Contextual modulation and blunted defensive responses to predators in head-fixed and freely moving mice

Marti Ritter[1], Lara Mariel Chirich Barreira[1], Lara Sach[1], Aileen Hakus[2,3,4], Sanra Kumsal Öktem[1], Ronny Bergmann[5], Anne Voigt[5], Dietmar Schmitz[5,6,7,8], Panayiota Poirazi[9], Matthew E. Larkum[1], Robert N. S. Sachdev[1]

[1]Inst. of Biol., Humboldt University of Berlin, Berlin, Germany;
[2]Grad. Ctr. of Life Sci., Humboldt University of Berlin, Berlin, Germany;
[3]Dept. of Psychiatry and Psychotherapy, Charité Universitätsmedizin Berlin, Germany;
[4]Intl. Grad. Program Med. Neurosciences, Charité Universitätsmedizin Berlin, Germany;
[5]Neurosci. Res. Ctr., Charité Universitätsmedizin Berlin, Germany;
[6]Bernstein Center for Computational Neuroscience Berlin;
[7]Einstein Center for Neurosciences Berlin, Charité-Universitätsmedizin Berlin, Germany;
[8]Max-Delbrück Center for Molecular Medicine in the Helmholtz Association Berlin, Germany;
[9]Institute of Molec., Biol., and Biotech., Foundation for Research and Technology -- Hellas, Heraklion, Greece.

Correspondence to: bs387ster@gmail.com; Matthew.Larkum@gmail.com; Poirazi@imbb.forth.gr

Short title: Innate defensive responses in head-fixed and freely moving mice
Number of pages: 49
Number of figures: 7
Supplementary figures: 10
Multimedia videos: 6
Number of words for Abstract: 254
Introduction: 620
Discussion: 1055
Total number of words: 7543

Additional Information: The following funding sources have supported this project: (1) Deutsche Forschungsgemeinschaft (DFG), Grant Nos. 246731133, 250048060 and 267823436 to ML; (2) DFG Project number 327654276 – SFB 1315 to ML; (3) European Commission Horizon 2020 Research And Innovation Program and Euratom Research and Training Program 2014–2018 (under grant agreement No. 670118 to ML); (4) Human Brain Project, EU Commission Grant 720270 (SGA1), 785907 (SGA2) and 945539 (SGA3) to ML; (5) Einstein Foundation Berlin EVF-2017-363 to ML; Einstein Foundation Visiting Fellowship EVF-2019-508 to PP; Humboldt Foundation Friedrich Wilhelm Bessel Research Award to PP.

Acknowledgements: We thank the Charité Workshop for technical assistance, especially Alexander Schill, Jan-Erik Ode and Daniel Deblitz. We also want to thank Melissa Long and the team of the ABPF for their help with animal handling and room planning. Finally, we thank Laura Schwarz of the Sainsbury Wellcome Centre and members of the Larkum lab for useful discussions about earlier versions of this manuscript.

1 Abstract

Behavioral responses to threats — such as fleeing, freezing, or fighting — can be either innate or shaped by learning and context. Here, we investigated whether mice exhibit fear of predators across four experimental contexts: one in a novel head-fixed condition and three in established, freely moving scenarios. In head-fixed mice, we measured the behavioral outcome and response to a live rat. Mice were water-deprived and habituated to walk on a treadmill that controlled a virtual environment and reward delivery. After meeting performance criteria, baseline data were collected in one session, followed by a test session in which the mice were exposed to a live rat. Despite the presence of the predator, most (5 out of 7) mice continued to forage at baseline levels; however, individual mice showed significant alterations in one or more of the following measures: running speed, pupil size, eye movement, and posture. To assess how behavioral context and physical restraint influence predator responses, we exposed 36 naive, freely moving mice to fear-inducing stimuli — including looming visual cues, rat odor, and a live rat. Even in these conditions, a substantial proportion of mice failed to exhibit classical defensive responses such as avoidance or escape. Notably, when presented with a freely moving rat, only about half of the mice displayed avoidance behavior. Together, these findings suggest that mice do not universally express innate fear behaviors such as avoidance or fleeing, even in ethologically relevant predator encounters. Instead, their responses appear to be context-dependent and variable, challenging common assumptions about automatic defensive reactions in rodents.

2 Introduction

Threat perception and appropriate reaction to a threat are necessary for survival. Incorrect assessment of a sound in a bush, or the strength of a barrier holding a vicious animal at bay can on the one hand lead to death or injuries, and on the other hand to anxiety even when there is nothing to fear. Earlier work on threat perception in both animals and human beings has focused on three aspects of threat and behavior: how the brain detects threats, responds to threats, and the conscious feeling of fear that occurs in threatening or dangerous situations (Beckers et al. 2013; Tovote, Fadok, and Lüthi 2015; Silva, Gross, and Gräff 2016; LeDoux and Brown 2017; Fanselow and Pennington 2017; Mobbs et al. 2019). These aspects of threat perception and the response to threats have most often been studied using Pavlovian conditioning, in which an innocuous stimulus, usually a sound cue is paired with an unpleasant noxious stimulus like an electric shock (Mowrer and Lamoreaux 1946). Over time the innocuous stimulus elicited the same behavioral defensive responses -- freezing, fleeing, changes in posture -- as the noxious stimulus and physiology, i.e. changes in pupil size and increases in heartbeat rate (Blanchard and Blanchard 1989). The advantage of this class of paradigms is that investigators have control over the causal conditions -- the stimulus -- and can measure the effect of stimuli on behavior. These approaches have been successful in elucidating the brain circuits involved in threat perception and those involved in generating the behavioral response to the threat (Phillips and LeDoux 1992; review by Tovote, Fadok, and Lüthi 2015). In recent years, a plethora of other paradigms have been developed using a variety of stimuli for inducing fear. Looming stimuli -- where a shadow grows above a rat or mouse, simulating a bird of prey readying to pounce -- can be threatening to rodents: they run, hide or freeze (Schiff 1965; Yilmaz and Meister 2013). Noxious odors -- fox or cat urine -- or loud sounds, heights, or larger rodents have also been used to elicit fear by introducing a "natural" threat (Gibson and Walk 1960; Mongeau et al. 2003; Farmer-Dougan et al. 2005; Silva, Gross, and Gräff 2016).

Our goal in this study was to design a head-fixed mouse preparation for prey-and-potential-predator interaction and test the outcome of this work against existing paradigms. In the head-fixed preparation we examined whether the need to forage for food affects the expression of fear. Earlier work suggested that rats prey on mice, and that mice are innately afraid of rats, i.e. that the presence of a rat elicits defensive behaviors in mice (Karli 1956; Panksepp 1971; Blanchard et al. 1998). Here we assessed whether head-fixed mice showed evidence of fear and changed their behavior, whether they continued foraging even in the presence of the rat. In our paradigm, mice ran on a treadmill to obtain a water reward and decided between "foraging" i.e. licking for reward, while faced with a threat, a living rat hovering over the lick tube. Surprisingly, even though mice showed some expression of fear in the presence of a rat, most head-fixed mice continued foraging even in the presence of the rat. During the interaction, all manners of sensory cues were available to mice -- mice could almost touch the rat, could smell, see and hear the rat moving and still most mice continued foraging. Even more surprisingly, when we used established standard paradigms designed for freely moving mice i.e. exposing mice to looming stimuli -- in the form of a rapidly expanding black disk designed to mimic the shadow of an approaching predator -- rat odors, or live rats, mice did not consistently show defensive fear behavior.

3 Methods and Materials

All experiments were conducted in accordance with the guidelines of animal welfare of the Charité Universitätsmedizin Berlin and the local authorities, the 'Landesamt für Gesundheit und Soziales'. Mice (n=43, 6-7 weeks old at start of experiments) on a C57BL / J6 background sourced from various breeding facilities and 5 rats were used in this study. For experiments involving head fixation, seven mice (4 male, 3 female) were sourced from the internal breeding facilities. In the following plots the male mice are enumerated as mouse 1-4, and the female mice as mouse 5-7. Because the primary outcome of this experiment, the blunted avoidance response to the presence of the live rat, was independent of the sex of the mice (see **Supplementary Figure 2-7**), for experiments with freely moving mice, male mice were sourced from in-house breeding colony, Janvier, or Charles River. Mice were housed socially with at least one other littermate. Animals were housed in a reversed 12-hour day / night cycle. Five female Wistar rats served as the predator stimulus and were kept in the same facility as the mice, but in another room. The mice and rats were kept in a 12h light cycle (mice 6am to 6pm, rats 5am to 5pm) and had ad lib access to food and water.

Surgical procedures for head-fixation

On the day of the surgery, adult C57Bl6 mice (n=7) weighing 20-40 grams were injected with Carprofen (5 mg/kg) intraperitonially pre-surgery, then deeply anesthetized with a mixture of Ketamine and Xylazine (Ketamine 12.5mg/ml, Xylazine 1 mg/ml, 10 µl/g dose) and placed on a heating pad maintained at 37°C. A local analgesic, Lidocaine (100 µl), was injected under the skin at the site of the incision. Lightweight aluminum headposts -- 3 cm long, 2mm thick, weighing 0.4 gm -- were affixed to the skull using Rely X and Jet Acrylic (Ortho-Jet) black cement (Ebner et al. 2019; Dominiak et al. 2019). Post-surgery analgesia was provided over 3 days, with Carprofen injections (5 mg/kg) intraperitonially and, if there were signs of post-operative pain, Buprenorphine (0.05-0.1 mg/kg) subcutaneously in addition.

Simulated foraging experiment

The simulated foraging environment consisted of a rigid-foam-based circular treadmill and a 30 cm long plexiglass tube with a 7 cm diameter (**Figure 1, Video 1**). The treadmill was lightweight enough that mice could move it effortlessly. It had a rubberized surface to increase the grip of the mouse on the wheel. The movement of the treadmill was read by an encoder. The output of the encoder was used to control the position of the plexiglass tube, which was mounted on a rail and controlled by a small stepper motor (**Figure 1**). To ensure safe and stable movement, and to minimize the stress on a rat held in the tube, the top speed of the tube was limited to 4 cm per second. The output of the encoder was also linked to the PC that controlled two large monitors positioned on each side of the treadmill that were used to stream visual stimuli tethered to the movement of the treadmill.

The tube holding the rat was coated with a red film limiting the ability of mice to see the position of the rat, or to see whether there was a rat in the tube (**Figure 1A**). Additionally, the front of the tube was covered with a disk which could be rotated by an electronic input (**Figure 1B)**. When a rat was in the tube, the disk could be used to selectively hide or show the rat during the simulated predator encounter. The disk could be rotated to allow odors through, or to block

all sensory stimuli from inside the tube. When the disk was in the open position, the rat could stick its nose out of the tube and almost touch the mouse when it was at the lick spout.

