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Characterization and comparison of visually induced defensive behavior in *Peromyscus*

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Acknowledgements

Just like a small zoo mouse hesitantly foraging in open field, I started this thesis not knowing what to expect. Little did I know that I would end up in the most open lab group I could have imagined. Each and everyone was truly amazing and incredibly helpful when I had questions. Even after the working hours they all formed one tight group and were not hesitant to include me in their activities. For this reason I would want to thank Karl Farrow for giving me the opportunity to be part of his impressive lab group and for investing his time in me. It is because of this experience that I learned the value of cooperating in a research lab, and of course to 'struggle and relax'.

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Samenvatting

Een essentiële taak voor veel dieren is het vermijden van roofvogels, wat voornamelijk gestuurd wordt door het visueel systeem. Afhankelijk van de habitat en levensloop vertonen verschillende diersoorten een verschillend gedrag. Deze studie streeft naar het karakteriseren van het visueel-geïnduceerd aangeboren defensief gedrag in twee nauwverwante soorten van de *Peromyscus* knaagdieren. Hiervoor wordt gebruik gemaakt van een controleerbare open omgeving waar verschillende dreigende stimuli gepresenteerd worden van bovenaf. Uit de data blijkt dat deze stimuli consistent defensieve responsen uitlokten. Verschillende stimuli die ofwel een overvliegende of aanvallende roofvogel imiteren, lokten verschillende defensieve strategieën uit in de species. Hoewel een overvliegende roofvogel een roerloze toestand uitlokte in beide species, zorgde een aanvallende roofvogel voor verschillende reacties tussen de soorten, namelijk roerloosheid of vluchtgedrag. Dit verschil in respons veranderde niet wanneer de stimulus parameters en de open omgeving werden aangepast. Deze verschillende responsstrategieën resulteren hoogstwaarschijnlijk van neurale verschillen in circuits die betrekking hebben op het visueel systeem en het defensief gedrag. Chemische inactivatie-experimenten ondersteunen de rol van een geconserveerde structuur in de middenhersenen, de superior colliculus, in het mediëren van deze gedragingen in deze soorten. Samengevat, data uit deze studie toont merkbaar verschillende reacties aan in twee nauwverwante *Peromyscus* soorten tegenover dezelfde visuele dreiging. Dit suggereert dat de neurale circuits die betrokken zijn bij defensief gedrag verschillen tussen deze twee species. Dit onderzoek vormt dus een basis voor evolutionaire studies van zulke essentiële neurale circuits.

Summary

Avoidance of avian predators is an essential task in many animals that is predominantly guided by the visual system. Different species may rely on different behaviors based on their habitat and life history. This study aims to characterize visually induced innate defensive behaviors of two closely related rodent species of *Peromyscus*. To accomplish this, a controlled open field environment was designed where different overhead threat stimuli were presented. It was found that these stimuli reliably induce defensive responses. Different types of stimuli that imitate either an overflying or attacking predator induced different defensive strategies in both species. While an immobility response was measured for an overflying predator in both species, an attacking predator induced different behaviors, i.e. immobility or flight, in each species. These response differences did not change when modulating stimulus parameters and the open field environment. The observed difference in response strategies between the species most likely results from neurological differences in vision-associated circuits that drive defensive behaviors. Chemical inactivation experiments support a role for a conserved midbrain structure, the superior colliculus, for mediating these behaviors in both species. In summary, the data obtained in this study show distinct response behaviors of two close *Peromyscus* relatives to identical visual threat, which suggests that the neural circuits underlying visually induced defensive behaviors are wired differently in the two species. This work thus forms a basis to study evolution of such essential circuitry.

Abbreviation list

DMSO	Dimethyl sulfoxide
PM	*Peromyscus maniculatus*
PO	*Peromyscus polionotus*
SC	Superior colliculus

1. Introduction

1.1 Problem definition

Sensory cues in the surrounding of animals can trigger physiological changes that induce innate defensive behaviors. These innate behaviors are essential for the survival of animals since they allow a fast and reliable avoidance of potential danger, as well as the detection and orienting towards food sources and mating partners.[1] Therefore, sensory systems are of great importance, including vision.[2] Visual information has proven to evoke innate defensive responses in a variety of animal species such as mice, rats, *Drosophila*, monkeys and zebrafish, which tend to be a conserved trait.[1-6] However, adaptations of behavioral strategies to different environments can be observed in closely related species,[7] which allows to study the evolution of behaviors and their underlying neural circuitry. The goal of this research is to study two closely related rodent species of *Peromyscus* to determine their behavioral differences to visual threat stimuli. Using a controlled open field environment and different ecologically relevant overhead threats, the defensive behaviors are characterized of the two closely related *Peromyscus maniculatus* and *Peromyscus polionotus*. This work addresses the different innate behavioral strategies between close relatives which will serve as a baseline to probe different neural circuit elements and their impact on behavior.

1.2 Neural circuitry associated with the visual system underlies innate behaviors

The processing and transmittance of visual information is supported by a complex interaction of organized neural circuits that support defensive responses to visual stimuli. Any visual information is observed by the eye where the retina detects and processes these signals.[8] The output neurons of the retina are the retinal ganglion cells.[9] More than 30 different types of retinal ganglion cells each send information about a different feature of the visual world to the brain, such as illumination, speed, size, orientation and directionality.[8,10-12] Such a large variety of different functional ganglion cell types constitutes a wide array of visual features to which the brain can subsequently act upon. As many as 40 different brain targets have been identified to be directly innervated by one or several retinal ganglion cell types, linking the perception of specific visual features to specific output activities.[13,14] Over the past decade, extended research focusing on the neurological foundation of visually induced innate behaviors has identified several brain areas mediating such responses. Of these, a highly retinorecipient midbrain area, called the superior colliculus (SC), is denoted as one of the key mediators due to its vast retinal input and behaviorally relevant output projections. In rodents, approximately 85% of the retinal output projects to the superficial layers of the SC, hence called the visual layers, while the deeper layers receive other sensory inputs and are associated with motor functions.[15,16] Neurons in the visual layers provide input to target areas, such as the lateral pulvinar and the parabigeminal nucleus.[17] These two targets have recently been demonstrated to promote specific visually induced behavioral responses in

mice, with the lateral pulvinar inducing immobility and the parabigeminal nucleus inducing flight.[18] These distinct targets are known to sample distinct retinal information from the SC with only a partial overlap between both structures.[19] As a consequence, information concerning distinct detected visual features can be selectively distributed in the brain to trigger specific behavioral responses.

1.3 Studying defensive behavior in mice

Earlier research regarding visually induced defensive behavior included real predators to evoke responses in animals.[20] Current studies, however, imitates the presence of predators by displaying a dynamic dark disc on a screen that is incorporated in a controlled testing environment.[18,21-24] This enables a good control over different parameters defining the visual stimulus such as speed, size, position, shape or color, which allows a good reproducibility of the experimental design. Two main classes of ecologically relevant stimuli have been developed and validated in mouse literature, i.e. the looming and sweeping stimulus.[21,23] A looming stimulus resembles an expanding disc representing an approaching object, and when presented as an overhead stimulus, it mimics an attacking avian predator. Sweeping, on the other hand, is a fundamentally different stimulus consisting of a laterally moving disc with constant size that mimics an overflying avian predator. In mice, these different stimuli have demonstrated to induce different innate defensive behaviors. Sweeping tends to evoke immobility while overhead looming induces both flight and immobility, with a predominance for flight towards a hiding spot.[21-23] Mice thus possess an innate defensive repertoire to respond to different types of visual threat stimuli.

Behavioral experiments with freely moving animals generally include one or several cameras around the setup to record the behavior of the animal. The video recordings are used to track the position and the speed of the animal. Behavioral responses can be quantified by either automatic annotation of the processed recordings, by manually scoring behavioral events, or by a combination of both.[3,20-23,25,26] Generally, immobility and flight behaviors are of interest when addressing defensive behaviors in mice towards visual stimuli.[3,21-23] When not manually annotated, these can be defined based on the extracted speed that was generated by the animal tracking, and on the directionality of movement such as entering a hiding spot during flight. Quantitative measures of these behaviors, such as the latency, duration, maximum speed or number of events, can be informative for the intensity of the reaction. In addition, to test the effect of visual stimulation on exploratory behavior, rearing events are generally manually scored.[21] By using both manual and automated approaches to study the behavior, the defensive repertoire of animals can be examined towards different visual threat stimuli.

1.4 Defensive behavior in mice are affected by experimental conditions

Although visual stimuli have demonstrated reliable responses in mice, these innate behaviors were observed to depend on multiple experimental parameters. An increase in sweeping speed was found to induce less immobility and more escape in mice.[23] On the other hand, an increase in looming speed was found to reduce escape. In addition to stimulus speed, several other parameters have demonstrated to affect defensive behavior, such as the contrast of the stimulus, its position in the visual field, inward shrinking instead of expansion, and the patterning of the background.[21] Defensive responses in mice are not exclusively affected by the properties of the stimulus, but are shaped by environmental information as well. The presence and absence of a hiding spot has demonstrated to modulate their escape behavior.[3,22] The awareness of such a hiding spot promoted shelter-directed escape even after a single exploratory shelter visit, while the absence of a shelter promoted a change in defensive strategy favoring immobility. Moreover, in the presence of a shelter, the animals demonstrated to flee towards the shelter following a straight trajectory, indicating their awareness of its location. Escape in mice is thus a goal-directed activity that is modified by knowledge related to the environment. These data suggest that defensive behaviors are influenced by both experimental conditions and stimulus parameters.[21,22]

1.5 Using closely related *Peromyscus* species

Peromyscus are small rodents found in North America inhabiting a variety of different territories ranging from Alaska to Central America. Comprising more than 50 different species that live in a variety of different habitats, they are known to be one of the most abundant and diverse groups of North American mammals.[27-29] In the early history of taxonomy, the majority of small mouse-like rodents were classified based on their external appearance under the genus *Mus*. Later, the species currently known as *Peromyscus* were reclassified multiple times under different names.[30] Due to their appearance, *Peromyscus* are also referred to as ‘deer mice’ or ‘white-footed mice’.[30,31] It should be noted that although they are referred to as ‘mice’, *Peromyscus* are more closely related to hamsters than to the commonly used genera *Mus* and *Rattus*.[32] Nevertheless, *Peromyscus* have demonstrated easy maintenance in laboratory settings similar to *Mus*, and they have been successfully used in various research topics including genetics, ecology, reproductive biology and parasitology, in which they have demonstrated several advantages over commonly used laboratory rodents.[27,33] The ecological diversity of this genus proves an interesting framework for evolutionary research concerning opposing traits in close relatives. Closely related species have demonstrated striking differences in a variety of aspects such as coat color, mating strategies, social behavior, temperament, spatial navigation and burrowing behavior.[7,25,27,34-36] For example, a genetic analysis on the inheritance of specific burrow traits in interspecific crosses of interfertile species enabled the detection of an evolutionary

underpinning of this behavior.[7] The possibility to perform interspecific crossing experiments renders *Peromyscus* a great model system to study behavioral evolution. Although a great amount of data has been documented for *Peromyscus* in various fields, making them “the *Drosophila* of the North American mammalogy”,[27] these animals have not yet been integrated in behavioral neuroscience investigating vision-based innate defensive behaviors. However, unpublished data from Hopi Hoekstra (Harvard University) suggest different innate behavioral phenotypes towards visual stimuli in two close relatives, i.e. *P. maniculatus bairdii* and *P. polionotus subgriseus*. These two species might therefore be useful to investigate differences in neural circuitry that underly visually induced behaviors. Based on this preliminary data, these two species are included in our research to study visually induced innate defensive behaviors.

1.6 Goals

The two closely related *Peromyscus* species *P. maniculatus* and *P. polionotus* will be used to study their repertoire of innate defensive behaviors against visual threat stimuli. This is achieved by testing the animals in a controlled open field environment in which they can move freely and which allows to present overhead visual stimuli. Three different stimuli will be used, i.e. looming, sweeping, and a stimulus combining sweeping with subsequent looming. These imitate either an attacking predator, overflying predator, or an overflying predator that suddenly attacks. Different variations of the stimuli are presented to address their effect on the behavior. For looming, experiments will be performed with (1) a single fast expansion, (2) a stimulus comprising several repetitions over multiple seconds of this single expansion stimulus and (3) looming in absence of a shelter. For sweeping, multiple sweeping speeds will be tested. Next, a sweeping disc at one of these sweeping speeds will be directly followed by the single fast expansion. In addition, one of these stimuli will be used to check the reaction consistency during habituation experiments. Finally, the neural work related to vision-based innate defensive behaviors is initiated by testing whether the superior colliculus is involved in these innate behaviors in *Peromyscus*. To do this, this structure will be chemically inactivated with muscimol and the behavior of these animals to visual threat will be subsequently tested. These experiments will provide information about different innate strategies towards danger in close relatives.

2. Materials and methods

Animals: All animals introduced in behavioral experiments were between 5 weeks and 16 weeks old. In-house colonies of *Peromyscus maniculatus bairdii* (PM) and *Peromyscus polionotus subgriseus* (PO; Harvard University) were used, along with PV-Cre and Ntsr1-GN209Cre mouse lines (The Jackson laboratory; Figure S0). Housing conditions were standardized in a polycarbonate cage containing wooden cage bedding, white cotton and an activity wheel. The animals were group-housed ad libitum separated by sex in a 12h/12h light/dark cycle. Animals included in muscimol inactivation were 7 weeks and 14 weeks of age for PO and PM respectively. All experiments were performed following protocol 165/2018 under ethical approval of KU Leuven.

Behavioral procedure: Behavioral experiments were performed in an open field setup in which the animals could move freely (L: 82.5 cm, W: 35.5 cm, H: 37 cm), which was equipped with a removable shelter (L: 11 cm, W: 20 cm, H: 12.5 cm; Figure 1A). Both the floor of the setup and the shelter consisted of transparent red Plexiglas. A screen (L: 82 cm, W: 35 cm; 2560 x 1080 pixels) covered the top of the setup to display overhead stimuli. Prior to testing individual subjects, the animals were habituated to the experimental room for minimally 30 minutes, after which they were introduced to an external wooden compartment (L: 23 cm, W: 9.8 cm, H: 20.2 cm) attached to the exterior of the setup. The animals were allowed to enter the open field independently via an opening (W: 7 cm, H: 7 cm) connecting the compartment with the open field, which was then closed with a lockable red Plexiglas plate. Animals not entering the open field within 2 minutes were gently guided to the opening. When experiments were performed in the absence of a shelter, the animals were introduced via the top of the setup to not anticipate the presence of an external hiding area. For *Peromyscus*, a 10-minute acclimatization period was provided in the setup, and mice were acclimated for 7 minutes. Visual stimuli were presented after the acclimatization period when the animal moved into the threat zone located at the opposite side of the shelter. When stimuli were presented multiple times, annotated as multiple trials, the inter-stimulus time was 7 minutes. During the experiment, group-housed tested animals were separated from the non-tested animals, and animals not entering the threat zone within 20 minutes after entering the open field were separated and retested the same day. Experiments were performed during the light phase, unless otherwise stated, and were recorded with a frame rate of 25 frames per second with a Manta G-419B camera positioned underneath the setup. Five minutes prior to testing, the setup was cleaned with 70% ethanol to eliminate residual odors of other animals. Naive animals were used to avoid habituation and animals were only handled when introducing them to the setup or during standard care taking.

Visual stimuli: The appearance of an aerial predator was simulated either by presenting an overhead black expanding disc, i.e. a looming stimulus (Figure 1B), or an overhead laterally

moving black disc, i.e. a sweeping stimulus (Figure 1C). The standard looming stimulus consisted of a sequence of 15 expansions each linearly expanding from a visual angle of 2° to 20° in 250 ms, after which the disc remained at the same size for an additional 250 ms. The time between disappearance of the disc and a successive expansion was 500 ms.[21] The sweeping stimulus consisted of a black disc with a visual angle of 4° moving along the length of the screen from the shelter to the threat zone at a constant speed of 21°/s.[23]

Muscimol inactivation of the SC: The inactivation of the superficial SC was obtained with microinjections of muscimol (Bodipy TMR-X Conjugate, ThermoFisher), which is an agonist of the inhibitory gamma-aminobutyric acid (GABA) neurotransmitter.[37] Animals were anaesthetized with isoflurane before and during the surgical procedure. Four spatially distributed microinjections of 100-200 nL (1mg/mL muscimol in dimethyl sulfoxide (DMSO)) were administered in the SC in both hemispheres. To avoid potential time associated effects impairing homogenous inactivation, sequential injections were alternated between both hemispheres. The behavior in the open field was tested within two hours after administration of the first injection. The behavior of the animals was retested one week after surgery without inactivation of the SC. Animals were treated with a combination of trimethoprimum and sulfadiazinum (emdotrim) during one week after surgery.

