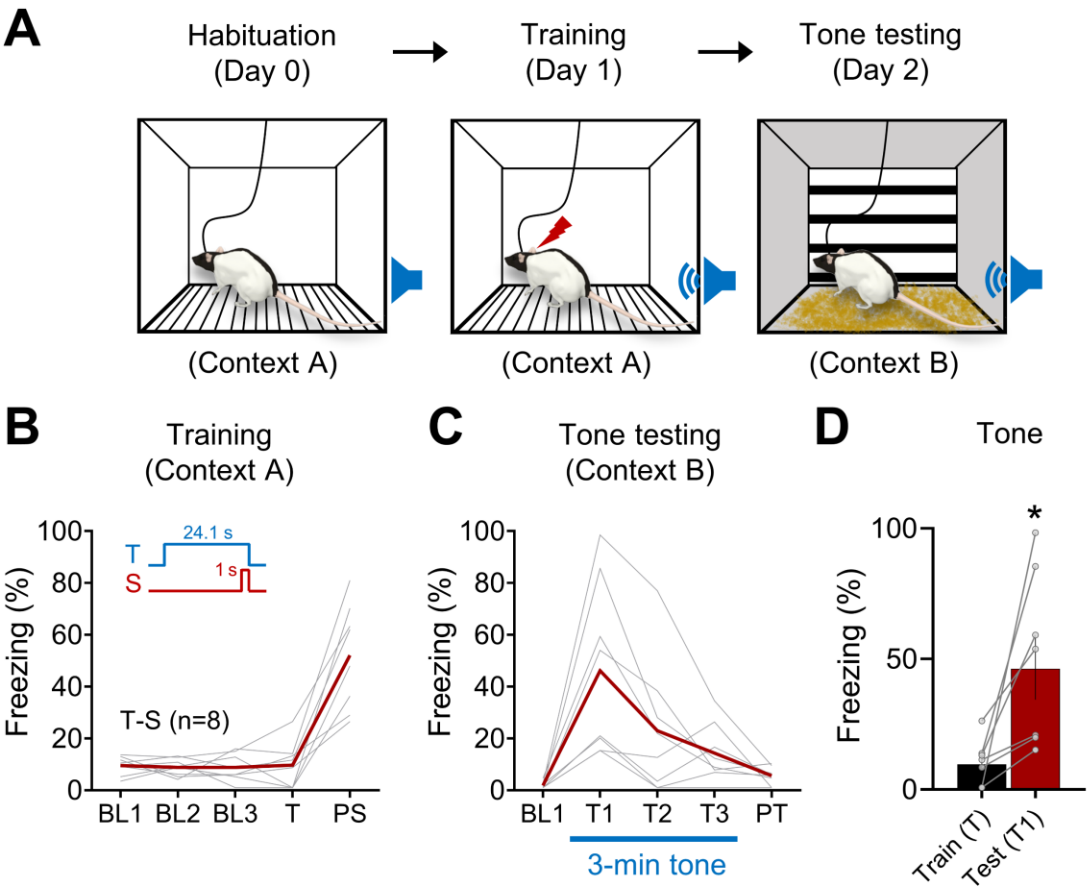

Figure 3.6. Auditory fear conditioning in a standard experimental chamber. (**A**) Illustrations of a rat implanted with wires subcutaneously in the dorsal neck/body region undergoing successive days of habituation (10 min tethered, conditioning chamber), training (a single tone CS-shock US pairing), and tone testing (context shift). (B) Mean (crimson line) and individual (gray lines) percent freezing data from 8 rats (4 females, 4 males) during training in context A: 3 min baseline (BL1, BL2, BL3); 23.1 s epoch of tone (T); 1 min postshock (PS). (**C**) Mean and individual percent freezing data during tone testing in context B: 1 min baseline (BL1); 3 min tone (T1, T2, T3); 1 min post-tone (PT). (**D**) Mean + SEM (bar) and individual (dots) percent freezing to tone CS before (Train, T) and after (Test, T1) undergoing auditory fear conditioning (paired *t* test; $t(7) = -3.188$, $p = 0.015$). * $p < 0.05$