Electronics and control of behavior

A finite state machine (Bpod r2, Sanworks LLC) monitored trial states, and the sequence of reward delivery and data acquisition. This state machine also controlled the high-resolution acquisition of movement traces from the encoder in the treadmill and triggered the cameras (recording at 100Hz) (Error! Reference source not found.). In addition to the state machine, a single-board PC (Raspberry Pi 4 by Raspberry Pi Foundation) was used for real-time control. The 1kHz output from the Bpod was down sampled to 25 Hz, then transferred to a microcontroller (Arduino Nano by Arduino.cc) which controlled stepper motors to translate this into motion of the plexiglass tube. If the mouse moved the treadmill faster than 4 cm / s then the output of the encoder was truncated to ensure that the tube followed smoothly. Note that the treadmill moved as fast as the mice needed; but if the treadmill moved at a high speed, the output was translated into a manageable speed for moving the lick spout, and the associated large tube that could contain a living rat.

The position of the disk at the front of the tube was also controlled by the single-board PC and microcontroller (Arduino Nano by Arduino.cc) and updated by stepper motors that rotated the disk. Finally, the single-board PC was used to generate the virtual visual environment tethered to the movement of treadmill.

The monitors used for displaying the streaming stimuli were also used as a go-cue at the beginning of the trial and an end-cue at the end. The streaming stimuli consisted of symbols that moved in synchrony with the treadmill. The screen flashed green at the start of a new trial and red at the end of a failed trial.

Habituation and training

After surgical operation to implant a head post, mice were habituated to being handled and head-fixed (**Figure 1C, D**). Training on the treadmill began, once mice tolerated head fixation for ~ 20 minutes. At this point, water intake for mice was monitored and restricted, but ensured to be at a level resulting in no more than 20% of weight loss per mouse compared to the weight before onset of water control. To ensure that all mice were available on the same experimental day when a rat was introduced, training continued for 3-4 weeks.

The initial training consisted of rewarding mice manually with Saccharose-sweetened water when they were head-fixed and they moved the treadmill in the correct / forward direction. Once mice moved the treadmill in the correct direction, training was automated. In the first days, there were no time constraints. Mice simply had to move at least 28.5 cm forward on the treadmill to receive the sweetened water reward. Five to seven seconds after reward delivery, the lickspout / tube contraption automatically moved to its starting position, away from the mouse and the next trial began. The beginning of a new trial was indicated by a sound cue (played by a buzzer at 50Hz) and flashing of the virtual reality screens.

In the next phase of training, a time limit was introduced. Mice had 30 seconds to move the treadmill at least 28.5 cm. Once mice learned to keep moving the treadmill to obtain a

reward, the duration of the trial was shortened to 12 seconds. When a cohort of mice achieved >65% successful trials under these conditions for 3 days, the control and experimental data were collected.

Live predator encounter

Once baseline control data had been collected on the next day (**Figure 1C-F**), a rat that had been habituated to handling and to the apparatus, was placed in the tube. The mouse was then head-fixed to the treadmill and experimental data were collected. The day after the encounter with the rat, a second day of baseline data was collected without the rat.

Experimental paradigm with freely moving mice

One cohort of twelve male C57Bl6/J mice and a second cohort of twenty-four male C57Bl6/J mice were used in these experiments. Mice were ordered from the in-house breeding colony, Janvier, or Charles River. For the first cohort the 3 female Wistar-rats as in the simulated foraging task were used, and for the second cohort 2 additional female Wistar-rats were used. Female rats were used to minimize the possibility of a rat biting through the holder or injuring a mouse (Karli 1956). We chose to use animals from multiple sources to verify whether mice obtained from our in-house colony showed unusually low threat responses.

Looming stimuli

To evaluate innate threat responses independent of the presence of a live predator, we applied the well-established looming stimulus paradigm. This paradigm is a standard for inducing fear in mice. The mice were solitary housed and habituated to changes in day-night-cycle for 5 days, then over the next four days, one mouse per source and day (resulting in 3 mice per day) was placed in a darkened room for an hour before experiments began. The purpose of the solitary housing of these mice was to ensure a minimum of unintended interaction between mice before each recording session. On the experimental day, mice were moved into a plexiglass arena (0.5 m x 0.29 m) and left to explore it for 10 minutes. The arena was cleaned with 15% Ethanol before each mouse was introduced. It contained a shelter, made of red plexiglass. When mice entered a pre-defined zone, a looming stimulus -- a shadow that expanded above the mouse -- was presented. The looming stimulus consisted of 5 presentations of a small black dot (3°visual angle) expanding rapidly over 200ms to its full size (50°) and then remaining at this size for 250 ms. The stimulus was repeated at least 90 s after the last presentation. The sequence of looming stimuli was repeated 10-14 times for each mouse over 45 minutes. Video data was collected at 60 Hz. Each stimulus presentation was triggered manually by a researcher supervising the experiment through a monitor located out of sight of the mouse, but within the same room as the setup.

Test with Rat odor

The mice used in the looming experiments were then used to examine place preference in the presence or absence of rat odors. Over two days two mice from each source, i.e. in-house, Charles River, and Janvier, per day (6 mice per day) were moved into the plexiglass arena one at a time. The arena was split in half with a small connector between the two sides. Mice were left to explore both sides of the arena for 10 minutes. Then the mouse was removed, and a rat was

brought into the arena, but could only explore one side of the arena. The rat was removed after 5 minutes, and the mouse was returned to the arena. Movement of the mouse in the two conditions was tracked offline with video acquired at 60Hz for the first cohort and 25Hz for the second cohort. The arena was cleaned before each mouse was introduced into the arena.

Assaying response to live rat

Finally, the same mice were placed in a modified version of the plexiglass arena (again 2 mice per group per day). The arena was divided in half by a 1mm thick metal mesh, with gaps that were just large enough for mice or rats to stick the tips of their noses through the mesh. Control data was acquired for five minutes, then mice were removed, and a rat was brought into the arena. Two minutes after introducing the rat, mice were introduced into the other side of the arena. Movement of the mouse was tracked offline with video acquired at 25-60Hz. The arena was cleaned before each mouse was introduced into the arena.

Behavioral analysis

Simulated foraging with a live rat encounter predator encounter

Behavioral measures including overall performance, speed of movement and a variety of movement parameters were tracked using high speed video at 100 Hz, over the course of the 3 days. Analog traces from the treadmill encoder, and the movement sequences of the mice were captured on two Basler cameras, one aimed at the body, and one aimed at the face. Movement and posture of the mice was tracked using DeepLabCut (Mathis et al. 2018). Behavioral phenotypes that were available for analysis in the control condition and in the presence of the rat included: 1) Flight, apparent as a backward movement on the treadmill. This movement pushed the tube holding the rat away from the mouse and showed up as negative deflection in the encoder output and leads to a failure in licking. 2) Freezing, i.e. the mouse stops moving, which would be apparent in the speed traces and lead to a failure of the trial. 3) Changes in mouse posture, hunching or stretching. 4) Changes in pupil size. 5) Changes in nose movement.

We performed the experiments in such a way that we were able to pair the behavioral phenotype of each mouse on the first recording day (before a rat was introduced) as a baseline to all further recordings of the same mouse.

Assaying threat response in freely moving mice

One of the three assays -- the looming stimuli -- was dependent on vision, two of them had olfactory components. In these experiments —looming, rat odor avoidance, and live rat exposure— the position of the mice was tracked using SLEAP (version 1.3.3). Three key body points were annotated along the midline of each mouse. For positional tracking, the pixel coordinates of the neck point were extracted and transformed into millimeter-based coordinates relative to the layout of each experimental arena. To reduce noise, positional data were clipped to the arena boundaries and smoothed using a sliding half-second window. Due to the side-view recording setup in the looming experiment (necessitated by an overhead screen), additional geometric corrections were applied. The X-coordinate was taken directly from the neck point, while the Y-coordinate was defined as the lowest Y-value among the three tracked points. An empirical downward shift of 20 pixels was then applied to the Y-coordinate. The adjusted coordinates were subsequently clipped to the nearest point within the arena polygon visible in

each frame. After these corrections the pixel-based position converted to millimeter-scale coordinates.

Because the perspective of the looming videos enforced a strong correlation between the X- and Y-position, only the X-position of the mouse relative to the shelter was used for further analysis. The absolute X-axis speed (relative to the shelter) was smoothed with an 11-frame window before being used to classify looms as either "fleeing" or "non-fleeing" events.

Statistical analysis

Simulated foraging with live a rat

Here we first assayed mouse behavior for evidence of innate fear -- i.e. freezing, fleeing, -- which would be evident in performance. This study was also designed to capture subtle changes in the behavior of mice -- speed of movement as mice approach rats, success rate, pupil size, body elongation nose movement -- in the presence of rats. To assay these changes in behavior, the data related to each behavioral feature (pupil size, speed of movement, nose movement, etc.) were first filtered with a rolling z-score across a one second window. Values with an absolute score above three were removed. The time series data was then smoothed with a half second window by way of a rolling mean.

In the case of the video data (DeepLabCut output) it was also necessary to perform initial filtering steps, according to the detected likelihood that a feature was discovered in a frame, and with an initial z-score filtering across the x- and y-position of the features. In statistics where we considered the pupil-diameter and pupil position, we also filtered the eight detected markers around the pupil with a modified z-score (Iglewicz and Hoaglin 1993).

We compared the behavioral items pairwise per mouse, between the day of the baseline recording and the following days. To achieve this, we summarized half-second wide bins relative to the beginning of the trial or the delivery of reward of the timeseries and compared these between the two conditions. These data were plotted to show the mean and standard deviation per feature and condition. Binned data for sessions with and without the rat were compared, and the difference between the mean and standard deviation and the significance of this shift shown as a bar plot. Significance was assessed for each half second bin. Additionally, for each behavioral parameter i.e. position, speed pupil size, eye position -- differences between sessions with and without the rat had to be significantly different for 6 consecutive bins. In the reward phase which lasts for a short duration, the pupil x-position, pupil diameter, body length, nose speed -- there had to be significant difference over at least 3 consecutive half-second bins.

The continuous mean and standard deviation of the timeseries was calculated with the formulas $\mu = \frac{\sum_{i=1}^{n} x_i}{n}$ and $\sigma = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(x_i - \mu)^2}$. These values were then used to calculate the difference between both statistics. We used the following formulas for this: $\mu_{diff} = \mu_{minuend} - \mu_{subtrahend}$ and $\sigma_{diff} = \sqrt{\sigma_{minuend}^2 + \sigma_{subtrahend}^2}$, with the subtrahend always being the baseline condition and the minuend representing either the rat encounter condition or the measurements from the day after the simulated rat encounter. To determine the significance of the shifts, we used the Mann-Whitney U-Test. The original thresholds in the figures were *=0.05, **=0.01, ***=0.001, and n.s. = not significant, which were then applied with a Bonferroni-correction corresponding to the number of bins considered in each subplot.