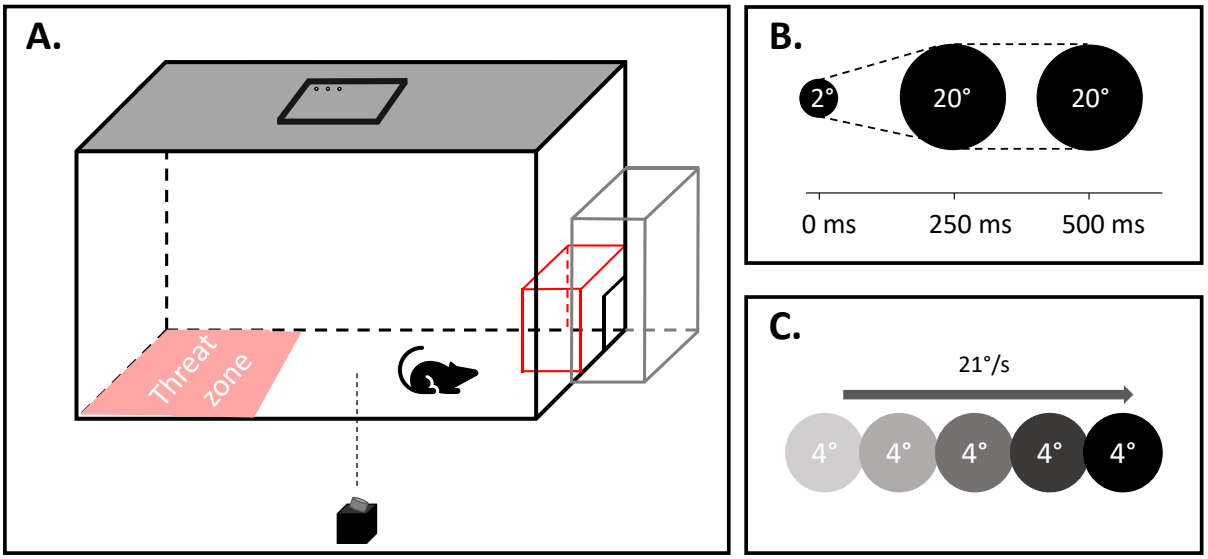


Figure 1. A. Schematic representation of the experimental setup. The black box represents the open field, the grey box the external entrance compartment and the red box the shelter. A screen covered the top of the setup. **B.** The looming stimulus. Visual angles at distinct time points in the expansion cycle are indicated in the disc. The standard looming stimulus comprised 15 repetitions with 500 ms between cycles. **C.** The sweeping stimulus. Grey shades illustratively indicate previous positions of the black disc that moved at constant speed with a constant size.

Data acquisition and processing: Trials were recorded at 25 frames per second (2048x1000 pixels) with a camera positioned underneath the setup. To determine the position of the animal, the recordings were processed using either Python v3.6.5[38] or DeepLabCut,[39] and RStudio v1.1.383[40] was then used to analyze the generated data (supplementary Figure S1) . When tracking animals in Python, a background subtraction was first performed using OpenCV (Open Source Computer Vision Library).[41,42] To minimize the salt and pepper noise, a median filter was applied which calculates the median pixel intensity within an 11x11 pixel area and locally applies this binary value to the corresponding pixels. Subsequent animal tracking was performed on the processed recording using the Tracktor package[43] tracking a contoured object covering an area corresponding to a predefined surface area range. When Tracktor failed to track the animal continuously, DeepLabCut was used to generate the speed and position profiles using unprocessed recordings. As an initial step for DeepLabCut tracking, a training set was created comprising a variety of frames to train the DeepLabCut model with. From both looming and sweeping experiments, 200 diverse frames were selected with a k-means algorithm which were then used to manually annotate the position of the animal. After 200.000 iterative training steps, the videos were automatically tracked based on the feature-extracting deep learning algorithm.

Both methods, i.e. using either Python or DeepLabCut, generated the position of the animal in the setup, which was converted to speed using the Pythagoras theorem. Velocities were expressed in cm/s after applying a transformation factor considering the distance to pixel ratio of the recording. When using DeepLabCut, additional information concerning the tracking accuracy was obtained when tracking animals, enabling the reprocessing of annotated low-probability positions with a likelihood below 99% to reduce speed outliers. These tracking artefacts were replaced using linear interpolation considering both the previous and the following high-probability position (Figure 2A). A general refinement independent of the tracking algorithm was obtained in RStudio by using a 3-window median approach to smoothen the generated speed data (Figure 2B).[44]

The position and speed data obtained by animal tracking enabled the automatic determination of flight episodes and shelter entry. Visual inspection of the resulting tracking data led to the empirical definition for flight as an episode at which velocity exceeded 25 cm/s over minimally 200 ms with the peak velocity exceeding 75 cm/s for *Peromyscus* and 45 cm/s for mice (Figure 2C). Shelter entry was confirmed when the position of the animal crossed the (x,y) threshold corresponding to the location of the shelter. Additionally, immobility episodes in the open field, i.e. occurring in the area outside the shelter, were manually checked in VideoPad v6.23 as immobile states with a minimal duration of 200 ms including minor movements that are not caused by explorative sniffing, grooming or a change in posture. Rearing events were also manually scored as episodes when the animal had

lifted both front legs off the floor. Because of small sample sizes and disturbed normality, data was treated non-parametrically. The behavior during stimulus presentation was compared with control periods prior to visual stimulation when the animal entered the threat zone when no stimulus was presented. Statistical significance was considered when $p<0.05$ with $ns>0.05>*>0.001>**$.

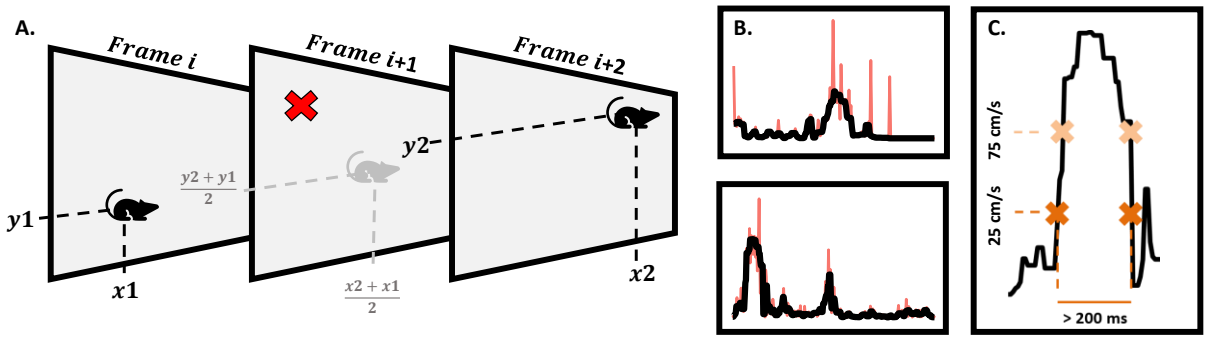


Figure 2. A. Linear interpolation (grey mouse) of low-probability positions (<99%, red cross) using the two nearest successive high-probability positions (black mice) generated with DeepLabCut. **B.** Examples of raw velocity data (red) superposed with the median-smoothened speed profile (black) generated with Python (upper panel) and DeepLabCut (lower panel). **C.** Empirical definition for the detection of flight in *Peromyscus*, with the peak value exceeding 75 cm/s while the speed exceeds 25 cm/s for minimally 200 ms. The peak value for mice was determined to exceed 45 cm/s.

3 Results

3.1 *Peromyscus* display innate defensive behaviors to different visual threat stimuli

3.1.1 Looming-induced responses differed between *Mus* and *Peromyscus* while sweeping induced similar behavior.

To characterize innate reactions in *Peromyscus* to visual stimuli indicating danger, two overhead stimuli described earlier in mouse literature were used to imitate the presence of aerial predators in a controlled open field environment.[21,23] These stimuli were a looming stimulus, which represents an attacking predator, and a sweeping stimulus, representing an overflying predator. Firstly, the efficiency of our setup was demonstrated by replicating published experiments with mice, and then explored the behavior in *Peromyscus*.

The behavior of mice was monitored upon the presentation of a repeated looming stimulus after an acclimatization period of seven minutes. During this period, all animals demonstrated exploratory behavior such as rearing and sniffing around the setup. The repeated looming stimulus was presented on a monitor on top of the setup and comprised a black disc undergoing a repetitive expansion of 15 cycles in 15 seconds (Figure 2B). This suppressed the exploratory behavior of the animals ($p=0.049$; Figure 3G) and reliably promoted a combination of immobility ($p=0.031$; Figure 3E) and flight ($p=0.031$; Figure 3F) behavior (Figure 3A,B). Immobility was initiated with a median latency of 0.2 seconds and was followed by flight with a median latency of 3.87 seconds relative to stimulus onset (Figure 3D). 80% of the animals engaged in shelter-directed flight within 5 sequential expansions, and one animal demonstrated escape to the area next to the shelter (Figure 3B,C). These results demonstrate that the reliable reaction of *Mus* upon repeated looming comprised a single sequence initiated by immobility and followed by escape, predominantly resulting in hiding in the shelter.

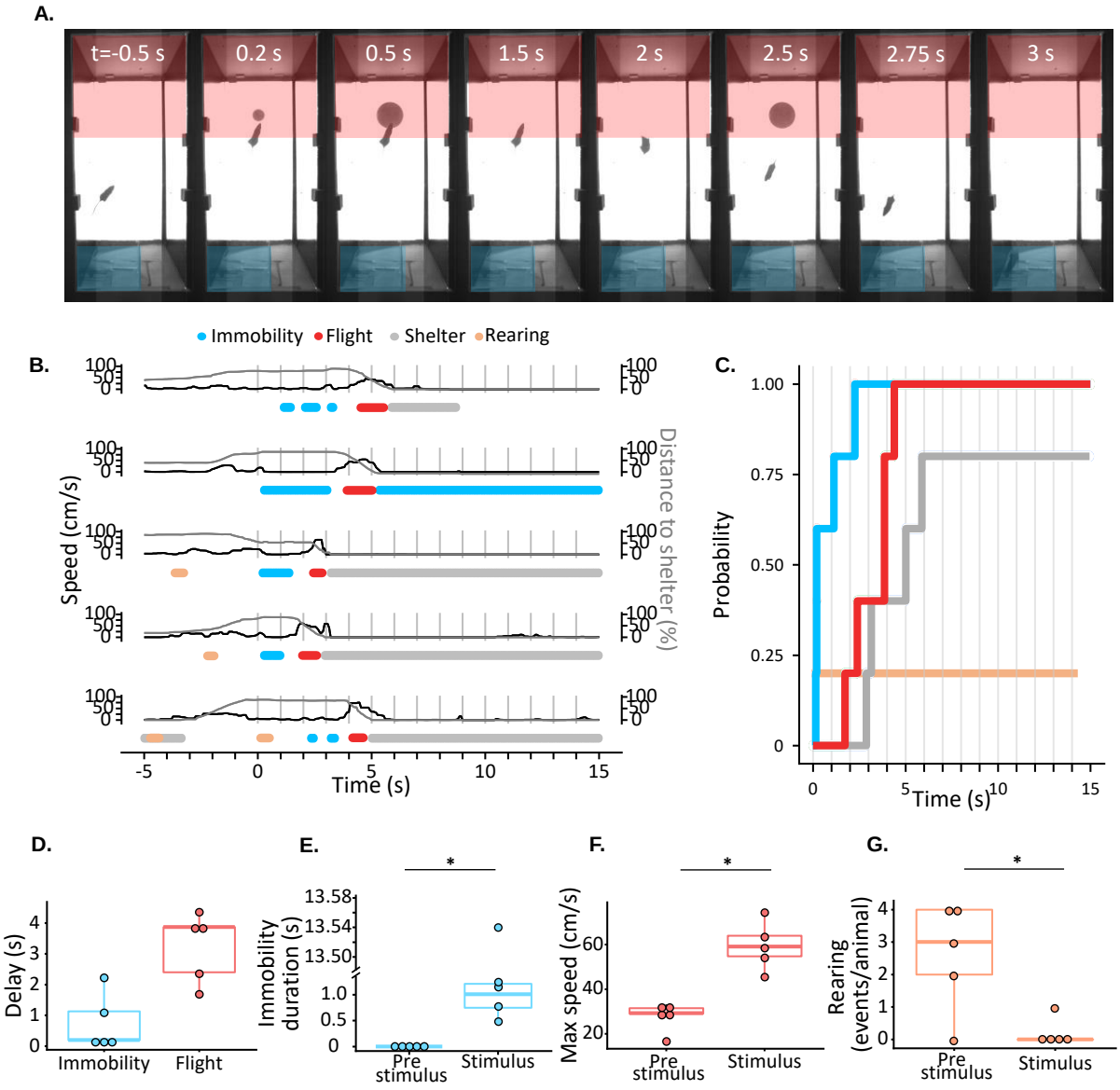


Figure 3. Behavior of *Mus* upon repeated looming (n=5). A. Example recording of the behavior of a mouse (Ntsr1-GN209Cre) in response to overhead repeated looming. The camera perspective visualizes the setup from underneath. Red: threat zone, blue: shelter. **B.** Reaction of mice. Each row corresponds to one animal. Running speed (black graph) and relative distance to the shelter (grey graph). The looming sequence was started at time 0. Grey vertical bars indicate the start of each looming dot. **C.** Cumulative probability for the initiation of immobilization in open field, flight, rearing and shelter entry. **D.** Latency of immobilization and flight relative to stimulus onset. **E.** Duration of immobilization during 15 s control period (Pre stimulus: median=0 s) and during looming (Stimulus: median=1.20 s; paired Wilcoxon test: p=0.031). **F.** Maximum speed during 15 s control period (Pre stimulus: median=29.20 cm/s) and during looming (Stimulus: median=59.08 cm/s; paired Wilcoxon test: p=0.031). **G.** Number of rearing events during 15 s control period (Pre stimulus: median=3) and during looming (Stimulus: median=0; paired Wilcoxon test: p=0.049).

Repeated looming also induced defensive behavior in *Peromyscus* (Figure 4A) and suppressed exploratory behavior as well (Figure 4E). However, their looming-induced response did not comprise the single reaction sequence consisting of immobility followed by

flight as displayed by mice. 80% of the *P. maniculatus* (PM) reacted with flight as the first response to looming (Figure 4A right), and a total of 90% demonstrated flight at a certain point during repeated looming (Figure 4A middle, S2B). Trajectories indicate that immediate flight is directed towards the shelter (Figure 4B,S2A). In total, 80% of the animals entered the shelter during stimulation (Figure 4A middle), either directly after initiating flight or with intermediate immobility events (Figure 4A left). Although immediate flight was observed in 80% of PM, repeated looming also frequently induced immobility following such events. Only 20% of the animals displayed immobility as initial response to looming, while a total of 70% had demonstrated immobility at a certain point during the stimulus (Figure 4A middle). In contrast to PM, 67% of PO reacted with immobility as first response to looming (Figure 4A right), and all the animals displayed immobility in open field at a certain point during the stimulus (Figure 4A middle). Also, cumulative distributions representing all immobility events during repeated looming indicate that PO engages more frequently in immobility than PM (Figure 4C), with a total duration lasting significantly longer than for PM ($p=0.016$; Figure 4F). Only 33% of PO reacted with flight as the initial response, and a total of 42% eventually demonstrated flight during the stimulus. Shelter entry was only observed in 33% of the animals. Cumulative distributions of flight for PO indicate that such events are only displayed during the early stage of repeated looming, and that its distribution reaches below that of PM (Figure 4D).