Table 3.1

Groups	T-S (8 males, 8 females)				O-S (8 males, 8 females)				T/O-S (8 males, 6 females)				T-O (4 males, 4 females)			
Measures	M, Med (IQR)	F, Med (IQR)	*z*	*p*	M, Med (IQR)	F, Med (IQR)	*z*	*p*	M, Med (IQR)	F, Med (IQR)	*z*	*p*	M, Med (IQR)	F, Med (IQR)	*z*	*p*
Baseline latency to pellet (s)	15 (12.8,20.2)	20.5 (18.4, 29.2)	1.472	0.161	14.2 (9.8, 26.1)	9.6 (8.3,30.1)	-0.473	0.645	20.2 (11.2, 27.5)	19.8 (8.3, 25.4)	-0.582	0.573	17.3 (12.3, 30.8)	15.8 (10.4, 33.3)	-0.289	0.886
Latency to Leave Nest (s)	18.9 (2.7, 66.5)	7.1 (2.4, 26)	-0.840	0.442	2.4 (1.8, 4.4)	1.9 (.5, 8.3)	-0.316	0.798	27.7 (17.3, 58.3)	10.7 (8.1, 31.6)	-1.420	0.181	7.3 (4.5, 14.9)	24.9 (8.9, 45.7)	1.307	0.200
Latency to Trigger Zone (s)	8.7 (5.5,11.9)	7 (4.6, 11.2)	-0.735	0.505	3.9 (1.1, 5.6)	7.9 (1.8, 10.9)	1.155	0.279	7.1 (3.6, 9.9)	5.3 (4.6, 7.3)	-0.775	0.491	6.5 (5.9, 14.1)	9.2 (6.3, 13.1)	0.581	0.686
Escape Latency to Nest (s)	2.9 (1.9, 5)	2.9 (1.5, 3.3)	-0.578	0.574	1.7 (1.1, 2.0)	2.7 (2, 3.6)	2.052	0.040	1 (0.8, 2.3)	1.8 (1.1, 1.9)	0.780	0.491	5.9 (2.6, 7.1)	0.9 (0.7, 1.6)	-2.021	0.057
Escape Distance (cm)	105.8 (99.2, 111.9)	109.9 (103.1, 130.7)	1.155	0.279	131.9 (117.6, 140.3)	142.5 (121.8, 158.5)	0.735	0.505	117.6 (111.1, 131.5)	125.8 (120.4, 130.1)	0.387	0.755	106.5 (99.8, 124.1)	104.5 (99.1, 108.8)	-0.577	0.686
Variance of Angles	0.19 (0.1, 0.8)	0.62 (0.3, 1.5)	1.575	0.130	1.7 (1.1, 2.1)	2.5 (1.3, 2.9)	1.890	0.059	1.5 (0.2, 2.6)	1.6 (1.1, 2.1)	0.129	1.000	0.3 (0.05, 0.5)	0.2 (0.1, 0.3)	-0.578	0.686
Latency to pellet (Pre-tone), D-1 (s)	13.2 (8.3, 156)	56.5 (24.9, 62.7)	1.471	0.161	160.7 (18.3, 300)	149.2 (67, 267)	0.053	1.000	122.5 (79.2, 300)	166.5 (91.8, 300)	0.264	0.852	15 (13.8, 18.8)	15.7 (10.1, 27.5)	0.000	1.000
Latency to Leave Nest, D-1 (s)	20.6 (7.6, 25.7)	9.1 (4.6, 17.6)	-1.575	0.130	41.8 (11.9, 242.2)	24.9 (7.9, 151.4)	-1.155	0.279	36.7 (23.1, 77.1)	95.3 (43.8, 188.3)	1.291	0.228	3.2 (2.8, 6.7)	3.2 (1.8, 3.6)	0.000	1.000
Latency to Trigger Tone, D-1 (s)	9.6 (2.7, 25.7)	3.9 (2.1, 11.5)	-0.840	0.442	12.3 (7, 22.5)	12.1 (5.3, 48.4)	0.192	0.902	24.8 (10.3, 32.7)	62.6 (22.5, 99.7)	1.512	0.164	9.8 (6.5, 19.4)	9.5 (7.7, 12.3)	0.146	1.000
Latency to pellet (Tone), D-1 (s)	15.9 (5.3, 4.3)	9.6 (5.6, 18.3)	-0.630	0.574	60 (21.7, 60)	52.3 (31.7, 60)	-0.231	0.878	60 (60, 60)	60 (56.5, 60)	-0.318	0.852	5.8 (4.4, 26.6)	4.9 (3.2, 5.3)	-0.577	0.686
Latency to pellet (Pre-tone), D-2 (s)	19.2 (7.6, 34.2)	11.7 (9, 37.5)	0.105	1.000	27.5 (11.1, 224.3)	50.5 (10.2, 107.6)	-0.263	0.798	27.2 (10.4, 93.2)	121.7 (33.5, 258.5)	1.616	0.108	8.2 (7.2, 13.4)	11.8 (7.8, 20.8)	0.866	0.486
Latency to Leave Nest, D-2 (s)	6.7 (4.9, 6.9)	4.7 (3.2, 9.1)	-1.051	0.328	9.4 (4.2, 46.9)	20 (4.9, 30.2)	0.210	0.878	17 (14.3, 31.6)	47.1 (10.4, 186.9)	0.775	0.491	3.1 (1.5, 5.2)	4 (2.4, 6.3)	0.866	0.486
Latency to Trigger Tone, D-2 (s)	5.7 (3.5, 9.1)	1.7 (0.6, 7.9)	-1.471	0.161	9.5 (1.5, 57.9)	6.8 (2.5, 19.7)	-0.579	0.613	17.7 (5.9, 32.5)	25.8 (13.9, 40.9)	0.878	0.435	4.9 (1.9, 12.3)	3.8 (2.8, 9.7)	0.000	1.000
Latency to Pellet (Tone), D-2 (s)	6.3 (5.6, 9.8)	4.7 (3.8, 8.1)	-1.682	0.105	35.8 (9.8, 60)	39.5 (15.5, 56.9)	0.160	0.878	60 (31.6, 60)	60 (46.7, 60)	0.566	0.662	4.1 (3.5, 6.1)	4.6 (3.3, 5.9)	-0.290	0.772
Success rate (%)	100 (71, 100)	100 (100, 100)	1.243	0.382	28.5 (0.0, 87.5)	33 (8.3, 8.5)	0.270	0.798	0 (0, 45.7)	0 (0, 16.7)	-0.567	0.662	100 (87.3, 100)	100 (100,100)	1.000	0.686
Trials to Success (count)	1 (1,1)	1 (1,1)	0.091	1.000	4.5 (1.3, 7.0)	3.5 (1.5, 6.3)	-0.535	0.645	7 (4.3, 9.8)	9.5 (5.8, 16)	1.171	0.282	1 (1, 1.8)	1 (1, 1)	-1.000	0.686