For the summary data, multiple mice into one group, the means and standard deviations of their individual bins were combined, under the assumption of independence between mice, with the formulas $\mu = \frac{\sum_{i=1}^{n} \mu_i}{n}$ for the mean and $\sigma = \sqrt{\frac{\sum_{i=1}^{n} \sigma_i}{n}}$ for the standard deviation of the

combined bin. The p-values were averaged, weighted by the inverse of the variance of the individual bins they originated from. This happened according to the formula $p_{group} = \frac{\sum_{i=1}^{n} 1/\sigma_i^2 \cdot p_i}{\sum_{i=1}^{n} 1/\sigma_i^2}$, with n corresponding to the individuals in the group. Due to the high number of single samples per bin, the estimate of significance was limited to the ethologically relevant threshold of 10% of the 5th to 95th percentile span of each behavioral feature, across animals and conditions.

Data analysis for freely moving mice

All analyses were performed blind to the source of the mice. When shown, the unblinded mouse ID follows the format "C{Cohort #}{Source}{Mouse #}," where the source is indicated as C (Charles River), I (Internal), or J (Janvier). For example, the ID *C2J1* refers to the first mouse from Janvier in cohort 2. No significant behavioral differences were observed among mice from the three sources. Detailed categorizations by experiment, cohort, and source are presented in **Supplementary Figure 9**.

Mice were classified as "fleeing" or "non-fleeing" based on the median of all loom-wise ratios comparing the mean speed during the 5-second looming visual stimulus to the mean speed in the subsequent 5 seconds. A threshold ratio of 2 was chosen as a conservative criterion for identifying clear fleeing behavior.

The same threshold (a two-fold change) was used to identify mice that exhibited ethologically meaningful changes in behavior in the odor-based experiments. Specifically, this included avoidance or preference for the rat-odor side of the arena, as well as avoidance of the region adjacent to the mesh in the live rat exposure condition. Mice falling below this threshold were classified as behaviorally unchanged.

Data analysis Software

SLEAP

Two different SLEAP (Pereira et al. 2022) bottom-up models (version 1.3.3) were trained to track the location of the mice in the verification experiments. The side view model for videos recorded in the looming stimulus experiments was trained on 225 masked frames (to crop the moving looming stimulus and reduce the complexity of the video). The top view model for videos recorded in the rat odor and presence experiments was trained on 430 frames.

DeepLabCut

A series of DeepLabCut models (version 2.1; Mathis et al., 2018) were trained to track key features of mice for behavioral analysis in the simulated foraging task. Separate models were trained for each anatomical landmark: **Body key points** (nose, shoulder, tail base): trained on 220 labeled frames for one cycle of 1,030,000 iterations. **Nose key points** (nose ridge, nose base, nose tip): trained for two cycles of 1,030,000 iterations each. The first cycle used 220 labeled frames; the second used 337. **Eye key points** (eight equidistant points around the pupil, starting from the 12 o'clock position, plus the left and right corners of the eye): trained over three cycles of 1,030,000 iterations each. The training sets consisted of 260, 466, and 629 labeled frames, respectively.

4 Results

Simulated foraging

This paradigm was designed to be easy for mice to perform but was also designed to minimize stress for both rats and mice. Once mice were habituated to head restraint and to obtaining reward by moving the treadmill they could perform 50-100 trials in a day, obtaining a total reward of 0.5 ml in a ~30-45-minute session. When mice started a trial, the lickspout was at a starting position ~30 cm from the mouse (**Video 1**, **Figure 1**). A go cue -- the flashing of the virtual reality monitors -- and a sound cue initiated the trial. A successful trial was one in which mice moved the treadmill 28.5cm in 12 seconds, with the last 1.5 cm of movement controlled automatically within the code. Two to four seconds after reward delivery, depending on the start of licking, the lick spout and red tube over it were reset to their starting position (**Figure 1A**).

Well trained, motivated mice start running on the tread mill immediately after licking the reward, even before the lick spout moved back to starting position. As mice improved their daily performance, adult female rats were habituated to being handled and to the apparatus. Once a cohort of mice were stable in their performance for 3 successive days -- reaching the threshold criteria in their performance -- the experimental data was collected over 3 consecutive days. On the first day control data was collected, on the second day the rat was introduced into the behavioral paradigm (**Figure 1A-C**). The third day was a post-rat control session. To maximize the potential for interaction between the head-fixed mouse and restrained rat, we used only one setting -- open-- on the disk that covered the mouth of the tube holding the rat. Note that in this setting mice could almost touch the rat on each trial and mice could see, smell and hear the rat moving in the tube as it came closer to the mouse. On each trial, with every mouse, rats could stick their nose out of the tube. When rats were in this position, their nose was effectively just above the lickspout for mice (**Video 1**).

Performance

Performance of each mouse was analyzed for three consecutive days: control / baseline day, day with the rat, and a post-rat day. Surprisingly, the success rate of most mice (5/7 mice) was unaffected by the introduction of the rat (**Figure 1F**). Two mice manifested a significant and dramatic decrease in their performance that persisted into the post-rat day. Next, we examined whether mice changed their behavior when the rat was introduced.

Movement speed on treadmill

One measure of performance is the rate of success; another measure was the speed with which mice moved the treadmill (**Figure 2; Video 2**). Mice could run or walk slowly and consistently to cover 28.5 cm in 12 seconds. Most mice learned to move at a consistent speed of around 0.2 m / s. This speed was mostly uniform and on average it stayed constant for most of the trial (**Figure 2B**), but the speed decreased abruptly as mice stopped moving to lick the reward spout (**Figure 2B**).

One issue that arose in monitoring and comparing the behavior of mice from day to day, was whether the position of the mouse on the treadmill changed across days. It was possible that mice were positioned at slightly different height relative to the treadmill, and this effectively

392 changed the speed with which mice moved (**Figure 2B**). To examine this, we selected frames
393 from different time points during each session and examined them for any obvious differences in
394 height of the mouse relative to the treadmill (**Figure 2A; Supplementary Figure 8)**. There were
395 no significant differences in the positioning of mice from day to day.

396 Next, we compared the speed of movement from control sessions (**Figure 2B**, green
397 traces) and sessions when the rat was present for individual mice. These data show that while the
398 majority of mice show no consistent significant changes in their movement speed in presence of
399 the potential predator (**Figure 2B**, blue traces), three out of seven mice showed significant
400 ($p<0.01$, Mann-Whitney U test; MWU) and consistent changes in their movement speed
401 throughout the session (**Figure 2C**). Their average speed was significantly different at the onset
402 of the trials when the rat was present. The two mice that decreased their movement speed in the
403 presence of the rat were slow on the treadmill from the beginning of the trial. One mouse ran
404 significantly faster in the presence of the rat. In the remaining mice, there were no consistent
405 changes in average speed (**Supplementary Figure 2, 3**). Taken together these data suggests that
406 some mice are strongly but differentially affected by the presence of the rat.

407 **Eye movement and pupil diameter**

408 To assess whether mice attend to the presence of the rat, we used DeeplabCut to track eye
409 movement and pupil diameter on the control day and on the day that the rat was introduced into
410 the tube (**Figure 3, Video 3**). We plotted the average position of the pupil in the horizontal axis
411 relative to the two corners of the eyes, over the course of the control session and in the presence
412 of the rat (**Figure 3A-C**). In five out of seven mice, there was a significant ($p< 0.01$, MWU)
413 change in the horizontal position of the pupil in the presence of the rat (**Figure 3B**, green traces);
414 in the remaining 2 there were no consistent changes in eye position. On average, the position of
415 the eyes of five mice was significantly different ($p < 0.01$, Mann Whitney U) during their reward
416 phase compared to the control days (**Supplementary Figure 4**). When the rat was present in the
417 tube and the mice were directly in front of the tube, on average, 3 mice positioned their eyes
418 more in the direction of the rat (looked right) and 2 looked away from the rat. These effects were
419 significant and were evident throughout the trial. Two mice showed no significant change in
420 their eye position (**Figure 3C**, right panels).

421 Next, we examined pupil size (**Figure 3D-F; Video 4**). Pupil size changes with changes
422 in lighting, or changes in parasympathetic or sympathetic system. To establish that light around
423 the face and head of the mouse remained consistent, we measured brightness and light intensity
424 around the eye and at another point on the head. There was no effect on the luminance / light
425 levels around the eyes or head when the rat was introduced.

426 But in the presence of the rat there were significant ($p<0.01$, MWU) changes in pupil
427 diameter in five out of seven mice (**Figure 3D, Supplementary Figure 5**), especially when mice
428 were close to the rat (Blue traces, **Figure 3E**). In these mice, the pupil diameter was consistently
429 smaller throughout the course of the trial and in four out of five of these mice the change in pupil
430 size persisted into the next session. The other 2 mice showed no significant changes in their pupil
431 diameter.

432 **Posture and facial movements**

To assess whether any other aspects of mouse posture or facial movement were affected by the presence of the rat, we measured mouse posture -- estimated by body-length -- and facial movements (**Figure 4A-C; Video 5, Supplementary Figure 6, 7**). In three out of seven mice there was a significant change in the pose when the mouse was confronted by the rat (blue). Additionally, in 2 other mice there was a significant increase in nasal movement speed in the presence of the rat (**Figure 4D-F; Video 6**).

Taken together this work suggests that head-fixed mice forage for reward, they do not flee but a diverse set of behaviors change when they are faced by the rat (**Supplementary Figure 9**). They move differently on the treadmill; they move their eyes and change their pupil size.

Next, we considered whether head fixation, thirst, and the need to forage affected threat perception or the mouses behavioral response to the threat. At the same time, we also assessed whether the mice in our colony were inherently less fearful. To address these issues, we used naive freely moving mice, from our colony and from two other sources.

Effect of looming stimuli

In these experiments, we used 36 naive mice, 12 mice were from our animal facility, 12 newly acquired from Charles River, and 12 from Janvier. Overall, it was not possible to determine whether the source of mice made a difference to the results, but mice obtained from our inhouse facility showed very similar behaviors as those obtained from external sources.

Mice were placed in an arena (**Figure 5A, B**), and when they entered a particular location in the arena, the looming protocol was initiated. A dark shadow enlarged above the mouse, simulating a bird of prey swooping over the mouse. For each mouse, the looming stimulus protocol was repeated 14 times. Post-hoc video analysis showed that none of the mice froze in response to the looming stimulus; seven out of 36 mice ran into the shelter (**Figure 5C, D**). The mice that ran into the shelter moved rapidly during the looming stimulus, and we verified the detection of fleeing behavior (**Figure 5E, F**), the remaining 29 mice did not show any significant and consistent movement toward the shelter. Overall, these results suggest that looming stimuli can reliably evoke fleeing in mice, but this effect is only observed in ~19% of the mice.