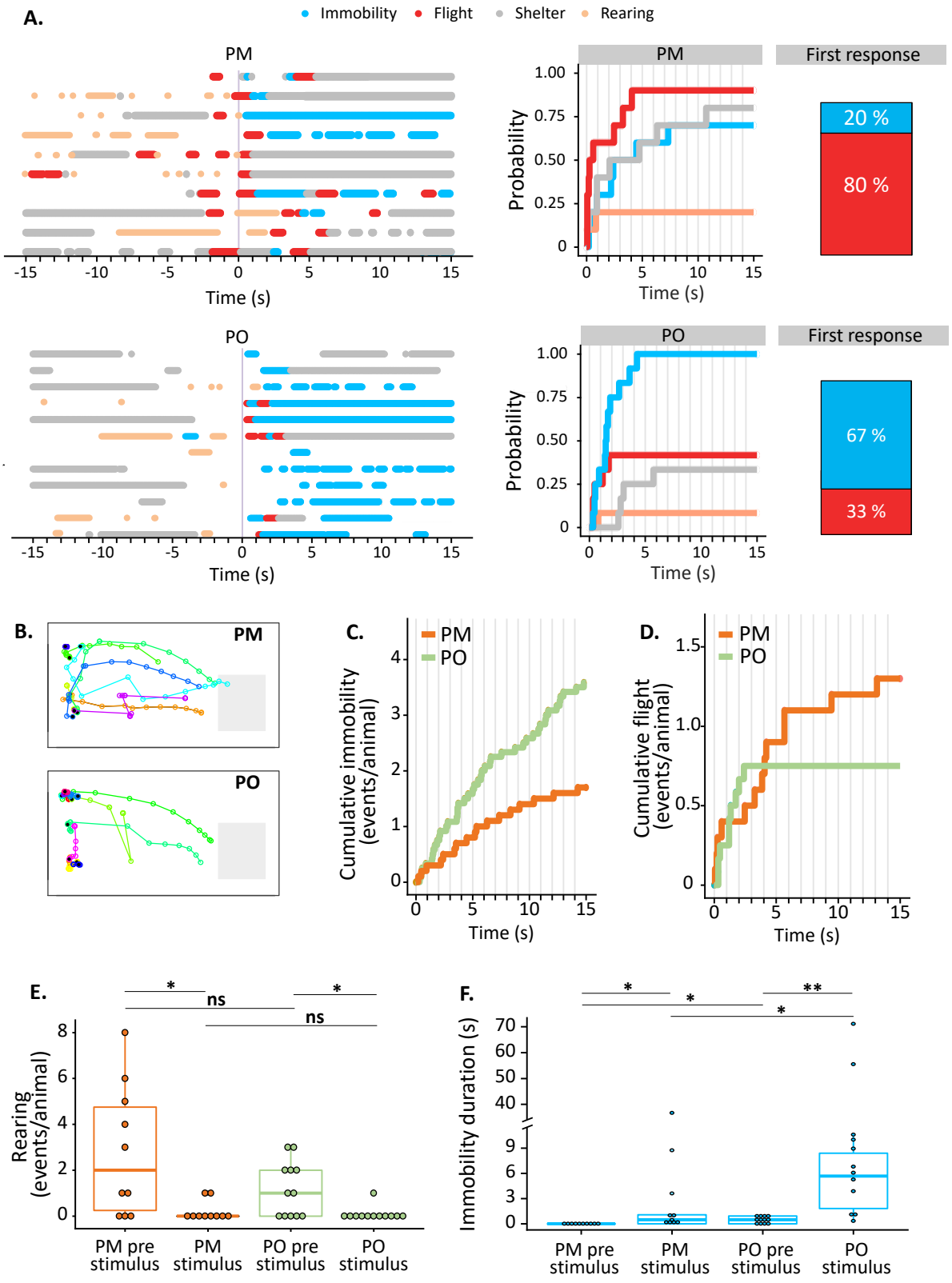


Figure 4. Behavior of *P. maniculatus* (n=10) and *P. polionotus* (n=12) upon repeated looming. A. Left column: reaction of PM and PO to repeated looming. First expansion started at 0 s (grey vertical bar) and 15th expansion at 14 s. Each row corresponds to one animal. Middle column: cumulative probability of the initiation of immobilization in open field, flight, rearing and shelter entry. Grey vertical bars represent individual expansions. Right column: amount of animals displaying either flight or

immobility as initial response to stimulus onset. **B.** Flight trajectories during the first second after stimulus onset. Different animals are indicated in different colors. Grey area represents the shelter and black dots indicate the starting point of individual trajectories. **C.** Cumulative distribution of all immobility events during repeated looming. Grey vertical bars represent individual expansions. **D.** Cumulative distribution of all flight events during repeated looming. **E.** Number of rearing events during 15 s control period (Pre stimulus: PM: median=2, PO: median=1) and during stimulus (Stimulus: PM: median=0, PO: median=0; paired Wilcoxon test: PM p=0.022, PO p=0.021; between species Wilcoxon test: pre stimulus p=0.222, stimulus p=0.471). **F.** Duration of immobilization during 15 s control period (Pre stimulus: PM: median=0 s, PO: median=0.47 s) and during stimulus (Stimulus: PM: median=0.70 s, PO: median=6.43 s; paired Wilcoxon test: PM p=0.022, PO p=0.0005; between species Wilcoxon test: pre stimulus p=0.002, stimulus p=0.016).

In a second step, the behavior of mice was characterized to a sweeping stimulus moving at an angular speed of 21 °/s (Figure 1C,5A), representing an overflying predator. Results suggested that sweeping induces an opposite behavioral response in mice than looming. All animals significantly increased immobility (p=0.005; Figure 5C,E) which was initiated with a median latency of 1.69 s (Figure 5D) and which persisted with a median duration of 1.59 s (Figure 5E). No flight was displayed, but instead, immobility was always followed by rearing (Figure 5B), which was not significantly suppressed by this stimulus (p=0.37; Figure 5F).

In *Peromyscus*, robust responses were also observed toward sweeping. Both species significantly increased immobility (PM: p=0.006; PO: p=0.006; Figure 6C), usually consisting of long lasting events (Figure S3), which was initiated with a median latency of 1.22 s and 0.98 s for PM and PO respectively (p=0.905; Figure 6B). In contrast to mice, sweeping-induced immobility was not systematically followed by rearing in *Peromyscus* (Figure 6A). There was no significant difference in total immobility duration between the two species (p=0.075; Figure 6C), but PM demonstrated significantly longer immobility than mice (Wilcoxon test: PM p=0.002, PO p=0.181). Probability curves giving the initiation of flight indicate that 20% of PM displayed running events suggestive for flight within 1.2 s following stimulus onset (Figure 6A). However, visual assessments of the corresponding recordings indicated that these animals had not yet perceived the stimulus and that their activity abruptly shifted to immobility when encountering the stimulus on its trajectory. On the other hand, 20% of PO displayed shelter-directed flight during sweeping, either following immobility or as initial response. Nevertheless, immobility was observed to be the main response in all animals which could persist for several seconds.

Taken together, mice and *Peromyscus* both demonstrate different defensive strategies towards different visual threat stimuli. Repeated looming induced bimodal behavior in most animals, however with a different reaction sequence depending on the species. The two *Peromyscus* species had opposed initial reactions to the onset of the stimulus and these differences were maintained during the remainder of the repeated looming stimulus. In contrast, a sweeping stimulus evoked immobility in both mice and *Peromyscus*, with a low

flight probability. These results thus indicate that different visual threats can induce different defensive strategies in different species.

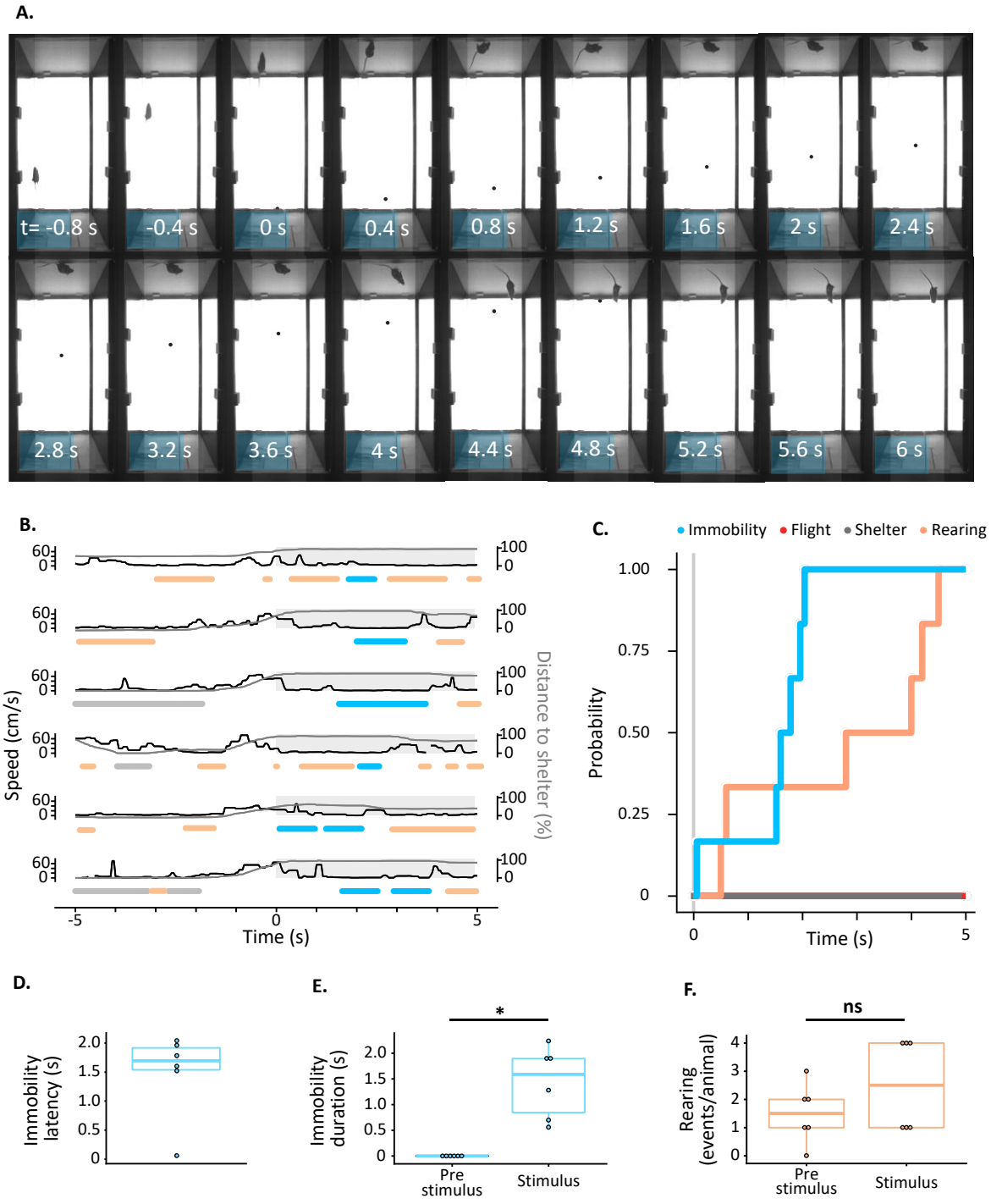


Figure 5. Behavior or *Mus* upon sweeping (n=6). A. Example recording of the behavior of a mouse (PV-Cre) in response to overhead sweeping at 21 °/s. Blue: shelter. **B.** Reaction to sweeping. Running speed (black graph) and relative distance to the shelter (grey graph). Each row corresponds to one animal. Grey boxes starting at 0 s indicate the presence of the sweeping stimulus. **C.** Cumulative probability of initiation of immobilization in open field, flight, rearing, and shelter entry. **D.** Latency of immobility relative to sweeping onset (median=1.69 s). **E.** Duration of immobilization during 5 s control period (Pre stimulus: median=0) and during sweeping (Stimulus: median=1.59 s: paired Wilcoxon test:

p=0.005). **F.** Number of rearing events during 5 s control period (Pre stimulus: median=1.5) and during sweeping (Stimulus: median=2.5; paired Wilcoxon test: p=0.37).

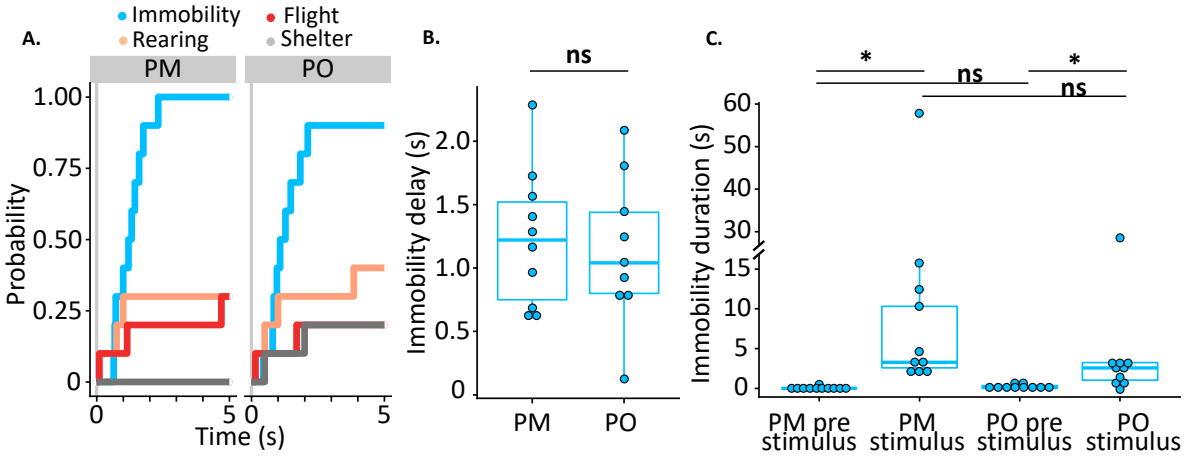


Figure 6. Behavior of PM (n=10) and PO (n=10) upon sweeping. A. Cumulative probability of the initiation of immobilization in open field, flight, rearing, and shelter entry. **B.** Latency of immobilization relative to sweeping onset (PM: median=1.22 s, PO: median=0.98 s; Wilcoxon test: p=0.905). **C.** Duration of immobilization during 5 s control period (Pre stimulus: PM: median=0 s, PO: median=0.15 s) and during sweeping (Stimulus: PM: median=3.94 s, PO: median=2.7 s; paired Wilcoxon test: PM p=0.006, PO p=0.006; between species Wilcoxon test: pre stimulus p=0.076, stimulus p=0.075).

3.1.2. One single fast expansion is efficient to induce defensive behavior in *Peromyscus*

To investigate whether a single expansion also triggers defensive responses in *Peromyscus*, one single expansion was presented that was identical to one expansion event from the repeated looming stimulus. This single expansion was efficient to evoke defensive behaviors in both *Peromyscus* species. Similar to the initial response to repeated looming, only 33% of PO reacted with flight to the single expansion (Figure 7C right), with a median latency of 0.2 s. The direction of their trajectories was not guided towards the shelter (Figure S4A), and none of the animals entered the shelter within three seconds after stimulus onset (Figure 7C left). Immediate immobility was observed in 50% of the PO animals with a median delay of 0.4 s (Figure 7D), which comprised only one single event in the majority of these animals (Figure S4B). One animal did not react to the single looming, even after multiple trials. In contrast to PO, only one animal for PM reacted with immobility as first reaction to looming. 80% reacted with flight directed towards the shelter upon stimulus onset, with a median delay of 0.22 s (Figure 7B, 7C left, 7E, S4A). However, all flight events for PM were terminated with immobility in the vicinity of the shelter with a delay that was twice the delay observed in PO (p=0.047; Figure 7B,7D). Consequently, only 20% of PM entered the shelter two seconds after stimulus onset (Figure 7C left). Instead, several immobility events in open field were observed in PM after flight, resulting in a total immobility lasting significantly longer compared to PO (p=0.041; Figure 7F).

A single expansion induced only one immobility event in the majority of PO, while repeated looming was associated with an increasing cumulative distribution of immobility (Figure 4C). In these animals, the total immobility duration in open field during repeated looming was significantly larger than for a single expansion ($p=0.008$; Figure S4D), without affecting the duration of individual immobility events ($p=0.273$; Figure S4C). In contrast, PM demonstrated multiple immobility events in response to both repeated looming and a single expansion with no significant difference in total immobility duration ($p=0.124$; Figure S4D) and duration of individual immobility events ($p=0.541$; Figure S4C). Moreover, repeated looming drastically increased the probability for shelter entry in both *Peromyscus* species. Although flight was directed towards the shelter for PM, only 20% had entered the shelter two seconds after onset of a single expansion. This is substantially lower compared to repeated looming where a total of 80% of the animals entered the shelter with 50% already entering the shelter within two seconds (Figure 4A). Similarly, no animals for PO entered the shelter within three seconds after onset of a single expansion, while 25% demonstrated to hide during this time for repeated looming.

Taken together, a single expansion of a black disc demonstrated to reliably evoke defensive behaviors in *Peromyscus*. The predominant behavior for each species is consistent with its behavior to repeated looming. However, shelter entry was reduced for a single expansion, compared to repeated looming.