Table 3.1. Sex differences analyzed across each behavioral test. Male (M) and female (F) median (Med) with interquartile range (IQR) in each experimental group and across all the behavioral measures presented in the study. There were no significant differences between male and female rats in all the measures except for the Escape Latency to Nest after owl-shock pairing in the O-S group (Mann-Whitney U tests; *z* and *p* values).

Discussion

It is generally believed (though never validated) that there is behavioral continuity of Pavlovian fear conditioning from the laboratory to real-life situations, and thus understanding the mechanisms of fear conditioning will have clinical relevance. The present study directly investigated whether fear conditioning readily occurs in naturalistic situations that animals are likely to encounter in their habitats. Standard fear conditioning in rodents takes place in small experimental chambers, and several studies have shown that a single tone CS-footshock US pairing (i.e., delay fear conditioning) reliably produces conditioned freezing in rats and conditioned tachycardia/freezing in mice (Stiedl and Spiess 1997). One-trial delay tone fear conditioning has also been demonstrated in human subjects using a loud white noise US and assessing conditioned skin conductance response (Guimaraes, Hellewell et al. 1991). However, in the present study, where rats are exhibiting a purposive foraging behavior (Tolman 1948) in a large arena, a delayed pairing of tone CS and dorsal neck/body shock US (tone-shock group) produced virtually no evidence of auditory (and contextual) fear conditioning across a range of CS durations (i.e., ISIs). A similar pairing of tone CS and looming owl (tone-owl group) also failed to produce auditory fear conditioning despite the owl US evoking robust fleeing UR. In contrast, foraging rats that experienced a looming owl and shock pairing (owl-shock group) later exhibited robust fear (escape) behavior to a novel tone presentation. In the tone/owl-shock animals, the escape behavior was uniformly observed across different ISIs, suggesting that the observed fear to the tone stimulus in this group may not be a Pavlovian response. These findings then point to a nonassociative sensitization (or sensitization-like) process, rather than associative fear conditioning, as playing a vital function in risky (i.e., predatory attack) situations that animals encounter in nature.