Avoidance of rat odors and rat presence

Next, we examined whether freely moving mice (n = 36) reacted to rat odors (**Figure 6A-C**). Mice were placed in an arena with a partition down the middle -- a partition that mice could and did traverse. Five minutes after putting mice in the arena, they were removed from the arena. A rat was introduced on one side of the partition -- a partition that rats could not traverse -- and then removed. The mouse was returned to the arena. Post-hoc analysis of the amount of time spent in each portion of the arena, revealed that mice either preferred the side that had contained the rat ($p<0.01$, MWU), or showed no clear preference for either side of the arena (**Figure 6C**).

Next, we used the same arena but closed off the wire mesh partition that separated the arena into two halves (**Figure 7A, B**). Once again naive mice were placed in the arena but were allowed to explore only one half of the arena, then they were removed, and a rat was placed on the right side of the arena. Mice were then returned to the arena. In these experiments, half the

mice avoided being close to the wire mesh that separated the rat from the mice (**Figure 7C-E**). The other half showed either no preference for one side or the other. Three mice preferred staying close to the mesh. These data suggests that mice do not innately display defensive behaviors in the presence of rats.

5 Discussion and conclusion

Our work shows that the obvious behavioral expressions of fear -- fight, flight or freezing -- are not easily elicited in laboratory mice. Specifically, the hardwired innate fear of a potential predator is not expressed in our experiment in head-fixed mice "foraging" for a reward. In fact, mice continue to forage even when a rat, the "predator", was in close proximity, within licking distance of the mouse. Furthermore, even when mice were free -- not head-fixed -- and able to move in their environment, looming stimuli did not elicit freezing responses and elicited escape to shelter responses in just a quarter of the mice. Finally, freely moving rats elicited an avoidance response in just half of the freely moving mice. Taken together our data suggests that the idea of an innate stimulus, that elicits a fixed and hardwired behavioral response is likely to be very sensitive to context; it might be learned or be history dependent.

Our goal was to reproduce a natural prey and predator encounter in a controlled setting where it is possible to monitor many dimensions of mouse behavior. In our simulated foraging paradigm, the movement of the mouse was digitally tethered to the movement of the reward spout and the tube that could contain the rat toward the mouse. Rats were only introduced into the paradigm for a single session, and only after mice displayed an expert level of understanding of the paradigm that allowed them to obtain a reward.

Our results with this paradigm are surprising. Even though the head-fixed mouse and the rat almost touched each other, most mice did not freeze or flee, and did not push the treadmill in the opposite direction to push the rat away, they simply continued to move to the reward spout. None of the mice showed classic defensive responses in the presence of rats. Instead, in the presence of rats, even though mice adjusted aspects of their movement and posture and two out of five mice showed a decrease in performance most continued toward the reward spout. Furthermore, even though most mice changed their behavior when the rat was introduced, the changes in behavior were not uniform. On average in the presence of the rat, mice did not uniformly run faster or slower, they did not hunch up or elongate, nor did they all look in the direction of the rat. Contrary to our expectations that the pupil would dilate, we observed a consistent constriction in pupil size -- a constriction that could not be explained by a change in luminance. Taken together this work suggests that head-fixed mice do not automatically display a set of defensive responses in the presence of a potential predator.

To examine whether foraging pressure, head fixation or the nature of our mouse colony could explain our results, we modified our experimental design to assess fear in freely moving mice that had ad lib access to food and water (Verma et al. 2016; Burnett et al. 2016). We replicated looming stimulus paradigms (Lenzi et al. 2022), used noxious stimuli, i.e. rat odor (Banik and Anand 2011), presented live rats (Karli 1956) and tested mice from 3 different sources. Surprisingly, looming stimuli elicited a flight response only in ~20% of the of mice; the smell of rats elicited no avoidance response. But the presence of a live rat in an adjacent

514 enclosure generated an avoidance response, even then in only half the mice (**Supplementary**
515 **Figure 10**).

516 Recent work suggests that the history of individual mice changes how they react to
517 looming stimuli (Lenzi et al. 2022; Wang 2020). In their work, socially isolated mice were more
518 likely to attempt to escape from the looming stimulus. In our work mice were socially isolated
519 for a few days before the experiments and still few mice showed escape or defensive behaviors.
520 These results are surprising given that we closely followed previously published paradigms
521 (Yilmaz and Meister 2013; Lenzi et al. 2022) where freezing and fleeing could be elicited. The
522 cohort of animals we used for these experiments was sufficiently large (36 animals), handled
523 identically, and was from diverse sources. It is possible that some aspects of how we habituate,
524 or house animals or how animal caretakers look after mice reduce fear and stress or reduce the
525 behavioral expression of fear in mice (Furlong, Richardson, and McNally 2016; Kallnik et al.
526 2007; Gouveia and Hurst 2017). It is also possible that just as in rats, the sexual identity of mice
527 affects how they respond in our behavioral paradigms (Gruen et al., 2015).

528 Our study was undertaken as an exploration of innate fear in head-fixed mice. Our
529 simulated foraging experimental design was novel. It had visual cues; it had a moving tube (that
530 could hold a rat) linked to the motion of the mouse on a treadmill. It had olfactory cues -- the
531 smell of a rat. It had auditory cues related to the motion of treadmill and the rat in the tube. The
532 behavioral approach was designed to measure fear, to measure how individual mice express
533 defensive responses -- how their behavior changed over ~50-100 trials, spread over 2 days in
534 response to a live rat. Even though mice continue to forage, even after rats come close to them --
535 almost lick them -- it is possible that for a short time, for a trial or a few trials, mice are in fact
536 afraid of rats, but they rapidly habituate to the presence of the rat. This kind of rapid learning
537 over the course of a few trials cannot be ruled out. But as we would be left with only very few
538 trials, potentially just a single trial per mouse, we could not perform these experiments on a scale
539 necessary to rule out these effects.

540 Our work suggests that mice adapt rapidly and learn to suppress their escape responses,
541 that even the response to an innate fear-inducing threat, such as presence of a predator can be
542 dynamically adjusted. It is possible that even innate processes need an appropriate sensory
543 stimulus to trigger or help teach the appropriate behavioral response. Is the innate response
544 genetically programmed, or do innate responses also need to be triggered by contextual stimuli
545 and learning? Our work suggests that even potentially innate processes are context dependent
546 and or they need a correct initial trigger (Heinemans and Moita 2022).

547

6 Acknowledgements

We thank the Charité Workshop for technical assistance, especially Alexander Schill, Jan-Erik Ode and Daniel Deblitz. We also want to thank Melissa Long and the team of the ABPF for their help with animal handling and room planning. Finally, we thank Laura Schwarz of the Sainsbury Wellcome Centre and members of the Larkum lab for useful discussions about earlier versions of this manuscript.

7 Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: http://doi.org/10.5281/zenodo.15264865. Extended data is available on request.

8 Code availability statement

The code used to generate the data shown in this study is publicly available. It can be found here: https://github.com/Marti-Ritter/contextual-modulation-and-blunted-defensive-responses-to-predators.

9 Ethics statement

All experiments were conducted in accordance with the guidelines of animal welfare of the Charité Universitätsmedizin Berlin and the local authorities, the ’Landesamt für Gesundheit und Soziales’.

10 Author Contributions

	Conceptualization	Data curation	Formal analysis	Funding acquisition	Investigation	Methodology	Project administration	Resources	Software	Supervision	Validation	Visualization	Writing – original draft	Writing – review & editing
MR	X	x	x		x	x	x		x	x	x	x	x	x
SÖ		x			x						x			x
LC	x	x			x	x								x
LS	x	x			x	x								x
AH	x	x			x	x				x				x
RB	x					x			x					x
AV	x	x			x	x		x						x
DS	x			x										x
PP	x			x										x
ML	x			x		x		x						x
RS	x			x		x	x	x		x			x	x

11 Funding

The following funding sources have supported this project: (1) Deutsche Forschungsgemeinschaft (DFG), Grant Nos. 246731133, 250048060 and 267823436 to ML; (2) DFG Project number 327654276 – SFB 1315 to ML; (3) European Commission Horizon 2020 Research And Innovation Program and Euratom Research and Training Program 2014–2018 (under grant agreement No. 670118 to ML); (4) Human Brain Project, EU Commission Grant 720270 (SGA1), 785907 (SGA2) and 945539 (SGA3) to ML; (5) Einstein Foundation Berlin EVF-2017-363 to ML; Einstein Foundation Visiting Fellowship EVF-2019-508 to PP; Humboldt Foundation Friedrich Wilhelm Bessel Research Award to PP.

12 Conflict of interest

The authors have declared no conflicts of interest.

13 Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

14 References

Banik, Avijit, and Akshay Anand. 2011. "Loss of Learning in Mice When Exposed to Rat Odor: A Water Maze Study." *Behavioural Brain Research* 216 (1): 466–71. https://doi.org/10.1016/j.bbr.2010.07.035.

Beckers, Tom, Angelos-Miltiadis Krypotos, Yannick Boddez, Marieke Effting, and Merel Kindt. 2013. "What's Wrong with Fear Conditioning?" *Biological Psychology* 92 (1): 90–96. https://doi.org/10.1016/j.biopsycho.2011.12.015.

Blanchard, Robert J., and D.Caroline Blanchard. 1989. "Attack and Defense in Rodents as Ethoexperimental Models for the Study of Emotion." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 13 (January):S3–14. https://doi.org/10.1016/0278-5846(89)90105-X.

Blanchard, Robert J, Mark A Hebert, Pier Ferrari, Paola Palanza, Rayson Figueira, D.Caroline Blanchard, and Stefano Parmigiani. 1998. "Defensive Behaviors in Wild and Laboratory (Swiss) Mice: The Mouse Defense Test Battery." *Physiology & Behavior* 65 (2): 201–9. https://doi.org/10.1016/S0031-9384(98)00012-2.

Branco, Tiago, and Gil Costa. 2020. *Mouse Running*. [object Object]. https://doi.org/10.5281/ZENODO.3926079.

Branco, Tiago, and Peter Redgrave. 2020. "The Neural Basis of Escape Behavior in Vertebrates." *Annual Review of Neuroscience* 43 (1): annurev-neuro-100219-122527. https://doi.org/10.1146/annurev-neuro-100219-122527.

Burnett, C. Joseph, Chia Li, Emily Webber, Eva Tsaousidou, Stephen Y. Xue, Jens C. Brüning, and Michael J. Krashes. 2016. "Hunger-Driven Motivational State Competition." *Neuron* 92 (1): 187–201. https://doi.org/10.1016/j.neuron.2016.08.032.

Dominiak, Sina E., Mostafa A. Nashaat, Keisuke Sehara, Hatem Oraby, Matthew E. Larkum, and Robert N.S. Sachdev. 2019. "Whisking Asymmetry Signals Motor Preparation and the Behavioral State of Mice." *The Journal of Neuroscience* 39 (49): 9818–30. https://doi.org/10.1523/JNEUROSCI.1809-19.2019.