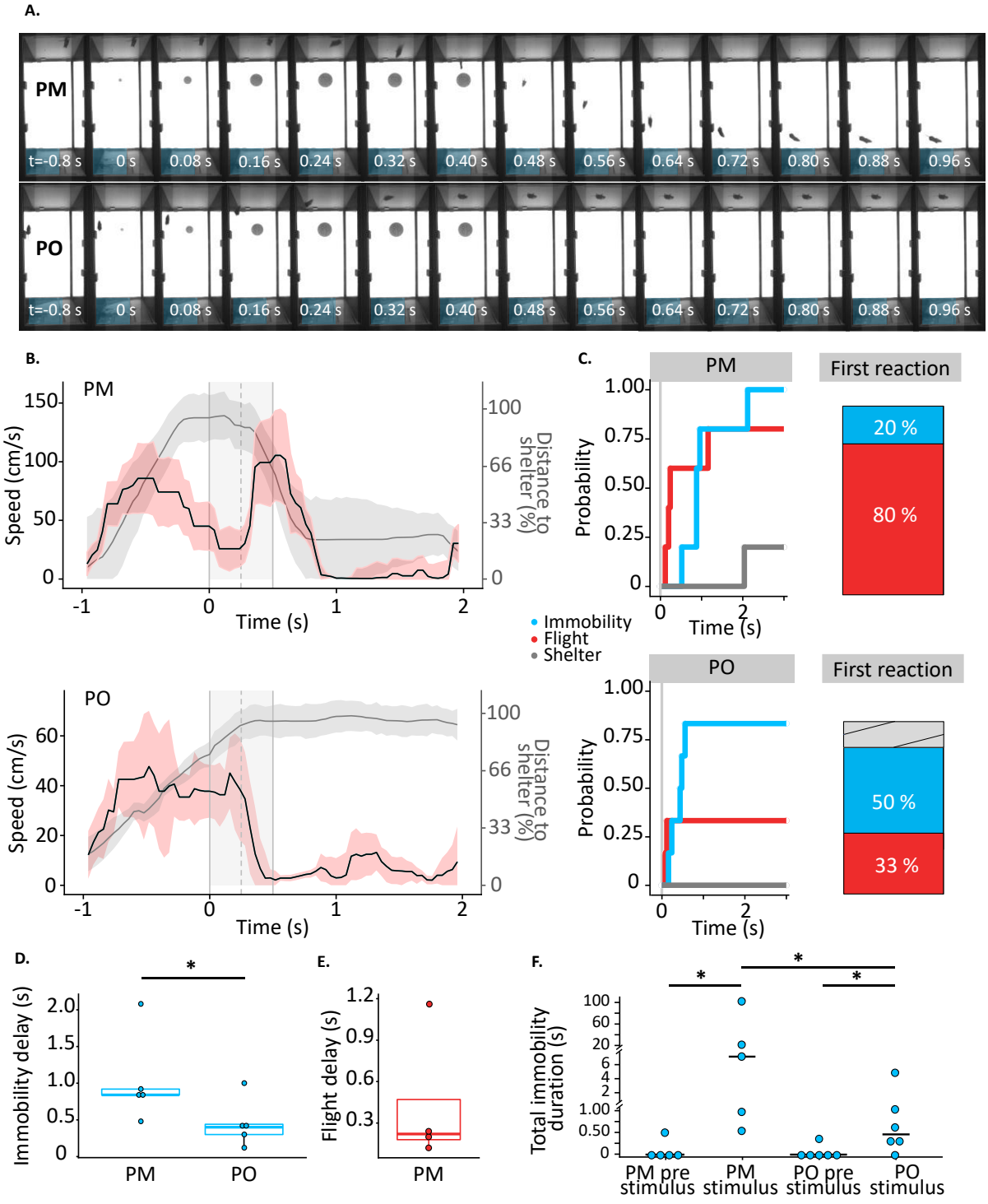


Figure 7. Behavior of PM (n=5) and PO (n=6) upon a single expansion. A. Example recordings for PM and PO in response to a single looming stimulus. **B.** Median speed (black graph) and relative distance to the shelter (grey graph) for all animals, with non-parametric bootstrapped (1000 iterations with replacement) standard error of the median (shaded areas). Grey area between the two grey lines indicates the presence of the stimulus. The expanding disc reached its full size at the dashed line. **C.** Left column: cumulative probability for the initiation of immobilization in open field, flight, and shelter entry. Right column: amount of animals displaying either flight or immobility as initial response. **D.** Latency of immobility for PM (median=0.84 s) and PO (median=0.4 s; Wilcoxon test: p=0.047). **E.** Latency of flight for PM (median=0.22 s). **F.** Total immobility in open field 15 s after stimulus onset for

PM (stimulus: median=7.32 s) and PO (stimulus: median=0.48 s; Wilcoxon test: p=0.041). Horizontal bars represent the median. Compared with total immobility during 15 s control period for PM (Wilcoxon test: p=0.031) and PO (Wilcoxon test: p=0.029).

3.1.3. A stimulus combining sweeping and looming only mildly alters the response towards a single expansion in *Peromyscus*

To test the response to an overflying predator that suddenly attacks, a stimulus was presented that consisted of a sweeping part that was immediately followed by looming. The black disc with a visual angle of 4° had a speed of 21°/s and was terminated after 3.94 s with a single expansion as used in single expansion experiments. All animals of each species reacted with immobility to the sweeping part of the stimulus (Figure S5). Similar latencies for immobility were observed compared to previous sweeping experiments (PM: Wilcoxon test p=0.272; PO: Wilcoxon test p=0.947) and no difference was observed between the two species (Wilcoxon test: p=0.268). All animals, with the exception of one PO, persisted immobility until the end of the sweeping period. For the subsequent expansion, only 13% of PM initiated flight with speed exceeding the 75 cm/s threshold, which is substantially lower compared to the 80% observed in single expansion experiments. Such post-sweeping flight events were absent for PO, while in single expansion experiments 33% of the animals reacted with looming-induced flight. However, for both species, the speed increased in reaction to looming to sub-threshold values in 63% and 33% of PM and PO respectively (Figure 8A,B), approaching the prevalence of flight observed in single expansion experiments (Figure 7C). These running events covered only a small displacement (Figure 8C) and were terminated with immobility in open field. Only one PO animal entered the shelter after a running event. The maximum running speed reached after expansion onset was lower in these experiments compared to flight episodes in single expansion experiments (PM: p=0.016; PO: p=0.2, Figure 8D). Sweeping-induced immobility persisted during expansion in 56% of PO and 25% of PM (Figure 8B), which is similar to the 50% and 20% prevalence observed in single expansion experiments. Although the total immobility duration was often increased for combined sweeping and looming compared to sweeping alone (Figure 8E), no statistical evidence was found for prolonged immobility (PM: p=0.943; PO: p=0.147). Taken together, defensive responses to looming were not altered qualitatively when following sweeping, however the velocity was quantitatively decreased during running events.

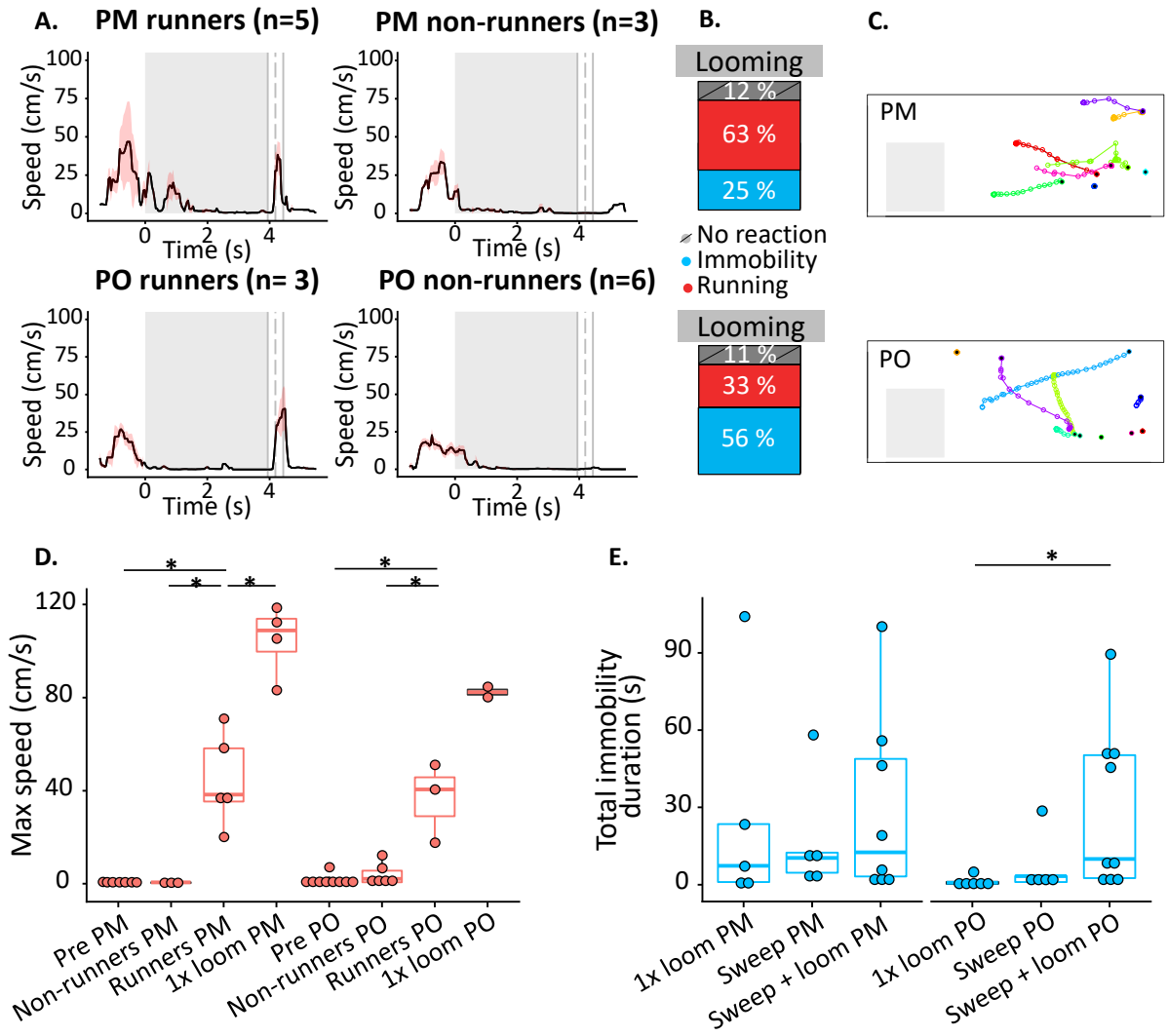


Figure 8. Behavior of PM (n=8) and PO (n=9) upon combined sweeping and looming. A. Median speed during the combined stimulus for animals displaying running activity (runners) and those that did not (non-runners). Grey area indicates the sweeping part. Two grey lines indicate the appearance and disappearance of the looming disc, with its full size reached at the dashed line. **B.** Amount of animals demonstrating either immobility or running activity in response to the expansion part. **C.** Trajectories during a 1 s time window after expansion onset. Black dots indicate the starting point of the trajectory. **D.** Maximum speed reached during the one second period prior to expansion (pre), by the (non)runner animals during post-sweeping expansion, and during single expansion experiments (1x loom). Runners PM (median=38.27 cm/s) compared with 1x loom PM (median=108.8 cm/s; Wilcoxon test: p=0.016). Runners PO (median=40.47 cm/s) compared with 1x loom PO (median=82.34; Wilcoxon test: p=0.20). Pre compared with runners (PM: Wilcoxon test p=0.002; PO Wilcoxon test p=0.015). Non-runners compared with runners (PM: Wilcoxon test p=0.036; PO Wilcoxon test p=0.024). **E.** Total immobility duration in single expansion experiments (1x loom), sweeping experiments (sweep), and combined sweeping and looming experiments (sweep+loom). Comparing sweep and sweep+loom (PM: Wilcoxon test p=0.943; PO: Wilcoxon test p=0.147). Comparing 1x loom and sweep+loom (PM Wilcoxon test p=0.749; PO: Wilcoxon test p=0.006).

3.2. Defensive behaviors in *Peromyscus* are only mildly influenced by different experimental conditions

3.2.1. Absence of a shelter only slightly affects defensive behavior in *Peromyscus*

To examine the influence of an available shelter on defensive behavior in *Peromyscus*, the repeated looming stimulus was presented in the absence of a shelter. In such an environment, PM displayed the same immediate responses as for trials with a shelter, with immediate flight and immobility initiated in 80% and 20% of the animals respectively (Figure 9B). The latency of flight was not significantly affected by the absence of the shelter (shelter: median=0.53; no shelter: median=0.24; p=0.271; Figure 9E) and flight was directed to the opposite side of the setup (Figure 9C). However, compared to the trajectories in the presence of a shelter (Figure 4B), immediate flight in absence of a shelter was not solely directed to the previous shelter location. Moreover, without a shelter, fewer animals entered the pre-shelter area during repeated looming (60% vs 80% when the shelter was present, Figure 9A). Cumulative event distributions show that PM engaged more frequently in both flight and immobility during repeated looming when no shelter was available (Figure 9D). Although the delay of immobility was reduced (shelter: median=2.27 s; no shelter: median=0.60 s) and the total immobility duration in open field was increased (shelter: median=0.7 s; no shelter: median=4.44 s), these differences were not significant (delay, Wilcoxon test: p=0.649; duration, Wilcoxon test: p=0.174; Figure 9E).

In PO, removal of the shelter increased the percentage of animals that reacted with immobility as the first response to looming compared to trials with a shelter (86% vs 67%; Figure 9B). There was no difference in both the latency of immobility (shelter: median=1.6 s; no shelter: median=0.6s: Wilcoxon test: p=0.238; Figure 9E) and the duration of immobility (shelter: median=6.06 s; no shelter: median=1.64 s; Wilcoxon test: p=0.104; Figure 9E). Animals entered the pre-shelter area later than when a shelter was available. 25% of the animals entered this area within 7.5 s in absence of a shelter, while 25% displayed their first shelter visit within 3 s in the other group (Figure 9A). Cumulative distributions for immobility events were very similar, and the cumulative flight distributions also reached similar values in both groups (Figure 9D).

In all previous experiments, animals were tested during the light phase of the 12h/12h light/dark cycle. A drastic change in cumulative flight was observed when PO was tested during the dark phase without a shelter. None of the animals displayed flight during repeated looming (Figure 9A). Immediate immobility was still observed in 80% of the animals, and one animal remained in the pre-shelter area during the stimulus (Figure S6). The initiation of immobility was not affected in this group (shelter: median=1.6 s; no shelter: median=1.72; Wilcoxon test: p=0.661; Figure 9E), neither was the duration of immobility (shelter:

median=6.064; no shelter: median=3.48; Wilcoxon test: p=0.145; Figure 9E). As noted for animals tested without a shelter during the light phase, entering the pre-shelter area was delayed (Figure 9A), and the cumulative distribution for immobility events also closely resembled that for PO with an available shelter (Figure 9D).

Taken together, these experiments demonstrate that removal of a shelter affected both the flight destination and cumulative responses in PM. The behavior of PO was only slightly altered, and when tested during the night flight was completely absent. Both species entered the pre-shelter location later during the stimulus than when a shelter was available.