The tone CS (3 kHz, 80 dB, ranging 9-86.6 s) and subcutaneous dorsal neck/body shock US (2.5 mA, 1 s) employed in the present study were effective in eliciting orienting and fleeing responses, respectively, and were presented to animals in the manner (i.e., a delay conditioning) that satisfied the stimuli saliency, intensity, surprising, and temporal contiguity requirements for conditioning (Rescorla 1988, Thompson and Krupa 1994, Fanselow and Wassum 2015). Indeed, the same dorsal neck/body shock served as an effective US to generate one-trial tone fear conditioning in a standard (small) conditioning chamber. Then, what can account for one-trial auditory fear conditioning, demonstrated in standard Pavlovian paradigms in rats, mice and humans (Guimaraes, Hellewell et al. 1991, Stiedl and Spiess 1997, Wilensky, Schafe et al. 2000, Lee, Berger et al. 2001), not emerging in animals that left the safe nest to forage for food in an open arena? It may well be that rats are not biologically predisposed to associate discrete CS and US in natural (complex) environments where amalgamation of hunger-driven, fear-driven and exploration-driven motivated behaviors are freely expressed. Indeed, in real-life, only a small minority of people experiencing trauma develop posttraumatic stress disorder (PTSD) and even with re-exposure to the same trauma there is low incidence PTSD (Somer, Zrihan-Weitzman et al. 2009, Palgi, Gelkopf et al. 2015). In contrast, standard experimental chambers may be conducive to fear conditioning because they are simple and limit the repertoire of behavior (Thorndike 1900), effectively bypassing a “biological boundary” that prioritizes less costly defensive responses over trial-and-error learning mechanisms. The absence of one-trial fear conditioning in a naturalistic setting may be analogous to “The Rat Park Experiment,” where rats housed in an enriched environment with plants, trees and social interaction resist drug addiction behavior evident in standard cage-housed rats (Alexander, Beyerstein et al. 1981, Gage and Sumnall 2019). Animals tested in naturalistic paradigms are given choices that do not force their behaviors into dichotomies (i.e., freezing or no freezing; drug craving or no drug craving). Allowing for an expanded behavioral repertoire, while more difficult to study, may thus yield a greater understanding of behaviors and their underlying brain mechanisms.

It should also be noted that fear encounters in real life generally occur in the presence of external agents or forms (i.e., predators/conspecifics in animals and assailants/combatants in humans), which is virtually nonexistent in standard Pavlovian fear conditioning paradigms. Thus, the effects of a discernable entity in associative fear learning have never been investigated. By simulating a realistic life-threatening situation, i.e., a looming aerial predator that instinctively elicited flight behavior followed by somatic pain, we found that rats engaged in purposive behavior utilize nonassociative sensitization as their primary defensive mechanism. The fact that the owl-shock and tone/owl-shock animals exhibited relatively nonlinear, erratic escape trajectories to the nest compared to linear escape trajectories in tone-shock animals (Fig. 3.3 F-I) suggests the intriguing possibility that the same dorsal neck/body shock US may be interpreted as a life-or-death (panic) situation in the presence of an external threat agent versus a mere startling (nociceptive) situation in the absence of an external threat agent. The erratic flight behavior in the presence of a looming owl may represent the penultimate stage of circa-strike, or “life-or-death,” behavior within the “predatory imminence continuum” theory (Fanselow and Lester 1988). Functionally, a sensitized fear system may intensify avoidance behavior, which in turn effectively transposes novel, neutral cues into “false positives” to prioritize survival in natural environment (Bolles 1970). In other words, nonspecific sensitization-based overestimation of danger may be a more prudent course for survival than relatively more specific association-based prediction of danger.