Ebner, Christian, Julia Ledderose, Timothy A. Zolnik, Sina E. Dominiak, Paul Turko, Athanasia Papoutsi, Panayiota Poirazi, et al. 2019. "Optically Induced Calcium-Dependent Gene Activation and Labeling of Active Neurons Using CaMPARI and Cal-Light." *Frontiers in Synaptic Neuroscience* 11 (May):16. https://doi.org/10.3389/fnsyn.2019.00016.

Fanselow, Michael S. 1994. "Neural Organization of the Defensive Behavior System Responsible for Fear." *Psychonomic Bulletin & Review* 1 (4): 429–38. https://doi.org/10.3758/BF03210947.

Fanselow, Michael S., and Zachary T. Pennington. 2017. "The Danger of LeDoux and Pine's Two-System Framework for Fear." *American Journal of Psychiatry* 174 (11): 1120–21. https://doi.org/10.1176/appi.ajp.2017.17070818.

Farmer-Dougan, Valeri, Seshanand Chandrashekar, David Stutzman, Kari Bradham, and James D. Dougan. 2005. "Fox Urine as an Aversive Stimulus: Modification of a Passive Avoidance Task." *The Journal of General Psychology* 132 (3): 313–20. https://doi.org/10.3200/GENP.132.3.313-320.

Furlong, Teri M., Rick Richardson, and Gavan P. McNally. 2016. "Habituation and Extinction of Fear Recruit Overlapping Forebrain Structures." *Neurobiology of Learning and Memory* 128 (February):7–16. https://doi.org/10.1016/j.nlm.2015.11.013.

Gibson, Eleanor J., and Richard D. Walk. 1960. "The 'Visual Cliff,'" April.

Gouveia, Kelly, and Jane L. Hurst. 2017. "Optimising Reliability of Mouse Performance in Behavioural Testing: The Major Role of Non-Aversive Handling." *Scientific Reports* 7 (1): 44999. https://doi.org/10.1038/srep44999.

Gross, Cornelius T., and Newton Sabino Canteras. 2012. "The Many Paths to Fear." *Nature Reviews Neuroscience* 13 (9): 651–58. https://doi.org/10.1038/nrn3301.

Heinemans, Mirjam, and Marta A. Moita. 2022. "Looming Stimuli Reliably Drive Innate, but Not Learned, Defensive Responses in Rats." https://doi.org/10.1101/2022.02.07.479432.

Iglewicz, Boris, and David C. Hoaglin. 1993. *How to Detect and Handle Outliers*. ASQC Basic References in Quality Control, v. 16. Milwaukee, Wis: ASQC Quality Press.

Isosaka, Tomoko, Tomohiko Matsuo, Takashi Yamaguchi, Kazuo Funabiki, Shigetada Nakanishi, Reiko Kobayakawa, and Ko Kobayakawa. 2015. "Htr2a-Expressing Cells in the Central Amygdala Control the Hierarchy between Innate and Learned Fear." *Cell* 163 (5): 1153–64. https://doi.org/10.1016/j.cell.2015.10.047.

Kallnik, Magdalena, Ralf Elvert, Nicole Ehrhardt, Daniela Kissling, Esther Mahabir, Gerd Welzl, Theresa Faus-Kessler, et al. 2007. "Impact of IVC Housing on Emotionality and Fear Learning in Male C3HeB/FeJ and C57BL/6J Mice." *Mammalian Genome* 18 (3): 173–86. https://doi.org/10.1007/s00335-007-9002-z.

Karli, P. 1956. "The Norway Rat's Killing Response to the White Mouse: An Experimental Analysis." *Behaviour* 10 (1/2): 81–103. https://doi.org/10.1163/156853956X00110.

LeDoux, Joseph E. 2014. "Coming to Terms with Fear." *Proceedings of the National Academy of Sciences* 111 (8): 2871–78. https://doi.org/10.1073/pnas.1400335111.

LeDoux, Joseph E., and Richard Brown. 2017. "A Higher-Order Theory of Emotional Consciousness." *Proceedings of the National Academy of Sciences* 114 (10): E2016–25. https://doi.org/10.1073/pnas.1619316114.

Lenzi, Stephen C., Lee Cossell, Benjamin Grainger, Sarah F. Olesen, Tiago Branco, and Troy W. Margrie. 2022. "Threat History Controls Flexible Escape Behavior in Mice." *Current Biology* 32 (13): 2972-2979.e3. https://doi.org/10.1016/j.cub.2022.05.022.

Mathis, Alexander, Pranav Mamidanna, Kevin M. Cury, Taiga Abe, Venkatesh N. Murthy, Mackenzie Weygandt Mathis, and Matthias Bethge. 2018. "DeepLabCut: Markerless Pose Estimation of User-Defined Body Parts with Deep Learning." *Nature Neuroscience* 21 (9): 1281–89. https://doi.org/10.1038/s41593-018-0209-y.

Mobbs, Dean, Ralph Adolphs, Michael S. Fanselow, Lisa Feldman Barrett, Joseph E. LeDoux, Kerry Ressler, and Kay M. Tye. 2019. "Viewpoints: Approaches to Defining and Investigating Fear." *Nature Neuroscience* 22 (8): 1205–16. https://doi.org/10.1038/s41593-019-0456-6.

Mongeau, Raymond, Gabriel A. Miller, Elizabeth Chiang, and David J. Anderson. 2003. "Neural Correlates of Competing Fear Behaviors Evoked by an Innately Aversive Stimulus." *The Journal of Neuroscience* 23 (9): 3855–68. https://doi.org/10.1523/JNEUROSCI.23-09-03855.2003.

Mowrer, O. H., and R. R. Lamoreaux. 1946. "Fear as an Intervening Variable in Avoidance Conditioning." *Journal of Comparative Psychology* 39 (1): 29–50. https://doi.org/10.1037/h0060150.

Panksepp, Jaak. 1971. "Aggression Elicited by Electrical Stimulation of the Hypothalamus in Albino Rats." *Physiology & Behavior* 6 (4): 321–29. https://doi.org/10.1016/0031-9384(71)90163-6.

Pereira, Talmo D., Nathaniel Tabris, Arie Matsliah, David M. Turner, Junyu Li, Shruthi Ravindranath, Eleni S. Papadoyannis, et al. 2022. "SLEAP: A Deep Learning System for Multi-Animal Pose Tracking." *Nature Methods* 19 (4): 486–95. https://doi.org/10.1038/s41592-022-01426-1.

Phillips, R G, and Joseph E. LeDoux. 1992. "Differential Contribution of Amygdala and Hippocampus to Cued and Contextual Fear Conditioning." *Behavioral Neuroscience* 106 (2): 274–85. https://doi.org/10.1037//0735-7044.106.2.274.

Schiff, William. 1965. "Perception of Impending Collision: A Study of Visually Directed Avoidant Behavior." *Psychological Monographs: General and Applied* 79 (11): 1–26. https://doi.org/10.1037/h0093887.

Scidraw. 2020. *Rat*. [object Object]. https://doi.org/10.5281/ZENODO.3925955.

Silva, Bianca A., Cornelius T. Gross, and Johannes Gräff. 2016. "The Neural Circuits of Innate Fear: Detection, Integration, Action, and Memorization." *Learning & Memory* 23 (10): 544–55. https://doi.org/10.1101/lm.042812.116.

Tovote, Philip, Jonathan Paul Fadok, and Andreas Lüthi. 2015. "Neuronal Circuits for Fear and Anxiety." *Nature Reviews Neuroscience* 16 (6): 317–31. https://doi.org/10.1038/nrn3945.

Verma, Dilip, James Wood, Gilliard Lach, Herbert Herzog, Guenther Sperk, and Ramon Tasan. 2016. "Hunger Promotes Fear Extinction by Activation of an Amygdala Microcircuit." *Neuropsychopharmacology* 41 (2): 431–39. https://doi.org/10.1038/npp.2015.163.

Wang, Hao, ed. 2020. *Neural Circuits of Innate Behaviors*. Vol. 1284. Advances in Experimental Medicine and Biology. Singapore: Springer Singapore. https://doi.org/10.1007/978-981-15-7086-5.

Yilmaz, Melis, and Markus Meister. 2013. "Rapid Innate Defensive Responses of Mice to Looming Visual Stimuli." *Current Biology* 23 (20): 2011–15. https://doi.org/10.1016/j.cub.2013.08.015.

15 Captions

Figure 1. *Experimental design, setup, and performance in the presence of a rat.* **A)** Side view of the mechanical components in the apparatus. The movement of a treadmill was tethered to the movement of a lick spout and a large red tube. When mice walked forward, the lick tube moved towards them, when mice moved backwards, the lick tube moved away. A flashing screen (part of a virtual reality display) and a sound cue signaled the start of a trial. Mice had 12 seconds to complete a trial, if they succeeded, reward was delivered within reach of the mouse, and a new trial was initiated by resetting the position of the spout to its starting position away from the mouse. **B)** Threat perception disc. The apparatus was designed to mimic four user defined interactive conditions with the large red tube positioned over the lick spout. The full view condition was one where the opening of the tube was completely open, and it was possible for a head-fixed mouse and the rat to almost touch each other. The olfactory condition was one where odors could be delivered but visual, tactile and other elements inside the tube were blocked. The visual condition was one where mice could see inside the tube. The opaque condition blocked all cues from inside the tube. Note that auditory cues were not filtered and that a fan at the end of the tube could be activated to extract smells from the red tube. **C**) Training procedures. Habituation consisted of head fixing mice on the treadmill and manually rewarding them when they moved forward spontaneously. Following this brief, 1- to 2-day habituation period, the reward delivery was automated. Reward was dispensed after mice had moved 28.5 centimeters-- the circumference of the treadmill. This movement was sufficient to move the lick spout into a position where mice could lick the reward as it was delivered. Over days the flashing light cue of the virtual reality screens, and the sound cue were added as go-cues to begin the trial. The duration of the trial was shortened, giving mice 12 seconds to reach the spout, or move at least 28.5 cm. **D**) Data acquisition. Once all mice of the cohort reached criterion (65% success rate) baseline data was acquired. In the next session, a live rat was placed inside the tube, and the experiment was repeated -- with the full interaction between the mouse and the rat. Finally, on a third day, reference data in the absence of the rat were acquired. **E**) Performance data. Of the seven mice used for these data sets, five showed no change in their performance in any of the three sessions. For the other two, performance was affected by the presence of the live rat. For both these mice, the reduction in performance persisted through the next day, post rat. Vector drawings of rat and mouse adapted from SciDraw (Branco and Costa 2020; Scidraw 2020).