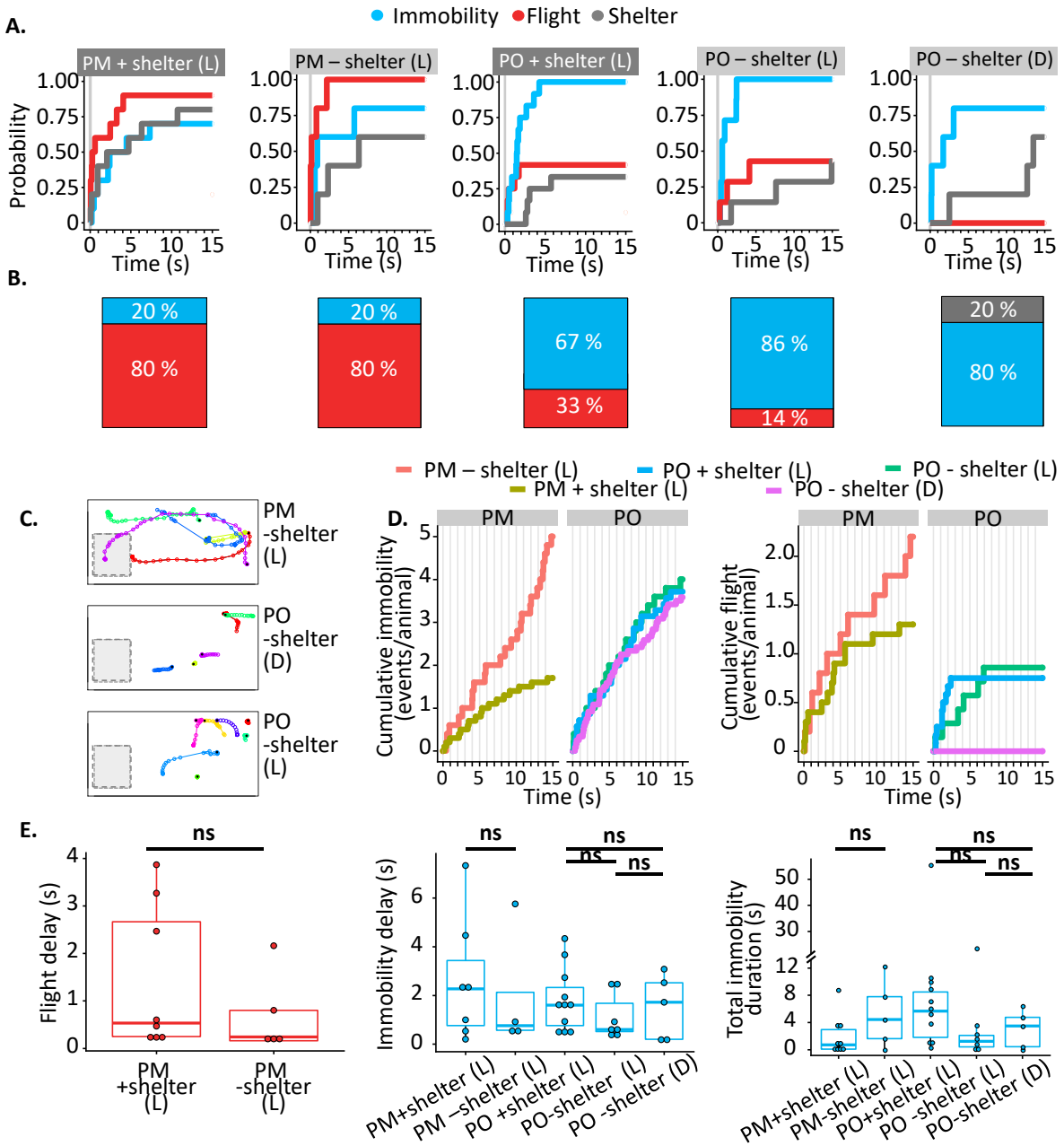


Figure 9. Behavior of PM and PO upon looming in absence of a shelter. *Peromyscus* were tested under different conditions: a plus and minus sign indicate the presence and absence of a shelter, and

L and D indicate light and dark phase. PM+shelter (L) (n=10); PM-shelter (L) (n=5); PO+shelter (L) (n=12); PO-shelter (L) (n=7); PO-shelter (D) (n=5). **A.** Probability of initiation of immobility in open field, flight and shelter entry. **B.** Amount of animals for each experimental group corresponding with those in A demonstrating either immobility, flight, or shelter entry as first behavior. **C.** Trajectories of animals after stimulus onset. The grey area indicates the pre-shelter location. Represented time window of 2s for PM and 1s for PO. Black dot indicates starting point of trajectory. **D.** Cumulative distributions of all immobility and flight events. Grey vertical bars indicate individual expansions of the repeated looming stimulus. **E.** Left: latency of flight for PM with (median=0.53) and without a shelter (median=0.24; Wilcoxon test: p=0.271). Middle: latency of immobility. Right: total immobility duration in open field.

3.2.2. Immobility is induced independently of the sweeping speed in *Peromyscus*

To examine the influence of sweeping speed on defensive behavior of *Peromyscus*, three different speeds, i.e. 5°/s, 21°/s and 84°/s, were presented for a black disc with a constant size of 4°. These different speeds reliably induced immobility in both species (Figure 10A). The median latency for the initiation of immobility increased for slower sweeping speeds (Figure 10B). However, only the reaction latency for PM significantly differed for different sweeping speeds (Wilcoxon tests: 5°/s-21°/s: p=0.042; 5°/s-84°/s: p=0.013; 21°/s-84°/s: p=0.017; for PO respectively p=0.604, p=0.176 and p=0.230). The total duration of immobility for PM did not differ for varying speeds, while the fasted speed in PO resulted in significantly reduced immobility compared to the slowest speed (Wilcoxon tests PM: 5°/s-21°/s: p=0.481; 5°/s-84°/s: p=0.818; 21°/s-84°/s: p=0.416; for PO respectively p=0.106, p=0.019 and p=0.075; Figure 10C). Comparing the two species, the immobility lasted significantly longer in PM during sweeping at the fastest speed only (p=0.004). Flight was less frequently observed during sweeping than immobility. Moreover, the flight probability seemed to reduce for higher sweeping speeds, with no flight detected for the highest speed (Figure 10A). Therefore, these results demonstrate that *Peromyscus* initiate flight less frequently for increased sweeping speeds, and that the probability of sweeping-induced immobility in these species is independent of the sweeping speed.

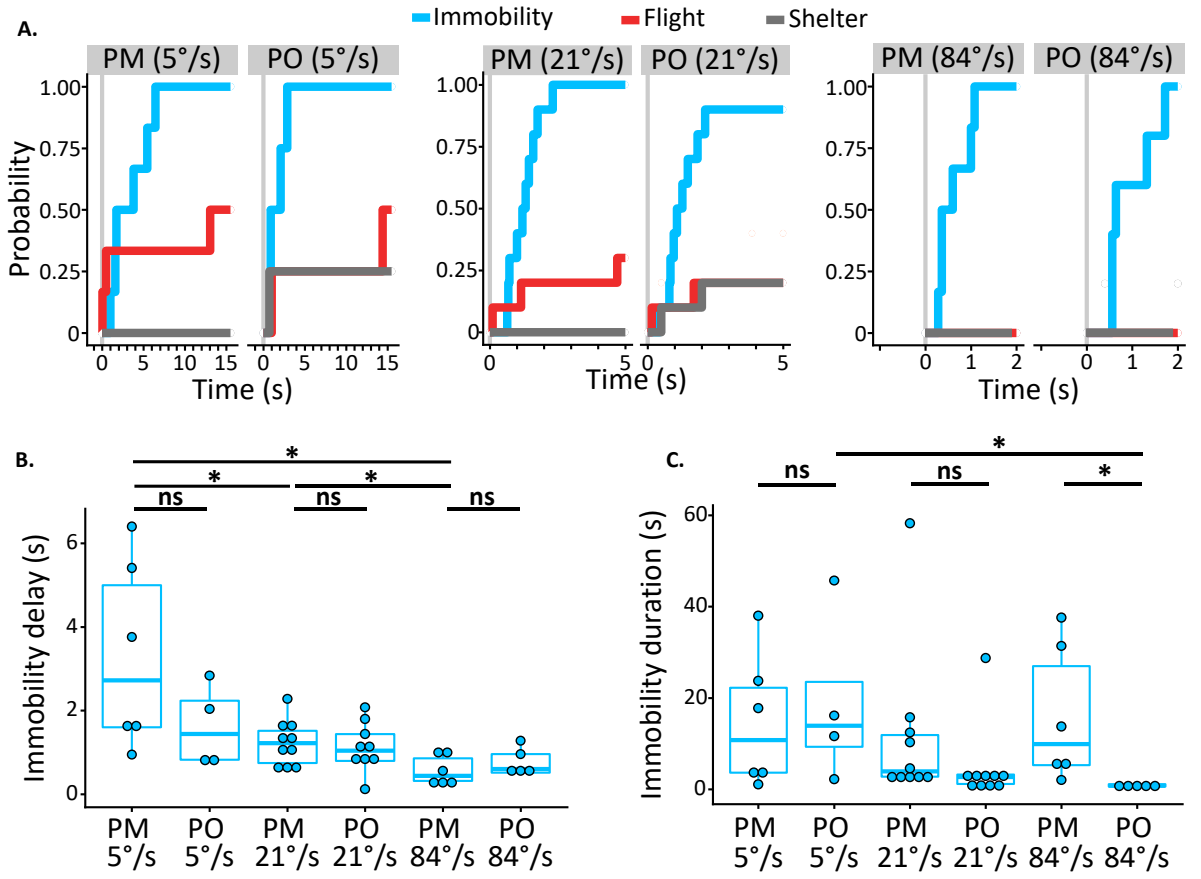


Figure 10. Behavior for PM and PO upon sweeping at 5 °/s, 21 °/s and 84 °/s. A. Cumulative distributions for the initiation of immobility in open field, flight and shelter entry, for PM 5°/s (n=6), PO 5°/s (n=4), PM 21°/s (n=10), PO 21°/s (n=10), PM 84°/s (n=6) and PO 84°/s (n=5). **B.** Median latency of immobility following the given group order: 2.72, 1.44, 1.22, 1.04, 0.44, 0.60 seconds. Comparing species per condition using a Wilcoxon test for 5°/s (p=0.257), 21°/s (p=0.905) and 84°/s (p=0.358). **C.** Median duration of immobility following the given group order: 10.75, 13.91, 3.94, 2.7, 9.92, 0.8 seconds. Comparing species per condition using a Wilcoxon test for 5°/s (p=0.914), 21°/s (p=0.075) and 84°/s (p=0.004).

3.3. *Peromyscus* demonstrate greater habituation than *Mus*

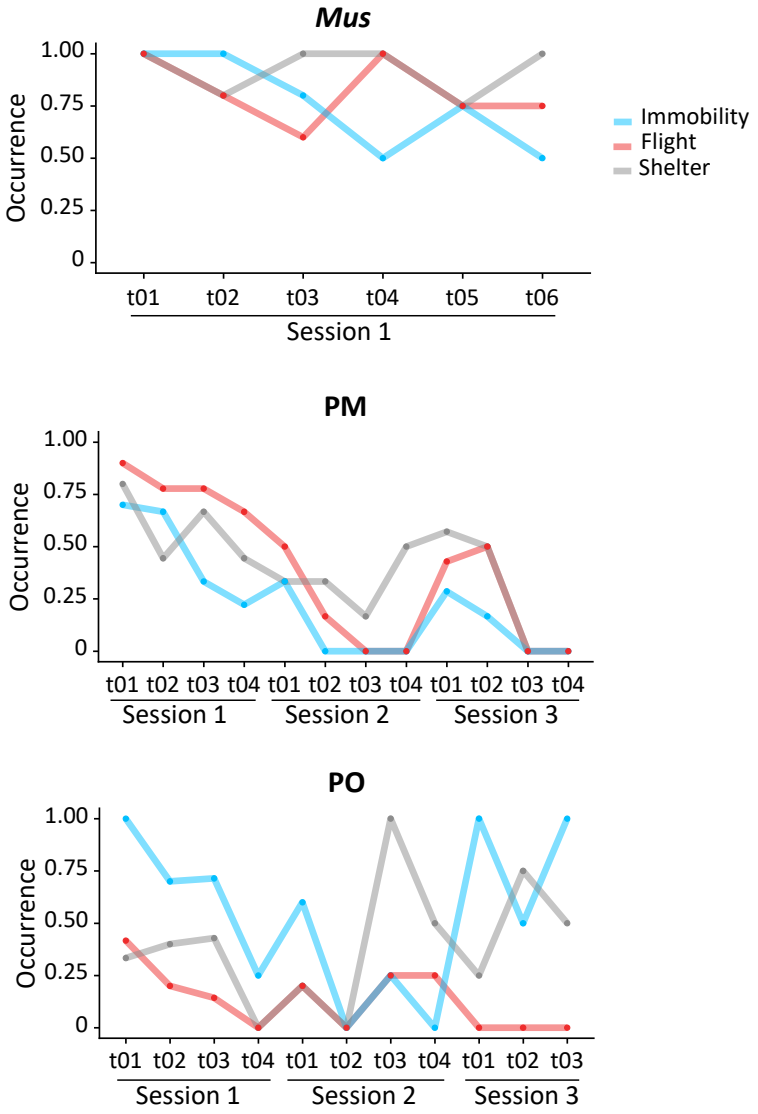
Repetitive testing can lower the effectiveness to induce defensive behaviors in mice.[21] To address this, the behavior of mice and *Peromyscus* was tested towards the repeated looming stimulus within different time frames (minutes, days and weeks).

Naive PO animals, in the first trial of the first session, all displayed immobility in response to repeated looming, and flight events and shelter entry were also observed in totally 42% and 33% of the animals respectively (Figure 11). The occurrence of these events changed when presenting the stimulus four times in the same session (also see Figure S7). The immobility and flight behavior were already decreased during the second stimulus presentation, and both were dramatically reduced during the fourth presentation reaching 25% and 0% respectively. One day later (session 2), immobility and flight increased only slightly to 60%

and 20%, however, responses quickly decreased again during further trials. One week later (session 3), all animals displayed immobility, but without displaying any flight events. These changes in occurrence were also accompanied with quantitative changes in the behavior (Figure S8). The total immobility duration for PO decreased over successive trials, along with the reaction span, which indicates the time between the first and last response displayed during the repeated looming stimulus. Both measures were already significantly reduced during the second exposure in the first session and they were still significantly reduced one week later.

As noted for PO, PM also habituated to the stimulus throughout repeated testing. 90% of the naive animals demonstrated flight, and immobility and shelter entry were noted as well in 70% and 80% of the animals respectively (Figure 11). During the first session, immobility behavior decreased more rapidly compared to flight, and the occurrence of shelter entry was fluctuating throughout the first session. One day later, immobility was the only response to increase, although only slightly. However, already during the second trial of the second session, this behavior was completely absent, accompanied by an absence in flight during the third trial. Both behaviors were partially recovered one week later, with 50% of the animals displaying immobility and 33% displaying flight, contrasting to the full qualitative recovery of immobility in PO after one week. Moreover, as noted for PO, responses also quantitatively decreased over trials for PM (Figure S8).

In contrast to *Peromyscus*, mice habituated less prominently during the first session. All three events, i.e. immobility, flight and shelter entry, fluctuated over consecutive trials, without reaching below 50% even after six stimulus presentations (Figure 11). The reaction span decreased less abruptly compared to *Peromyscus*, and the reaction latency significantly increased over trials (Figure S8). Taken together, these experiments demonstrate that *Peromyscus* habituate already in an early stage during repeated testing, and that their habituation is more prominent than for *Mus*.



 # Animals (*Mus*)

Trial	Session 1
t01	5
t02	5
t03	5
t04	4
t05	4
t06	4

 # Animals (PM)

Trial	Session 1	Session 2	Session 3
t01	10	6	6
t02	9	6	6
t03	9	6	3
t04	8	4	1

 # Animals (PO)

Trial	Session 1	Session 2	Session 3
t01	12	5	4
t02	10	4	4
t03	7	4	2
t04	4	4	0

Figure 11. Habituation for *Mus* and *Peromyscus*. The occurrence of immobility, flight and shelter entry represents the fraction of animals displaying the corresponding behavior. Session 1, 2 and 3 were performed on day 0, 1 and 7. Session 2 and 3 were started with available animals from the previous session. Within a session, animals were excluded from further trials (trials are indicated with 't0') when they did not enter the threat zone within 20 minutes. Ntsr1-GN209Cre mice were used.

3.4. Chemical inactivation of the superior colliculus impairs visually induced defensive behaviors in *Peromyscus*

The SC has been identified as a key mediator in visually induced defensive behavior in mice and other species.[15,16,18] To indicate the role of this midbrain structure in *Peromyscus*, bilateral microinjections of muscimol, an agonist of the inhibitory neurotransmitter GABA, were administered in PM and PO.

The repeated looming stimulus was presented followed by sweeping at 21 °/s, with an inter-stimulus time of seven minutes. Behavioral experiments were performed between one and maximally two hours after the first muscimol injection. After surgery, several animals

demonstrated great episodes of inactivity during the acclimatization period in the setup. In this case, both stimuli were still presented, but these animals were excluded from analysis (PM: looming: 5/13, sweeping: 2/11; PO: looming: 5/13, sweeping: 5/13). One week later, all animals were retested without administration of muscimol to serve as internal controls.