Some caveats, however, must be considered in the present naturalistic study of fear conditioning. First, although neither the tone-shock group nor the tone-owl group showed overt manifestations of fear conditioning to the tone (as measured by fleeing or freezing in the arena) that prevented a successful procurement of food, the possibility of physiological (e.g., cardiovascular, respiratory) indices of fear (Steimer 2002) cannot be excluded in these animals. If so, the *presence* of tone-elicited fleeing and foraging termination behaviors in owl-shock and tone/owl shock animals versus the *absence* of tone-elicited fleeing and foraging termination behaviors in tone-shock and tone-owl animals may reflect differences in the magnitude (rather than presence-absence) of fear conditioning. Second, the erratic escape trajectory behavior exhibited by owl-shock and tone/owl-shock animals may be indicative of rapid associative processes at work (Fanselow 2018). For example, the immediate-shock (and delayed shock-context shift) deficits in freezing e.g., (Fanselow 1986, Landeira-Fernandez, DeCola et al. 2006) provide compelling evidence that postshock freezing is not a UR but rather a CR to the contextual representation CS that rapidly became associated with the footshock US. In a similar vein then the erratic escape CR topography in owl-shock and tone/owl-shock animals might represent a shift in ‘functional CR topography’ (Fanselow and Wassum 2015) resulting from the rapid association between some salient features of the owl and the dorsal neck/body shock. A rapid owl-shock association nevertheless cannot explain the owl-shock animals’ subsequent fleeing behavior to a novel tone (in the absence of owl), which likely reflects nonassociative fear. Third, there are obvious procedural differences between standard fear conditioning versus naturalistic fear conditioning. In the former paradigm, typically ad libitum fed animals are placed in an experimental chamber for a *fixed time* before receiving a CS-US pairing (irrespective of their ongoing behavior). Thus, the CS duration and ISI are constant across subjects. In our study, hunger-motivated rats searching for food must navigate to a *fixed location* in a large arena before experiencing a CS-US pairing (instrumental- or response-contingent). Because animals approach the US trigger zone at different latencies, the CS duration and ISI are variable across subjects. A more pertinent question is whether “procedurally pure” laboratory Pavlovian fear conditioning can possibly occur in real world settings, where behaviors of animals and humans are largely purposive/goal-oriented (Tolman 1948). Indeed, Bouton (Bouton 2007) articulated that, “Outside the laboratory, stimulus [Pavlovian] learning and response [Instrumental] learning are almost inseparable.” Lastly, tone fear conditioning might not have transpired in our foraging apparatus because the shock-induced pain was targeted to the dorsal neck/body region. As stated before, this is unlikely given that the same dorsal neck/body shock US effectively supported single trial tone fear conditioning in a standard conditioning chamber. Though predators would not direct their attacks underneath the paws of small prey animals, the possibility of a footshock US supporting tone fear conditioning in the foraging apparatus, however, cannot be excluded.

Clark Hull (Hull 1929) has posited that Pavlovian fear conditioning offers biological utility by circumventing a “bad biological economy” of defense reaction always necessitating injury. This prevailing view that ascribes preeminent importance of fear conditioning as the primary defensive mechanism is likely to be a theoretical simplification and provides an incomplete picture of fear, as its function in a natural environment may be rather limited (i.e., lacks face validity). It may well be possible to produce fear conditioning in naturalistic settings with further CS-US trials, varying the CS and US intensity/duration or applying footshock but then this too would be a bad biological economy as such specific parameter-dependent learning would dramatically reduce biological fitness. It is also important to recognize inconsistencies in the literatures, such as clinical studies that have reported that patients with anxiety disorders, such as phobias, have trouble recalling the particular pairing of the fear event with its aversive consequences (Lazarus 1971, Öhman and Mineka 2001). The increased utilization of naturalistic fear paradigms that simulate dangers that animals and humans encounter in real life will enable us to clarify, update, and revise fear concepts derived largely from fear conditioning studies and in doing so facilitate future progress in the treatment of fear disorders.

Chapter IV General Conclusions

Summary of Results

The experiments presented above have analyzed and manipulated different components of foraging and fear behaviors in the rat to further our understanding of how rats successfully forage in the wild. Beyond an ethological interest, these results can aid in the advances of fear and anxiety research in humans. Most of the brain structures in the rat have been evolutionarily conserved in the human brain (Burwell, Witter et al. 1995, Lu, Zou et al. 2012), and in order to study these structures rats should be placed in paradigms that most accurately simulate the habitat they evolved within.