Figure 2. *Movement speed in the presence of rat.* **A**) Representative images of mouse position from three sessions. The images show the position of a mouse at 3 different times on three different sessions. These kinds of images were used to assess whether the head-fixation height or position of the mouse were stable from day to day. Plots for each of the seven mice are in **Supplement-figure 8**. **B**) Speed of movement on the treadmill. The median and confidence interval of the speed of movement in baseline -- control day -- recordings (black trace) and the session with the rat present (blue) show the two outcomes of having the rat hovering over the lick spout. The left plot (trial phase) shows no change in velocity throughout the trial. In another mouse there was a momentary reduction in speed at the beginning of the trials (right panel, trial phase). When the mice were at the lick spout, they stopped moving (see reward phase). Significance was assessed with a Mann-Whitney U-test (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). See methods for additional filtering applied to significant effects. **C**) Pie chart grouping mouse behavior. Mice could be divided into two main groups, one that showed significant changes in

743 speed and another that showed no change in speed. One mouse significantly increased its speed
744 during the approach to the tube with the rat, 4 mice showed no significant change of speed while
745 approaching the rat, and 2 slowed down significantly.

746 **Figure 3**. *Pupil movement and pupil size in the presence of the rat.* A) Schematic showing the
747 analysis of horizontal pupil position. The x-position was defined relative to the center of the
748 pupil and the corners of the eyes. Movement of the eyes could be nasally oriented (toward the
749 rat) or oriented to the back of the head away from the rat. **B)** Plots for a mouse that positioned its
750 eyes toward the lick tube (nasally) as it moved toward the lick spout (left plot) and positioned its
751 eyes to look forward toward the rat, when the mouse was stopped at lick spout (right plot). The
752 second set of plots on the right show eye position for a mouse which showed no significant
753 change in x-position while in front of the rat. **C**) Pie chart grouping mice. Five mice positioned
754 their eyes significantly differently in the presence of the rat (n=5). Two mice showed no change.
755 Three mice looked toward the tube holding the rat more often, and two looked away from the rat.
756 **D**) Schematic of the pupil diameter analysis. The diameter was calculated based on points
757 labeled by DeepLabCut. **E**) Example plots from two mice, one showing a significant reduction in
758 pupil diameter in presence of the rat (black trace is control session, blue trace was with rat
759 present) the other one showing no effect. **F**) Pie chart grouping mice. Mice could be divided into
760 two groups, mice that showed no change in pupil diameter and a second group of five mice that
761 had a consistent reduction in pupil diameter in the presence of the rat.

762 **Figure 4.** *Changes in posture and facial movement in the presence of a rat.* **A**) Schematic
763 showing analysis of posture using a tail to nose distance. **B**) Body-length diagrams for two mice.
764 The mouse on the left showed no significant change in posture when in front of the rat, the one
765 on the right was significantly more hunched up in the presence of the rat. **C**) Pie chart grouping
766 mice. Mice could be divided into three groups: 4 mice showed no change in body length in the
767 presence of the rat, and 2 mice were more hunched up at the lick tube in the presence of the rat,
768 and one elongated its body at the lick spout in the presence of the rat. **D**) Schematic showing the
769 analysis of facial movement / nose speed. The nose position was detected with DeepLabCut and
770 normalized to the distance between the corners of the eye, to account for mouse size and
771 position. **E**) Facial movement. The left plots show data from a mouse that did not significantly
772 change the movement speed of its nose even when it was directly in front of the rat. The right-
773 side plots show movement data from a mouse that increased the movement speed of its nose
774 significantly, during the reward phase. **F**) Pie chart grouping mice. Most mice showed no
775 significant change in nose speed even when they were directly in front of the rat.

776 **Figure 5.** *Effect of looming stimuli on the behavior of freely moving mice***. A)** Experimental
777 protocol for the looming stimulus paradigm. **B)** Representative annotated video frame. Black
778 bars denote frame boundaries, and the white area marks the masked region used to isolate the
779 tracking area. The transformed arena outline is overlaid. **C)** Scatterplot showing the median
780 shelter-relative speed for each mouse across all loom presentations. The behavior classification
781 threshold (speed ratio = 2) is indicated by a dashed black line. Individual cohorts are marked
782 with distinct symbols, and behavioral groups ("fleeing" vs. "non-fleeing") are color-coded. The
783 global distribution of all average speeds is shown as a background contour plot. Seven of 32
784 mice met the criterion for consistent flight behavior (see Methods). We avoided defining a
785 "freezing" group based on these parameters, as the trial-wise speed distribution did not show a
786 clean separation as it did for the "fleeing" animals. **D)** Strip plot of average speed ratios per

mouse. The median ratio for each animal is shown as a black horizontal bar. Mice were classified as "fleeing" if their median ratio exceeded 2. Numbers in brackets next to mouse IDs indicate how many individual looms exceeded the plot's y-axis limit. Mouse *C1I3* completed only seven looms, having remained in the shelter after the seventh presentation. **E)** Time-aligned traces of shelter-relative X-position for all loom events from mice in the "fleeing" group. The median and 95% confidence interval are shown as a bold line and surrounding shaded region, respectively. **F)** Same as in E, but for loom events from mice in the "non-fleeing" group. Vector illustrations of the rat and mouse in this and subsequent figures were adapted from SciDraw (SciDraw, 2020a; Costa, 2020).

Figure 6. *Avoidance of rat odors.* **A)** Protocol of rat odor avoidance experiment. **B)** Same as Fig. 5B, but for the rat odor avoidance experiment. **C)** Scatterplot mapping mouse-wise preference for the clean area before and after the rat was introduced to the "rat odor" area. The behavior class criteria are shown as dark black dashed lines, while the 2 cohorts of animals used in the experiments are marked with different symbols. The resulting behavior groups are shown with three different colors (avoiding the rat odor in blue, which did not occur); and no change in preference (gold) and preferring the rat odor (green). Six out of 32 mice preferred the area with rat odor after the rat was introduced. **D)** Overall distribution of positions of mice in the arena, belonging to the group of mice "preferring" the rat odor, before the rat was introduced. **E)** Same as D, but for the position distribution after the rat was introduced.

Figure 7. *Avoidance behavior in the presence of a live rat.* **A)** Protocol of rat presence experiment. **B)** Same as Fig. 5B, but for the rat presence experiment. **C)** Same as Fig. 6B but for the ratio of avoidance of the area close to the mesh without and with the rat present. All three possible behavioral groups were observed (avoiding in blue, preferring in green and no change in yellow). Eighteen out of 32 mice avoided the mesh when the rat was present, while 3 mice preferred to explore the mesh when the rat was present. **D)** Same as Fig. 6D but applied to mice that avoided the mesh once the rat was present, showing positions before the rat was present. **E)** Same as D but showing the overall position distribution while the rat was present.

16 Figures

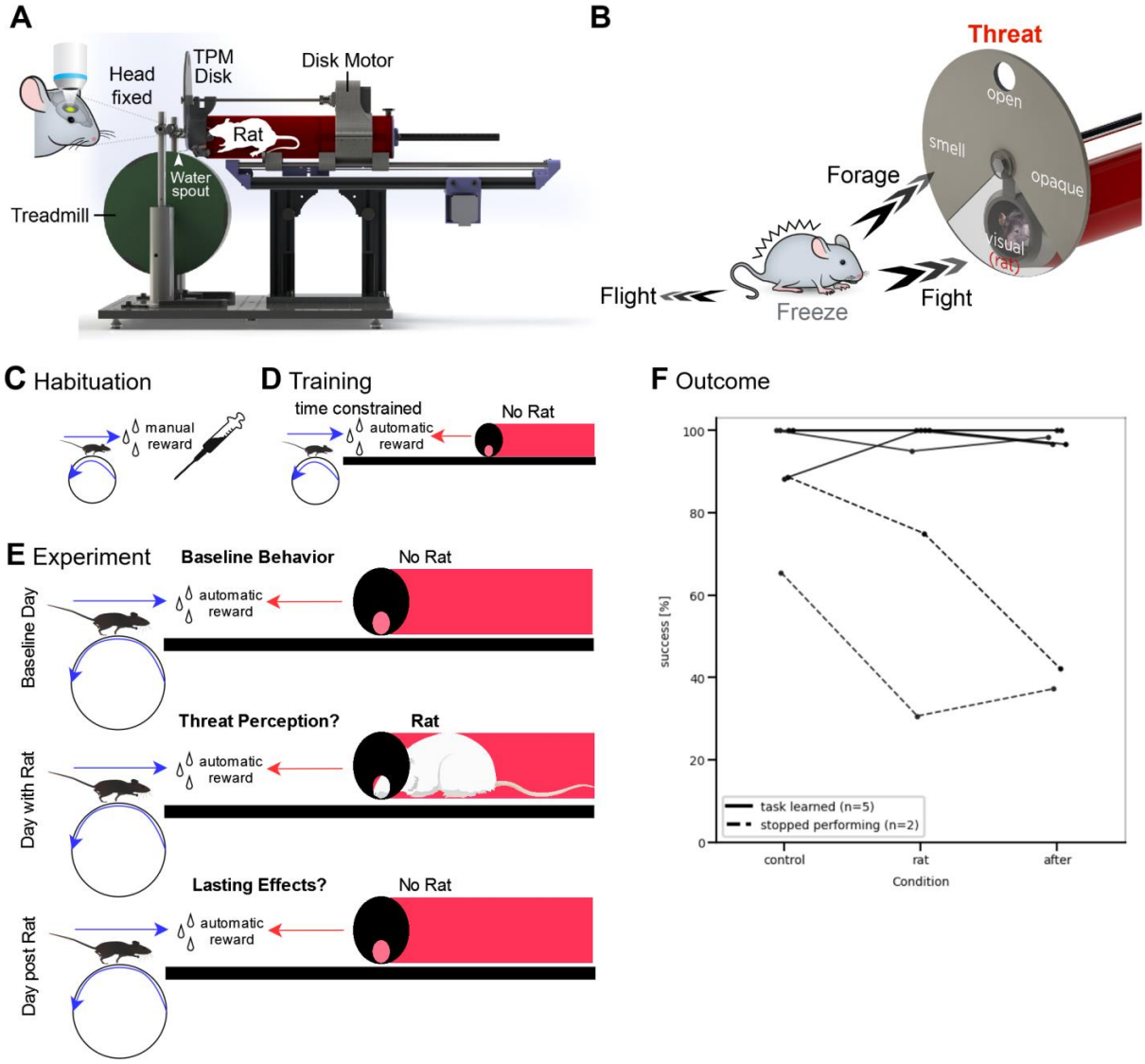


Figure *1*. *Experimental design, setup, and performance in the presence of a rat.*