Repeated looming did not induce flight in both PM and PO after treatment with muscimol, while 73% of PM and 15% of PO displayed such events one week later (Figure 12A). No significant reduction was observed in the maximum running speed prior to stimulus presentation after muscimol administration (Wilcoxon test: PM p=0.442, PO p=0.109). For PM, already 63% of the muscimol-treated animals reacted with immobility one second after stimulus onset, which was similar to the 73% observed for the control group (Figure 12A). While the initiation of immobility was not affected for PM in response to looming (Wilcoxon test: p=0.99), the total duration of immobility was significantly shorter when treated with muscimol (muscimol: median=0.56 s; control: median=2.81 s; Wilcoxon test: p=0.001; Figure 12B). In PO, there is a clear difference in immobility between the two groups as only 25% of the treated animals displayed immobility, compared to 75% for the control group. Although the median duration of immobility was decreased with muscimol, this difference did not reach statistical significance (PO muscimol: median=0.36 s; PO control: median=1.32 s; Wilcoxon test: p=0.137).

Sweeping did not induce flight in muscimol-treated PM and PO, and only few events were noted in the control groups. A clear reduction in sweeping-induced immobility was present in both species. 56% of muscimol-treated PM displayed immobility during sweeping, in contrast to 100% of the control animals, with a significant reduction in total immobility duration (muscimol: median=1.16 s; control: median=3.96 s; Wilcoxon test: p=0.029; Figure 12B). On the other hand, none of the muscimol-treated PO animals displayed immobility, but this behavior was fully recovered one week later.

To address the impact of the surgical procedure on the behavior, two PO animals were included that were injected with DMSO instead of muscimol. Neither of these animals reacted to repeated looming after injection with DMSO, but both displayed immobility events one week later (Figure 12D). In contrast to looming, both animals reacted with immobility to sweeping, with a delay around 2.2 s and a duration of 3.4 s (Figure 12C,D). This is in contrast with the irresponsiveness to sweeping in PO after muscimol injections (Figure 12C).

Taken together, muscimol injections in the SC impaired defensive behaviors in both PM and PO in response to looming and sweeping stimuli. The probability of flight and immobility initiation was decreased for both stimuli, as well as the total immobility duration in open field. DMSO injections were associated with an absence of response towards looming, but maintained responses to sweeping.

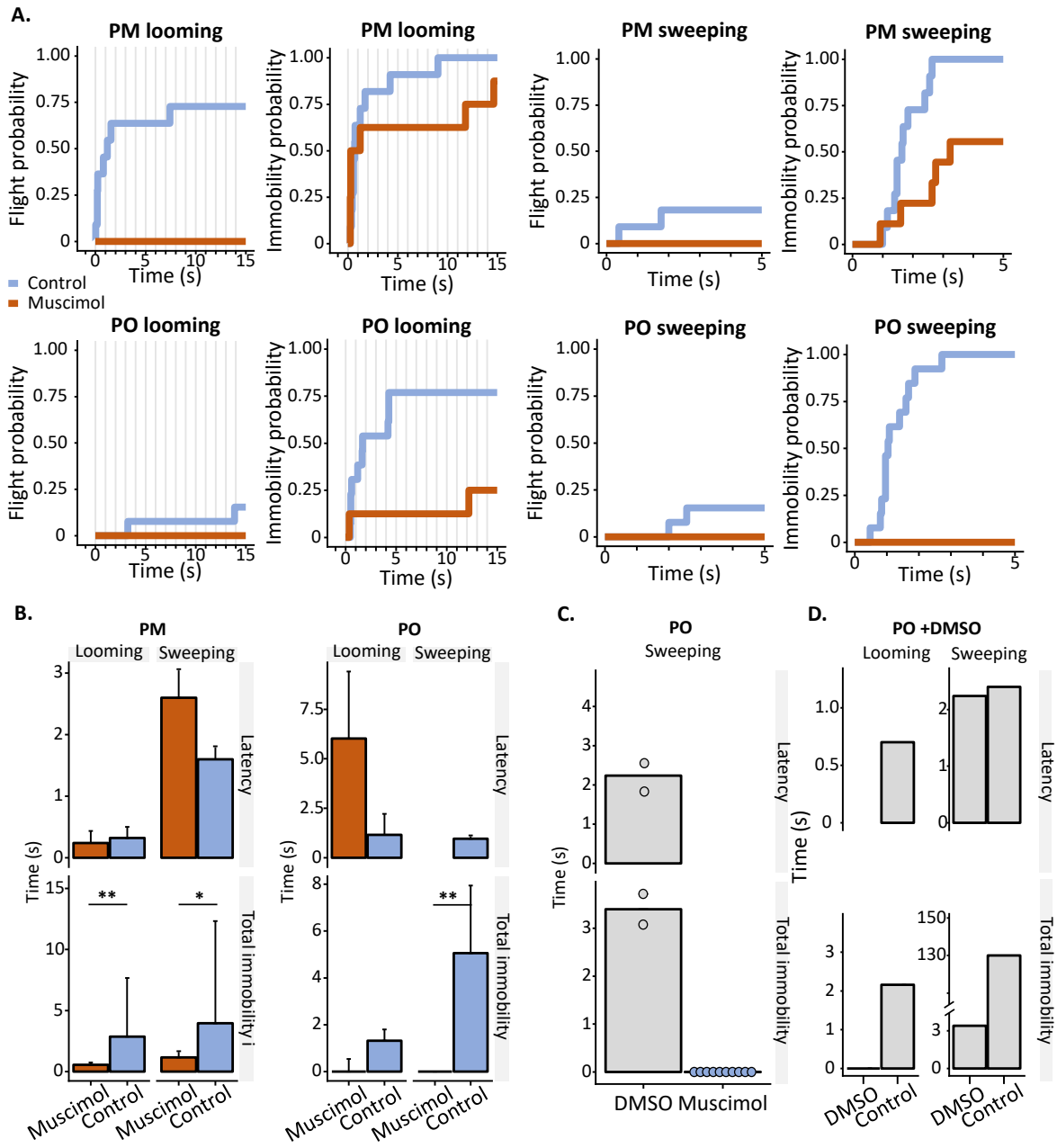


Figure 12. Muscimol inactivation of the SC in *Peromyscus*. A. Cumulative distributions of the initiation of flight and immobility in open field upon repeated looming (PM: control n=13, muscimol n=8; PO: control n=13, muscimol n=8) and sweeping (PM: control n=13, muscimol n=9; PO: control n=13, muscimol n=9). **B.** Quantitative measures for the latency of response and the total immobility in open field when treated with muscimol and one week later (control). Bars indicate the median and error bars indicate the non-parametric bootstrapped (1000 iterations with replacement) standard error of the median. **C.** Comparison between DMSO-treated (n=2) and muscimol-treated PO in response to sweeping. Bars represent the median. **D.** Quantitative measures for PO when treated with DMSO and one week later (control). Bars represent the median.

4. Discussion

4.1 *Peromyscus* enable to study different defensive behaviors in close relatives

In this research, an open field setup was used to study the repertoire of defensive behaviors to various overhead visual threat stimuli. In a first stage, mice were included to test their responses towards these stimuli as previously demonstrated in literature. In a second stage, two *Peromyscus* species were tested to address behavioral differences between close relatives.

In mice, different overhead visual stimuli mimicking either an approaching or an overflying predator reliably triggered different defensive strategies. Repeated looming triggered a sequential response comprising immobility followed by shelter-directed flight, while sweeping induced immobility followed by rearing. Although both stimuli reliably triggered these reactions, different responses have been reported in literature. Looming was noted to induce either sub-second flight or long lasting immobility,[21] while for sweeping only immobility has been reported.[23] Despite the use of similar experimental conditions as stated in literature, different responses observed in this research might result from the use of different mouse strains, a different circadian phase in which the experiment was performed, or different handling of the animal prior to testing. It has also been reported that different housing facilities can influence the behavior of mice, which might also support these observed differences.[21] Nevertheless, the established experimental design provided an efficient and reliable approach to study visually induced defensive behaviors.

Peromyscus demonstrated defensive behaviors towards overhead visual stimulus that were different from mice. Since habituation was observed in an early stage of testing, reported results are based on the first stimulus presentation. Both *P. maniculatus* and *P. polionotus* reacted with immobility to sweeping, independently from the sweeping speed. This is a striking difference with studies that reported an increase in flight probability in mice for increasing sweeping speeds.[23] Interestingly, looming evoked opposing reactions in the two *Peromyscus* species. *P. polionotus* displayed immediate immobility in response to a single looming stimulus, while *P. maniculatus* reacted with immediate flight that was followed by immobility in the open field. Repeated looming did not affect these immediate responses, but it affected the behavior during a later stage by increasing immobility in *P. polionotus*, lowering the probability of immobility directly following flight in *P. maniculatus*, and by stimulating shelter entry in both species. The latter behavior was reduced when removing the shelter from the setup. Moreover, the prominent flight of *P. maniculatus* in this case was not guided any more towards the pre-shelter location, indicating that the flight destination is influenced by an available hiding spot. Looming-induced species-specific behaviors persisted even when presenting a combined stimulus consisting of a sweeping part that was directly

followed by looming. This indicates the strong intrinsic character of their stimulus-associated defensive responses. It has been addressed in mice, and many other animals, that such defensive behaviors are mediated by the superior colliculus. Muscimol inactivation experiments supported that this evolutionary conserved brain structure might mediate visually induced defensive behaviors in *Peromyscus*.

4.2 Muscimol inhibition of the SC

Muscimol is an agonist of the inhibitory neurotransmitter GABA.[37] By injecting it in the superior colliculus, this area is reversibly inactivated which provides an approach to address its engagement in defensive behavior. In these experiments, several animals were completely inactive after injections of muscimol. This observation has been reported before in rats which were inactive for a long period of time after administration of muscimol in the superior colliculus.[37] These animals were excluded from the experimental group, and they were found to recover well like the other animals. The resulting experimental group displayed no flight and a decreased immobility towards both repeated looming and sweeping. It has been suggested that the effect of muscimol arises quickly after injection while persisting for several hours, after which it is completely reversed.[9,45] Indeed, one week after muscimol injections, defensive behaviors were found to be restored. However, to address the impact of the surgery on the behavior of the animals, two *P. polionotus* were injected with DMSO as an exploratory test. This substance was used as dissolving agent for the muscimol and it has proven not to influence neuronal responses in rats, rendering it a proper control.[46] Both DMSO animals reacted with normal immobility to sweeping, which was not observed in any of the nine muscimol-treated animals. The absence of sweeping-induced defensive responses in the muscimol group therefore results from the chemical inhibition, acknowledging the engagement of the SC in mediating defensive behavior towards sweeping. In contrast, none of the DMSO animals reacted to looming after surgery. This suggests that the procedure itself might selectively affect the responsiveness to this stimulus. However, since four spatially distributed microinjections were administered in the SC, it is very unlikely that only neurons mediating the detection of looming stimuli were damaged. This is supported by several muscimol-treated animals that still displayed immobility in response to looming for both species. Additionally, any unknown aversive effects of DMSO in *Peromyscus* on such looming-sensitive neurons in the control animals would also be reflected in the muscimol-treated animals. It might thus be possible that this unresponsiveness of DMSO control animals is not associated with impaired neurological activity, but rather a coincidental result. Since these two DMSO animals recovered faster after surgery than the muscimol animals, they were tested relatively earlier. The eyes of these animals might still have been partially covered with hydrating gel from the surgery, impairing an adequate observation of the first stimulus. During the inter-stimulus period, the

gel could have been removed by grooming, restoring sight. To further address the impact of surgery on looming-induced defensive behavior, more animals should be tested of both species. Also, additional analysis should show the accuracy of the injections and the spread of muscimol in the treated animals.

4.3 Improvements to the current approach

The overall low number of animals included in different experiments is a recognized limitation. In total, around 145 animals were used in this study generating cohorts of 5 to 13 animals. In literature, cohorts including approximately 10 mice have been widely used in behavioral studies.[3,20-23] Since it would be beneficial to reuse the same cohort animals, this study addressed the habituation over several trials. Both *Peromyscus* species habituated more than mice over successive trials, and this already occurred at an early stage. Therefore, only the first trial was considered throughout this research, similar to other studies.[21] This habituation experiment, however, is incomplete and might be continued in the future. Since the three major sessions, i.e. at day 0, 1 and 7, each comprised multiple trials, the habituation effect over sessions might be influenced by the repeated trials. Future experiments might therefore focus on repeated testing over multiple sessions with only one exposure to the stimulus each. Additionally, it would be worthy to investigate if the exposure to one stimulus type affects the behavior towards another type of visual stimulus. Experiments including the combined sweeping and looming stimulus suggested that the speed of looming-induced flight is affected when the inter-stimulus time between the two stimulus parts was minimal. The question arises whether this still counts when testing different stimuli over different sessions. This might help to increase the amount of data for animal cohorts.

Finally, the experimental design might not be suited for a detailed quantitative description of abrupt velocity changes in *Peromyscus*. In this research, the onset of the overhead visual stimulus was manually triggered when the animal entered the thread zone at the opposite side of the shelter. Although this was efficient to present a stimulus during a running episode for mice, *Peromyscus* moved substantially faster in the setup reducing the timing accuracy of stimulus presentation. In this way, the stimulus often appeared when the animal already reached the end of the setup. Although this still evoked reliable defensive responses, it is not favorable when investigating detailed changes in speed profiles. Real life animal tracking might aid in the automatic presentation of visual stimuli in the future. Another approach is to use a treadmill or ball on which the animal's running activity is not restricted by the physical boundaries of the open field setup. Therefore, the animals can prolong their running events which enables a more functional anticipation on the speed of the animal to present visual stimuli.

4.4 Linking differences in innate behaviors to habitat and neural circuitry

Naive animals that had never been exposed to the visual stimuli reliably reacted with species-specific responses. This indicates that such defensive behaviors are most likely innate and that they are probably mediated by dedicated neural circuits. The different behavior for these close relatives to the same looming stimulus is not a unique characteristic. They have already demonstrated different species-specific traits such as burrowing and mating behavior. For example, *P. maniculatus* is known to be polygamous and it makes simple nests, while *P. polionotus* makes complex burrows and is a rare example of a monogamous rodent species.[7,25,27] Such behavioral differences, including defensive behavior, might resemble evolutionary adaptations to different habitats. As demonstrated for other mammals,[35,47,48] the distribution and diversity of *Peromyscus* were influenced by various geographical events such as sea-level changes, glacial and pluvial cycles, population expansions and mountain range elevations.[49-52] These events guided the separation of *P. maniculatus* and *P. polionotus* which are presently encountered in grasslands and scarce field areas respectively.[7] The observed differences in defensive behavior in looming experiments might relate to the natural habitat of these species. Initiating immobility upon the approach of an avian predator is reasonable for *P. polionotus* when moving in a scarce field. In the absence of a nearby hiding spot, escape might be ineffective and could additionally render the prey more visible. In contrast, initiating shelter-directed flight might be effective for *P. maniculatus* living in a habitat with dense vegetation that supports hiding. Additionally, their similar immobility response to a sweeping stimulus would render both species less visible to an overflying predator. Therefore, the probability to provoke subsequent attack might reduce.

Such reasonable evolutionary adaptations in defensive behavior could originate from different mechanisms such as genetic modifications, epigenetics or learning. However, since the animals were naive to the experimental stimuli, a learning process is very unlikely to shape the observed defensive behaviors in these species. Also, both species were kept in the same controlled animal facility, which could exclude that different environmental effects introduce different epigenetic changes in these species. The most reasonable mechanism supporting these species-specific differences would be based on genetic variability.

It has already been demonstrated that the contrasting burrowing behavior of *P. maniculatus* and *P. polionotus* results from distinct adaptations in different genes.[7,35] Likewise, differences in defensive behavior might result from a genetic variability between these species that is then translated in neurological differences that support these behaviors. It has been demonstrated in mice that circuits through the SC consist of hard-wired pathways that connect distinct sets of relevant retinal ganglion cells to targets that mediate defensive

behavior.[19] In these hard-wired pathways, distinct downstream areas, i.e. the lateral pulvinar and the parabigeminal nucleus, which are involved in either immobility and flight behavior, are innervated by specific collicular cell types.[15,18] Differences in such hard-wired circuitry might form the foundation for the different looming-induced defensive behaviors observed in *Peromyscus*. It could be hypothesized that retinal ganglion cells might innervate the SC differently in these species. Consequently, the same visual information would be transmitted to different downstream circuits, which then evokes different behavioral responses in the two species. Secondly, collicular cell types might project to different targets that mediate different behaviors. Specifically, looming-sensitive neurons in the SC might be preferentially innervating the lateral pulvinar in *P. polionotus* resulting in looming-induced immobility. In contrast, innervation of the parabigeminal nucleus might be preferred in *P. maniculatus* resulting in flight. Thirdly, these circuits might be modulated by non-retinal inputs that shape responses differently to other stimuli. This might occur on the level of the SC itself or on the downstream brain areas. The mechanisms proposed in these hypotheses might also occur simultaneously, and this list could possibly be further expanded. Since it is impossible to determine the internal wiring solely based on external observations, these hypotheses are purely speculative.