The first study in Chapter 2 built and validated a novel aerial threat paradigm to compare 3D aerial threat with the traditional 2D looming stimuli. It was also the first laboratory study to examine looming defense responses (LDR's) in a large arena with rats performing competing motivated behaviors (foraging vs. fleeing). Traditional looming studies are usually performed in smaller chambers (30 cm^3 boxes) with a screen on top that plays animations of expanding discs. The study in chapter 2 enlarged every aspect of previous research to better represent a more natural and dynamic environment that allows for flexible behaviors from the rat. It also compared the effects of the rats seeing a specific shape above them (projected onto the ceiling), or just the quick decrease in light that would occur from the expanding disc by projecting the image onto the floor. Only projecting distinct shapes above the rat (mainly the expanding disc) led to frequent LDR's, showing that just the decrease in light is not sufficient to drive a fleeing response. After being presented with multiple looming stimuli (expanding disc, sweeping bar, large bar), all female rats and most of the male rats fled from the expanding disc, and no freezing was observed. Unfortunately rats also rapidly habituated to the looming threat, with many retrieving the food pellet after fleeing a few times, meaning the paradigm’s practicality for neural recordings that require repeated trials is low. For a foraging rat however, the rapid habituation to overhead stimuli would be useful when in areas of high foliage where leaves or branches moving overhead may trigger an LDR, but if every time an object meeting the trigger criterion appeared overhead and the rat ran away or froze it would be impossible for it to forage successfully. In the same arena a life-like owl (3D looming stimulus) plunged down at rats foraging for food pellets and led to a more intense and longer lasting fear response than 2D looming stimuli. All rats, regardless of sex, failed to retrieve the pellet on the first day and habituation required multiple days of exposure. In addition, a contextual fear of the foraging area formed over time. The owl was also hidden from the rat until it ‘attacked’, which may have also added to the ambiguity of when the rat would encounter the owl.

As testing continued, sex differences developed in the fear response to the owl and contextual fear of the foraging area. Female rats had significantly increased latencies retrieving the pellet when under threat of owl-attack and also during baseline pre-owl trials. The male rats, while failing to retrieve the pellets for the first few days of owl exposure, were all able to retrieve the food pellet by day 5 and quickly foraged during baseline pre-owl trials. Like humans, rats are also sexually dimorphic and certain behaviors, or disorders, are displayed differentially between the two populations. In humans, women have been found to consistently have higher diagnosed rats of anxiety disorders (generalized anxiety disorder, panic disorder, PTSD) than men significantly across all age groups (Jalnapurkar, Allen et al. 2018). Rodent fear research however, usually trends towards male rats displaying higher fear and anxiety behaviors (Maren, De Oca et al. 1994). The larger issue for sex differences research in rodents, is the alarmingly low rate of any experiments examining female behaviors at all. With such a low sample-size to report from, many meta-analyses examining rodent sex differences in fear and anxiety have little to work with (Kokras and Dalla 2014). Fortunately, more researchers have begun utilizing female rats as much as males (whether voluntarily or mandated), and it may be as more behaviors, strains, and development periods are analyzed, more stable findings of rodent sex differences will advance. Although male rats may freeze more when placed in an evolutionarily irrelevant chamber that artificially limits behavior, when faced with an evolutionarily relevant dynamic task with competing motivations (exploration and hunger vs. predator avoidance), other sex differences will be revealed.

To begin understanding the brain structures involved in the expression of LDR's, the basolateral amygdala (BLA) and superior colliculus (SC) were inactivated or lesioned respectively during the same foraging task with the owl. The silencing of both areas eliminated the fear response while leaving intact the rat's ability to forage and retrieve food pellets. While both muscimol and electrolytic lesions have been used for decades to investigate the functions of brain structures, more recent techniques would be beneficial in providing more detail at a circuit level. For example, Wei et al. (2015) used an optogenetic retrovirus tracer to determine there are multisynaptic connections between the SC and BLA that are required for the fear response to a 2D expanding disc. Because of its multi-day habituation period allowing for many trials, the risky foraging task using the owl could also be utilized in single-unit and local field potential recordings within these structures.