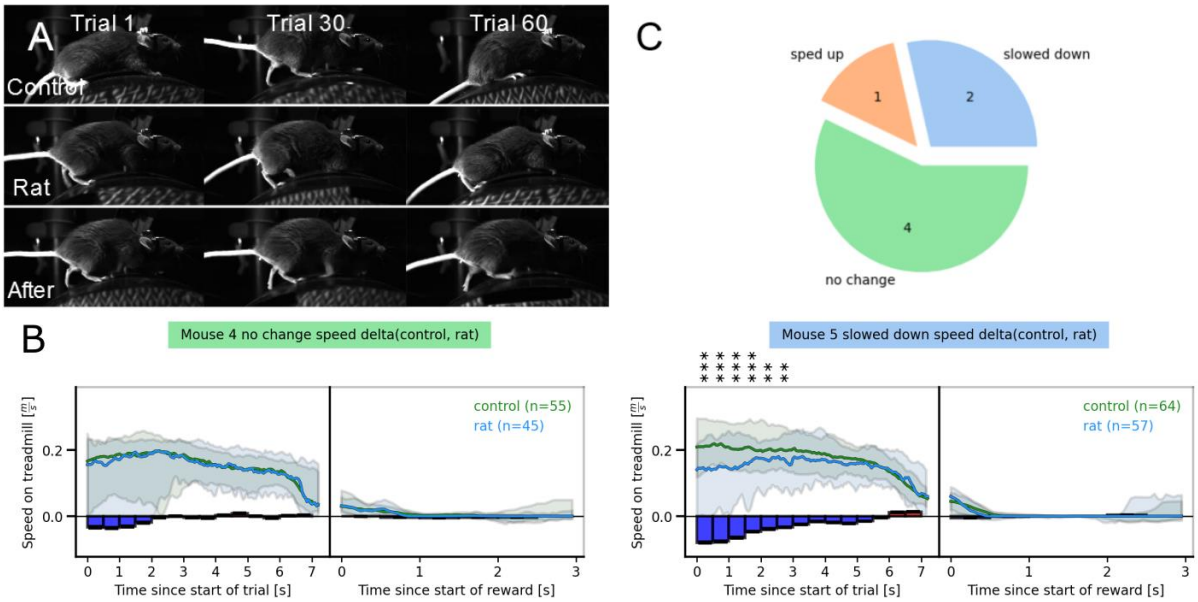


Figure 2. *Movement speed in the presence of rat.*

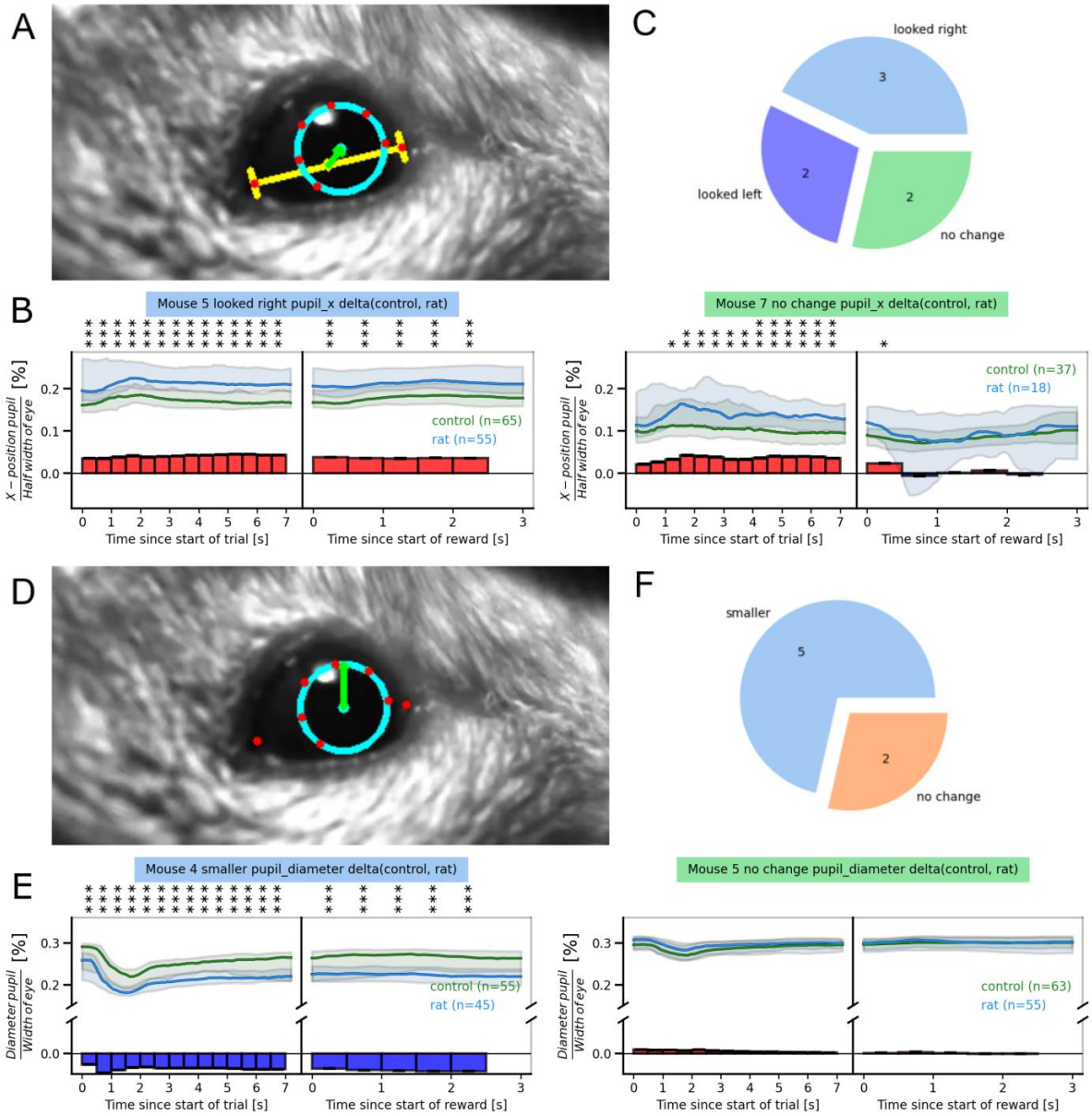


Figure 3. *Pupil movement and pupil size in the presence of the rat.*

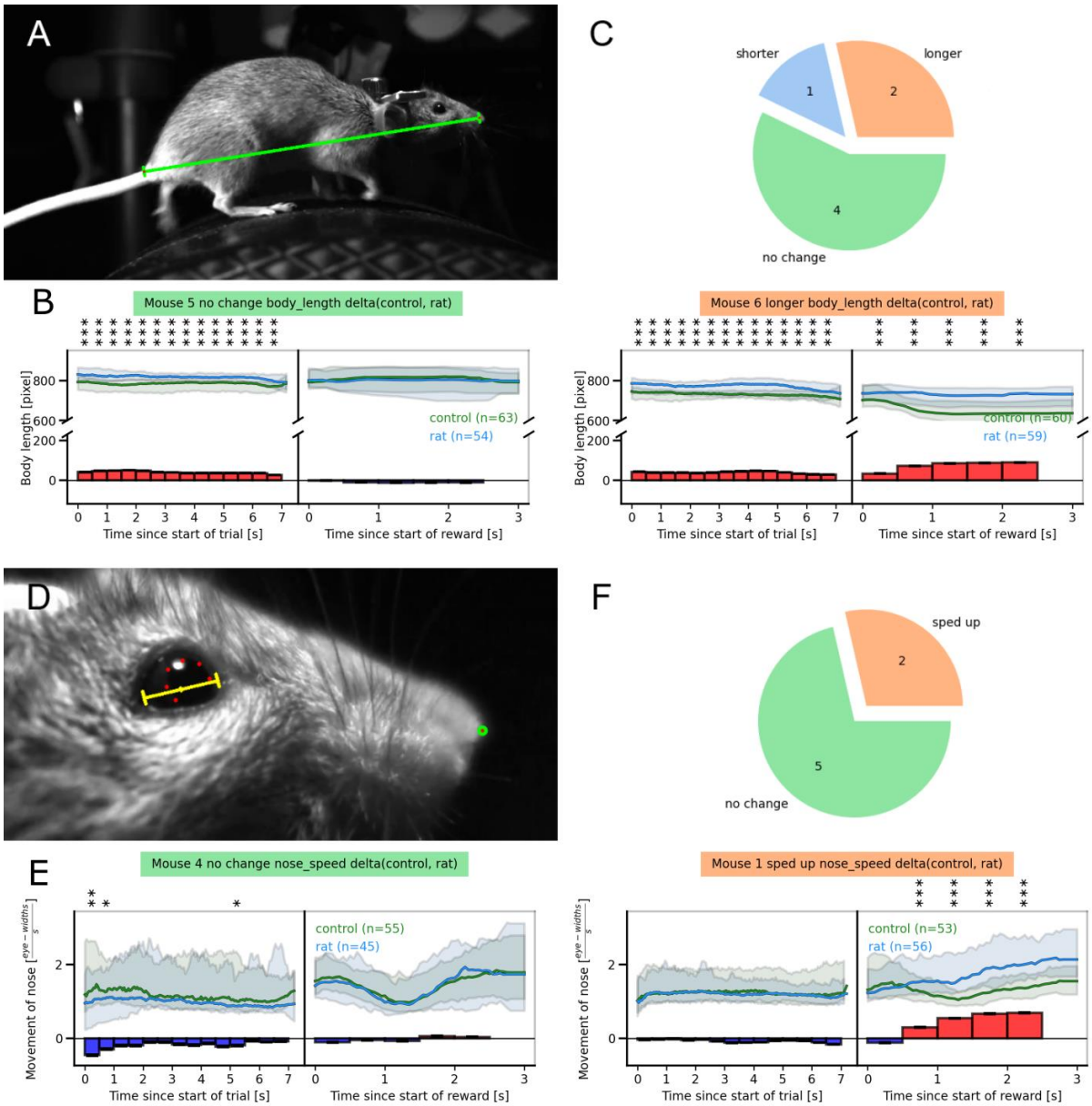
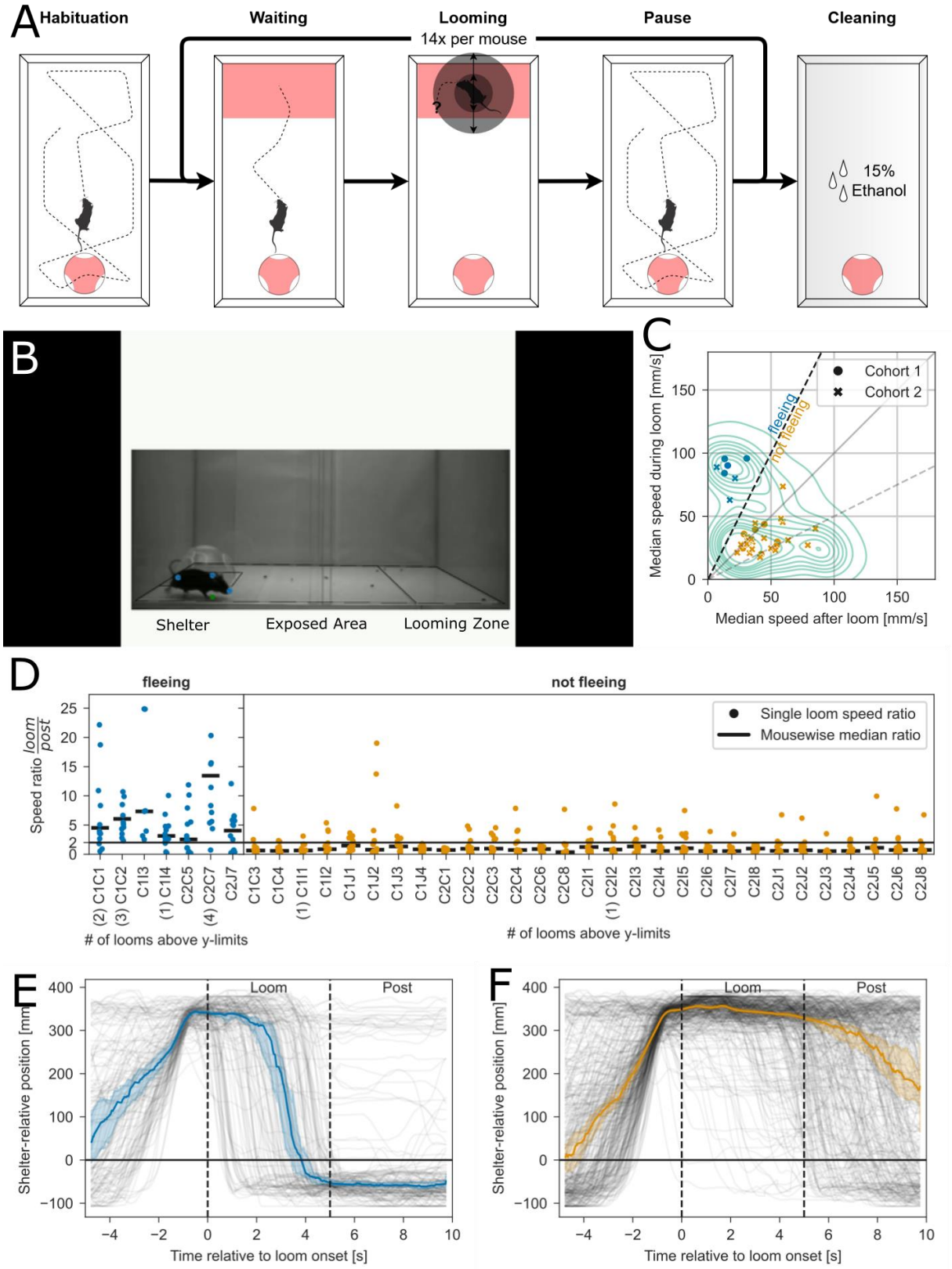
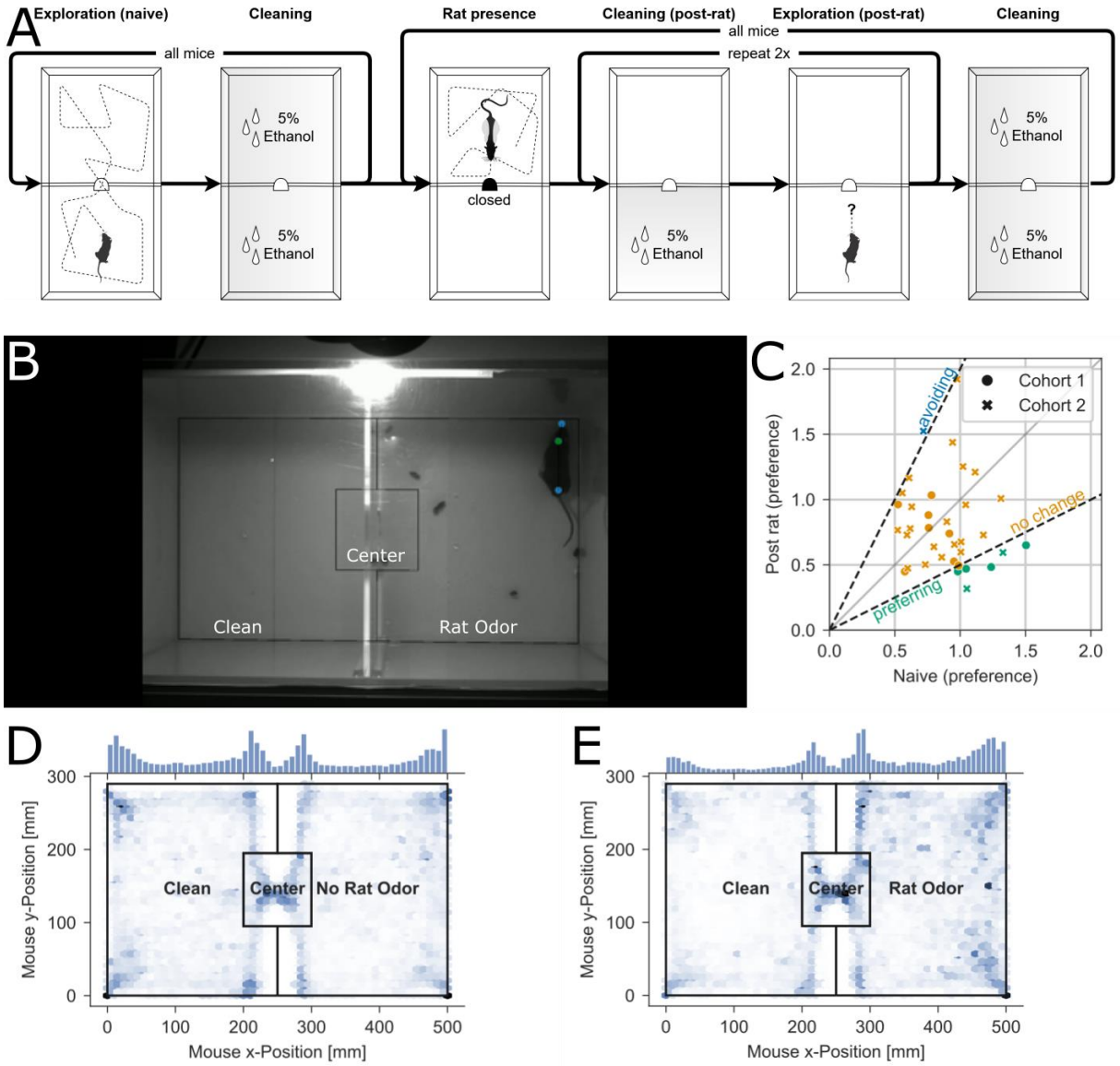