4.5 Future perspectives

The experimental design developed in this research realizes a straightforward and easy approach to study the behavior in small rodents. Using different ecologically relevant stimuli, visually induced innate behaviors can be examined. By controlling the projection perspective of the stimulus relative to the animal along with various stimulus parameters, the animals can be studied in the future also in a predation or hunting context. In either of these studies, circuit manipulation might be easily incorporated to address neural mechanisms involved in such contexts. In this light, the two studied *Peromyscus* species especially render them a promising model system for studying the neurological basis of visually induced innate behavior. This is supported by the finding that these close relatives are characterized with different looming-induced defensive strategies, that might originate from different hard-wired circuits. The development of transgenic *Peromyscus* should allow new insights in circuit specificity associated with innate behaviors.

5. Conclusion

Ecologically relevant visual stimuli indicating danger reliably triggered defensive responses in closely related species of the rodent genus *Peromyscus*. Despite displaying similar responses towards stimuli indicating an overflying predator, an interesting difference was observed in the response mechanism towards an approaching predator. These species-specific responses were only mildly affected when changing the experimental conditions, indicating their strong intrinsic character. This data strongly suggests that neural circuits underlying these defensive behaviors are wired differently in these species, providing an interesting basis for studying evolution of such circuits. Initial experiments involving chemical inactivation of the superior colliculus already suggest that this conserved midbrain structure might mediate visually induced defensive behavior in these species. This research proposes an interesting rodent model system for studying the neurological basis of defensive behavior.

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Appendix

Risk assessment

The animal experiments should be performed while considering the safety of the handler, the animals and the coworkers in the lab. Firstly, suitable lab protection should be worn to prevent the spread of contamination via normal clothes. Hands should be washed after performing experiments that involve animals, and used animal cages should not be opened for a long period of time. These precautions reduce contact between allergens for both the handler and coworkers. When allergy symptoms develop, one should contact his medical doctor. Secondly, to avoid sudden aggression of the animals during handling, animals should always be carefully approached. Animals are preferentially handled when they are not markedly annoyed and they should always be handled with respect. When bitten by an animal, one should disinfect the contact area and check what treatment the animal underwent to know whether additional actions should be considered. Thirdly, precautions should be taken to avoid the escaping of animals during experiments. Therefore cages of grouped-housed animals should be closed as soon as possible, and *Peromyscus* should always be handled in an additional large arena. These species are particularly skilled in escaping quickly from a standard home cage. The large arena then allows the animals to escape from the cage, without being able to escape in the room. If an animal escapes, its moving area should be contained as much as possible to facilitate catching the animal and to reduce the chance for the animal to escape the lab. Fourthly, when cleaning the experimental setup with 70% alcohol, one should be mindful about the surrounding electronics, especially the sockets in close vicinity to the setup. Lastly, a careful handling is required of the sharp tools used to isolate the brains of the animals. When cut, one should disinfect the wound immediately and determine what substance he or she came into contact with to check if further actions should be taken.

Different chemicals are used during this research that could potentially harm the user and coworkers. Isofluraan: could damage the cardiovascular system upon repeated exposure and lowers the attentiveness, therefore diffusion in the air should be minimized. 70% ethanol: flammable and should be removed from sockets. Sodium azide: poisonous salt that should always be treated with the proper personal protection equipment (PPE). Paraformaldehyde: irritating liquid for the respiratory system and skin, and can cause severe eye damage when not wearing the proper PPE. This substance is also carcinogenic and impairs pregnancies, therefore it should always be handled in the fume hood. Muscimol: poisonous when orally administered and has the potential to affect the nervous system. When working with these chemicals, one should always wear the appropriate PPE and take the proper precautions to avoid contact with coworkers. When coming in contact with these chemical, one should rinse the contact areas extensively at the designated locations in the lab.

Figure S0. Animals used in the study. Left: *Peromyscus maniculatus bairdii* (PM), middle: *Peromyscus polionotus subgriseus* (PO), right: *Mus musculus*.

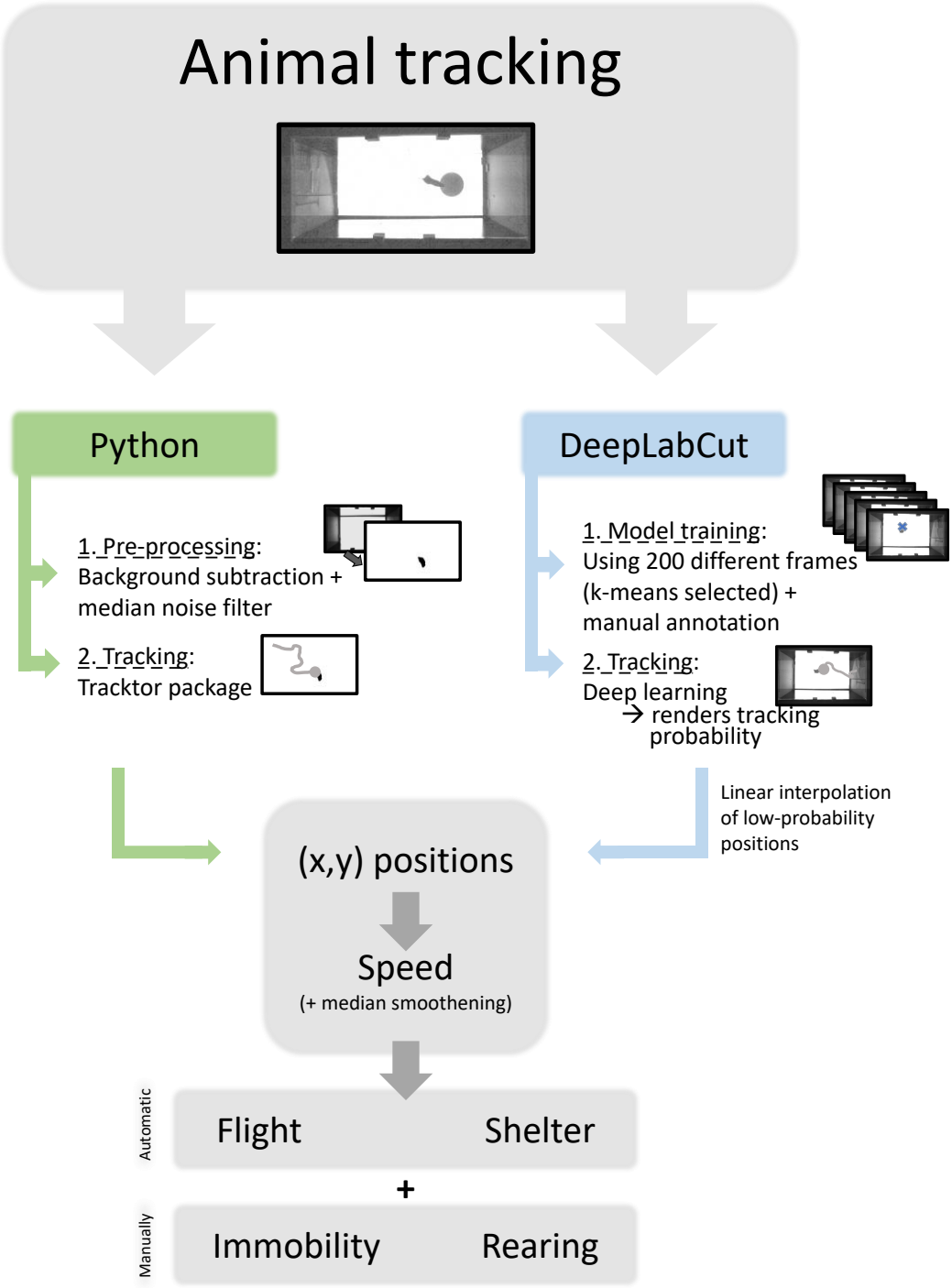


Figure S1. Animal tracking flowchart. Either one of the two methods were used. When using Python, an initial processing step was performed to generate trackable background subtracted recordings. When using DeepLabCut, the deep learning model was trained using 200 different annotated frames prior to tracking. Both methods generated the position of the animal in the setup which was used to automatically determine flight and shelter entry. Immobility and rearing were manually scored using VideoPad.

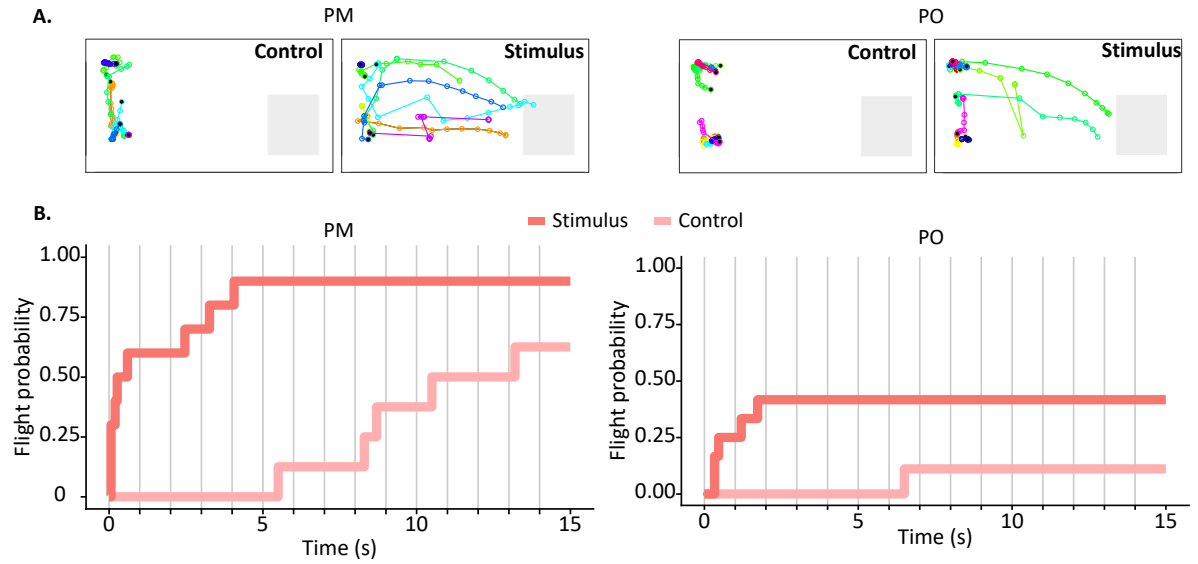


Figure S2. Flight of PM and PO in repeated looming experiments. This figure is related to Figure 4 in the main text. **A.** Trajectories during a 1 s time window after entering the threat zone without (control) and with visual stimulation (stimulus). Different animals are indicated with different colors and the black dot indicates the starting point of the trajectory. **B.** Flight probability after entering the threat zone without (control) and with visual stimulation (stimulus).

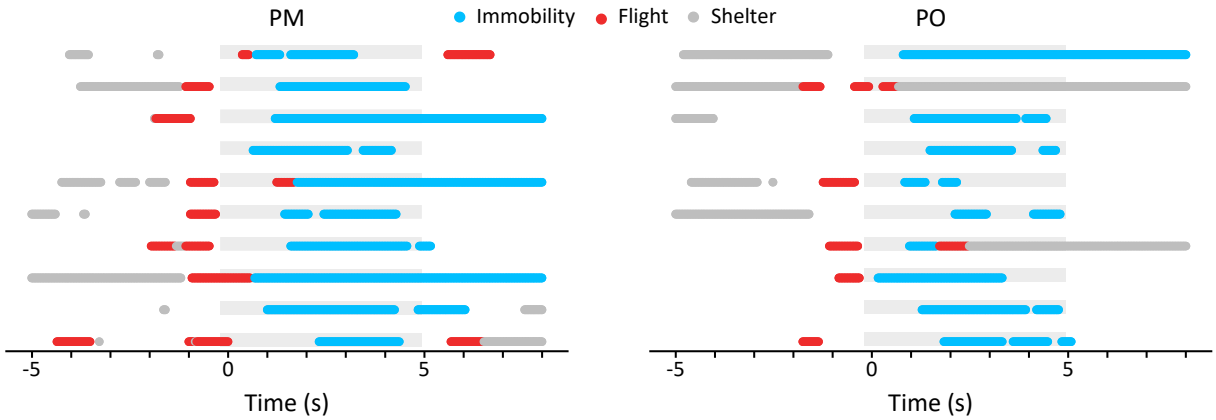
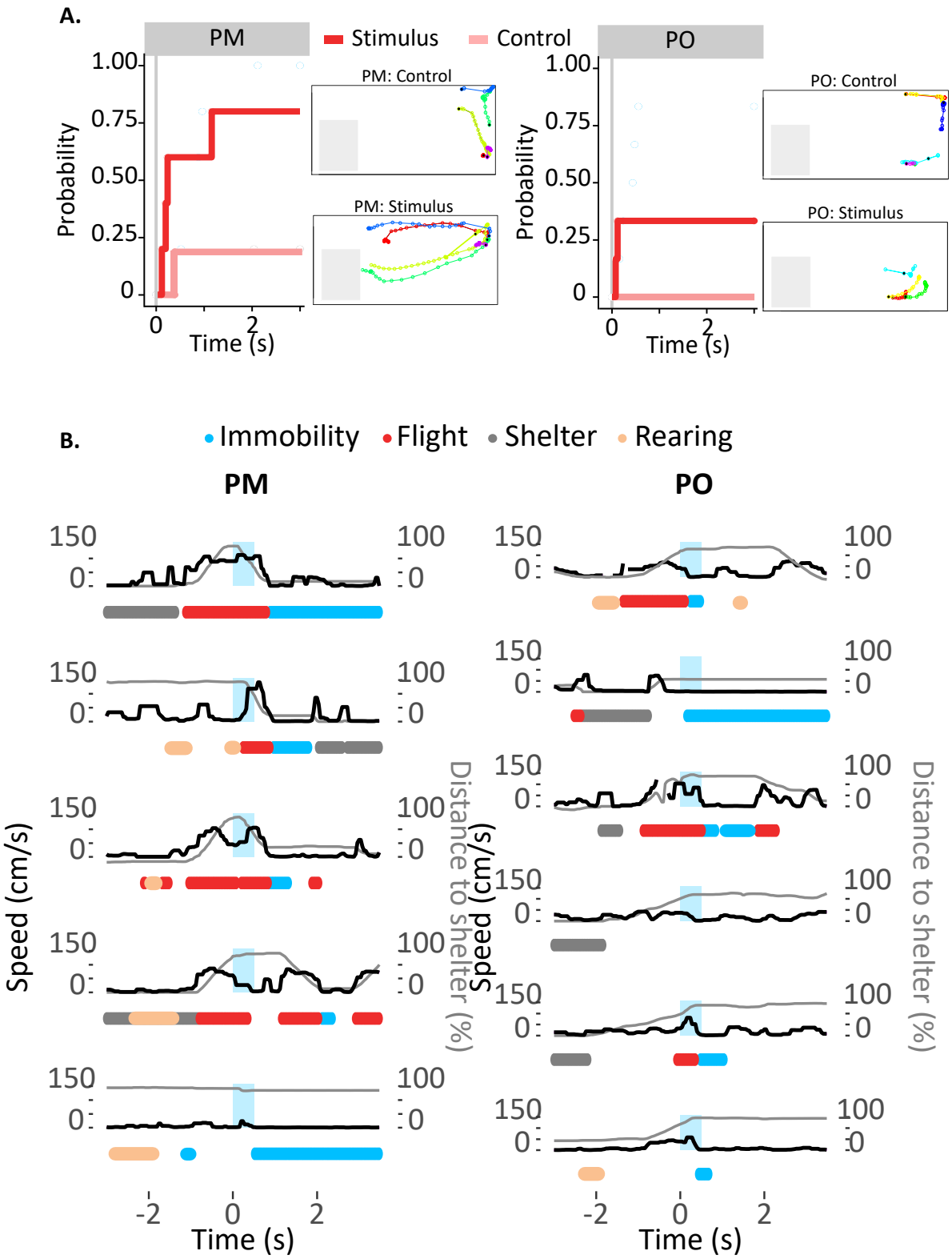


Figure S3. Reaction of PM and PO to sweeping. The stimulus used was a black disc with a visual angle of 4° sweeping at 21°/s (grey area on graphs). Each row represents one animal. This figure is related to Figure 6 in the main text.