The third chapter of the dissertation attempted to validate the use of Pavlovian fear conditioning in a naturalistic setting. It is clear from decades of research that rats quickly and reliably pair a footshock (US) with a pure tone (CS), and then respond to that tone again later with fear behaviors (CR). What is less clear however, is how useful that learning would be to a wild rat foraging for food and encountering the same types of stimuli. No distinct cues, especially pure tones that play for 30 seconds, exist in nature that would reliably predict a predator strike. The only cues that may fall under this category would be predator scents (fox and cat urine), which rats already react to defensively without any learning required. Rats' innate defensive abilities alone are what allows them to avoid predator strikes. In this study, foraging rats were presented with a tone (CS) before foraging began, then when they reached a certain distance from the nest area, were delivered with a shocked on the back (US). According to Pavlovian fear conditioning, these rats should now freeze when hearing the tone again, as this tone now represents a distal predatory-threat (Fanselow and Lester 1988). Instead, many of the rats in this group showed no fear response and continued to forage successfully. Surprisingly, the most naturalistic of the groups, rats that heard no tone, but were attacked by the owl without warning that coincided with a predatorily relevant back-shock, did show a strong fleeing response to the tone during testing the next day. A sensitization-like response may occur from the traumatic predator attack the previous day, so now any novel stimuli presented to the rat is also encoded as a dangerous stimulus. If a rat was lucky enough to escape a predator strike in the wild that most likely had no predictive cues, it too would be better-off avoiding any novel and salient stimuli when required to forage again.

Foraging, Fear, and Translational Relevance

Although Pavlovian fear conditioning has been vital in our understanding of how different types of fear information can be associated, represented through long-term memory, and all of the biological processes involved, it has many limitations when it comes to aiding in the research of human anxiety disorders (Flandreau and Toth 2017). Take for example one of the most prominent and debilitating anxiety disorders, Post-traumatic stress disorder (PTSD), where patients experience extreme anxiety, avoidance, sleep-disturbances, and hyper-arousal after experiencing a traumatic event U.S. Department of Veteran Affairs (2022) .Feeling the emotional effects after a traumatic event is completely normal and is most likely a part of the process in recovering from a traumatic event. It is not until these symptoms have continued for at least three months after the event are people diagnosed with PTSD, and in some cases, symptoms may reappear 6 months or more later (National Institute of Mental Health). Rat studies of PTSD often times utilize fear conditioning to a specific context or tone 24 hours or a few days after the rat experiences its traumatic event (undamaging footshock), that requires specific cues to trigger a fear response.

A major facet of PTSD in humans is its generalizing nature to all facets of a patient's life: a soldier that was in battle overseas that has returned home is now living in a completely new context without any cues from their traumatic experience, but cannot continue with their life because of PTSD (Carr 2011) . Rats are readily able to distinguish between tones that have been paired with shock, safety (no shock), and reward all in the same context with the amygdala differentially firing for each cue (Muramoto, Ono et al. 1993). Rats also quickly extinguish to a CS after a few presentations (Bouton 2005), whereas PTSD patients can live with the disorder for years with no relief. Chronic stress has been shown to enhance the fear response in the rat from fear conditioning and leads to extinction resistance for extended periods of time (Long and Fanselow 2012), but the diagnosis of other stress disorders in humans pre-traumatic event has no correlation with the development of PTSD (Bryant 2011). Together these discrepancies may outweigh the translational relevance in the continued use of Pavlovian fear conditioning for the study of PTSD. Fear conditioning instead, is akin in how it should be perceived as is eyeblink conditioning: An extremely useful and stereotyped paradigm in studying the molecular and cellular changes the brain goes through to learn and remember a certain event that elicits an observable behavioral response. Also, like eyeblink conditioning, Pavlovian fear conditioning while easily obtained under sterile laboratory conditions, may serve no purpose in aiding in the rat's survival in dynamic-nature.

As machine learning technology advances and more complicated behaviors are automatically coded with high output, more laboratories should begin adopting paradigms that allow for a rat's full behavioral repertoire. Foraging tasks can be modified to not only study fear, but complex decision making, social interactions, longitudinal, and developmental experiments. Wireless technologies too are advancing that allow for remote optogenetic control and remote tetrode recordings for hours at a time, so that much larger arenas can be built that support all of the natural behaviors in the rat. With these findings, providing evolutionarily relevant explanations of human brain operations undergoing similar tasks becomes a straightforward process.

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