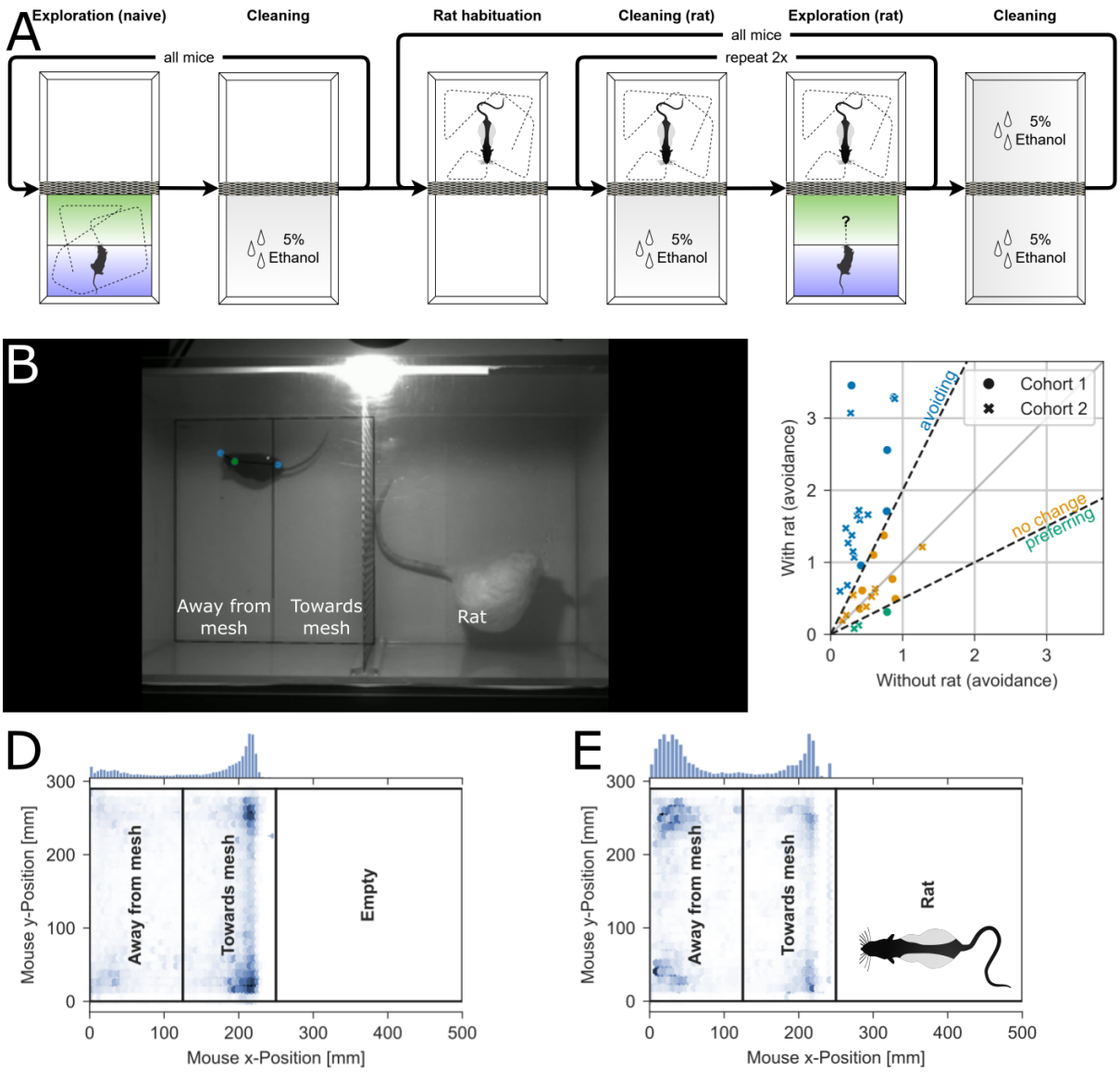
Figure 4. *Changes in posture and facial movement in the presence of a rat.*



820 **Figure 5.** *Effect of looming stimuli on the behavior of freely moving mice*



821 **Figure 6**. *Avoidance of rat odors.*



822 **Figure 7.** *Avoidance behavior in the presence of a live rat.*