A.
PM
Stimulus
Control
PO
Probability
1.00
0.75
0.50
0.25
0
Time (s)
PM: Control
PM: Stimulus
PO: Control
PO: Stimulus
B.
Immobility
Flight
Shelter
Rearing
PM
PO
Speed (cm/s)
Distance to shelter (%)
150
100
-2
0
2
Time (s)

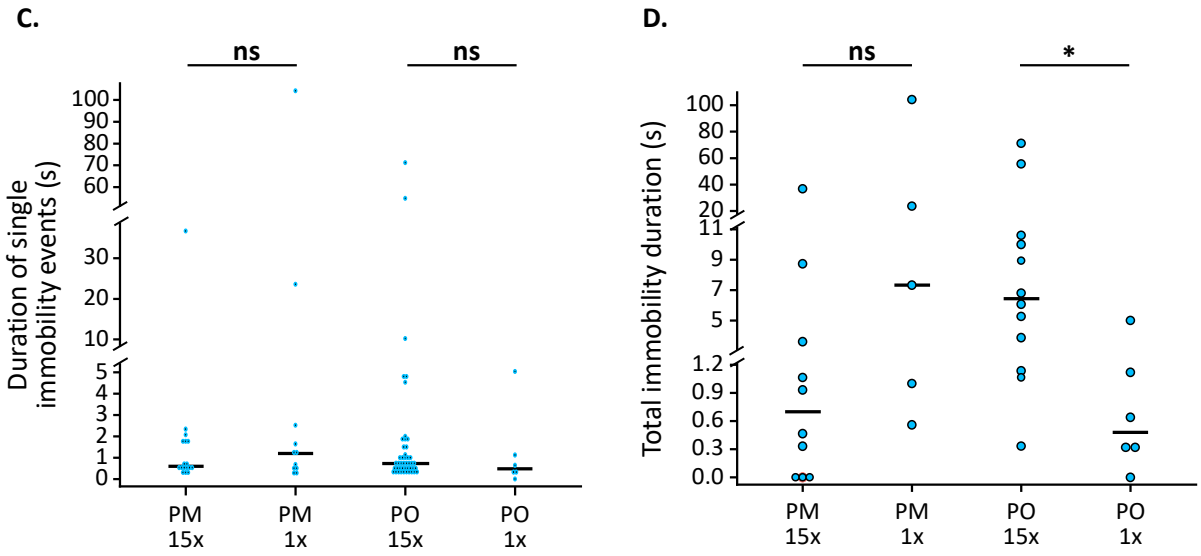


Figure S4. Behavior for PM and PO upon single looming. This figure is related to Figure 7 in the main text. **A.** Flight probabilities and trajectories of animals within a 2 s time window after entering the threat zone without (control) and with visual stimulation (stimulus). Grey area represents the shelter and black dots indicate the start of the trajectory. Different animals are indicated with different colors. **B.** Behavior upon stimulus presentation (blue rectangular area at 0 s). **C.** Duration of individual immobility events during repeated (15x) and single (1x) looming. Median duration for PM for repeated looming is 0.6 s and for single looming 1.2 s (Wilcoxon test: p=0.541). Median duration for PO for repeated looming is 0.732 s and for single looming 0.48 s (Wilcoxon test: p=0.273). Medians are represented by black horizontal bars. **D.** Total immobility duration during repeated (15x) and single (1x) looming. Median duration for PM for repeated looming is 0.7 s and for single looming 7.32 s (Wilcoxon test: p=0.124). Median duration for PO during repeated looming is 6.43 s and for single looming 0.48 s (Wilcoxon test: p=0.008). Medians are represented by black horizontal bars.

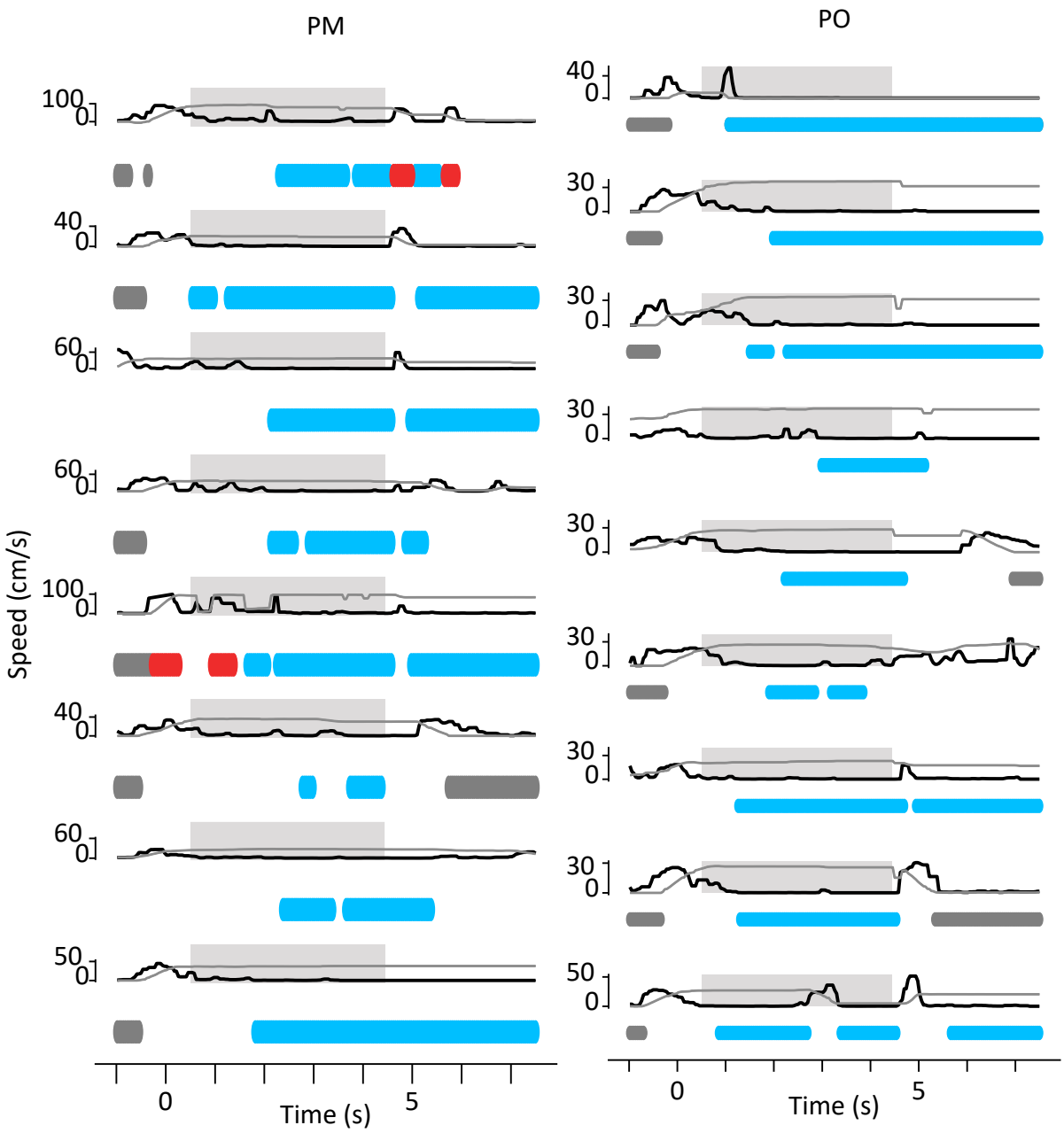


Figure S5. Behavior for PM and PO upon a combined sweeping-looming stimulus. Blue: immobility, red: flight, grey: shelter. Speed (black graph) and relative distance to the shelter (grey graph) are giving upon sweeping (grey area) immediately followed by one single expansion (the end of the grey area). This figure is related to Figure 8 in the main text.

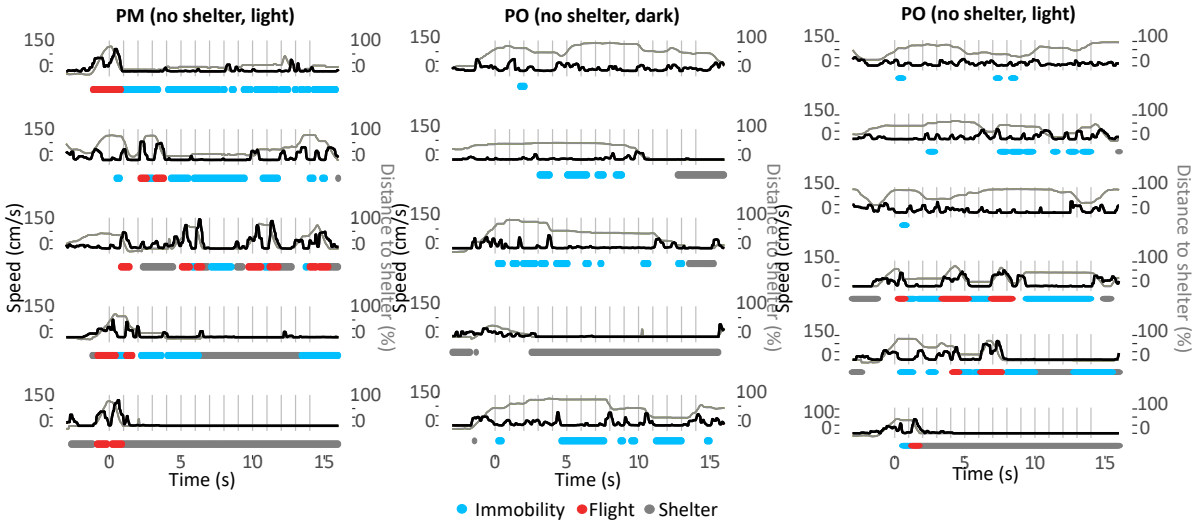


Figure S6. Behavior for PM and PO upon looming in absence of a shelter. Each row represents one animal. Vertical grey bars indicate individual expansions of the repeated looming stimulus. This figure is related to Figure 9 in the main text.

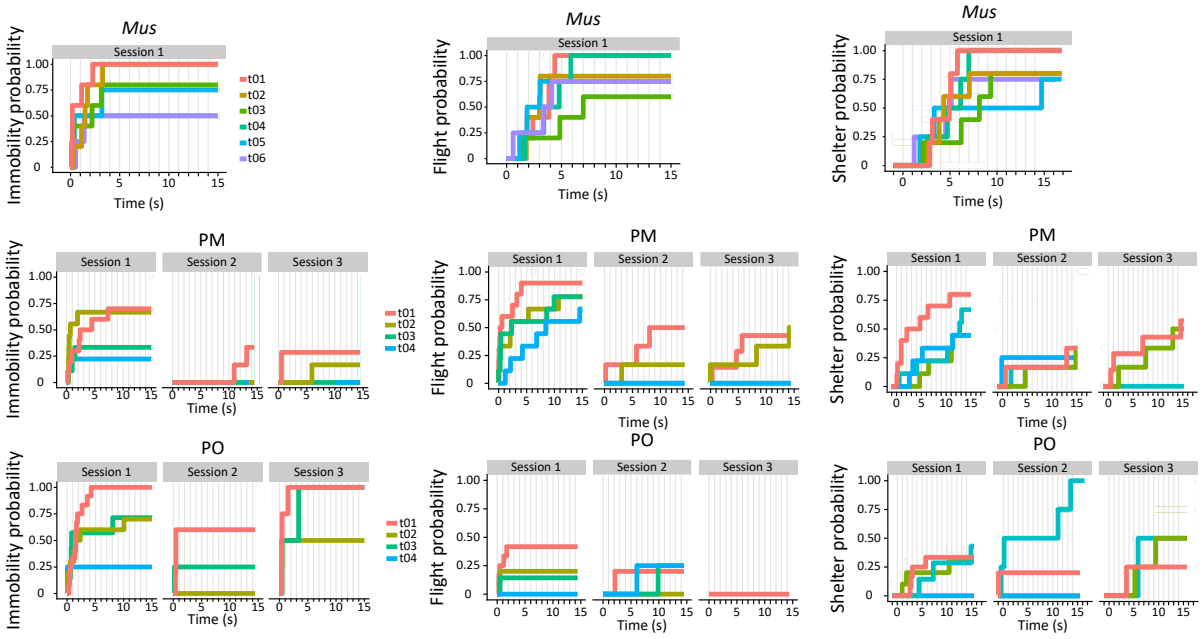


Figure S7. Habituation for *Mus* and *Peromyscus*. Changes across the different species (row wise) are demonstrated for immobility, flight and shelter entry (column wise). Consecutive trials (denoted with 't0n') are plotted in the same session panel with different colors. This figure relates to Figure 11 in the main text.

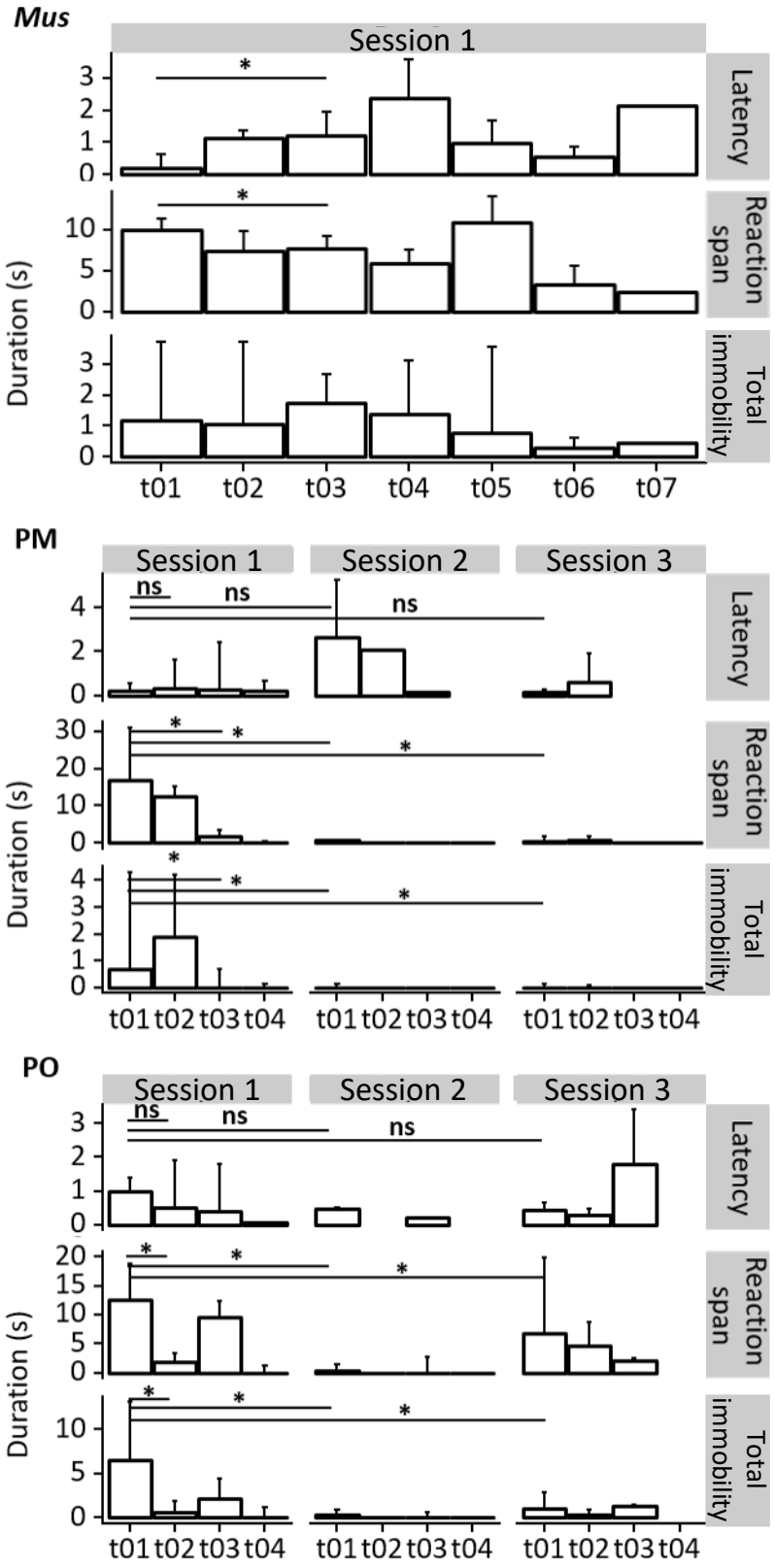


Figure S8. Quantitative changes during repeated testing for *Mus* and *Peromyscus*. Median values are giving with the standard error. Latency represents the delay upon the first response. Reaction span represents the time frame between the first and last response displayed during the stimulus. Total immobility represents the total duration of immobility in

open field that was initiated during stimulus presentation. Significance levels were obtained using a gamma-based generalized linear model incorporating two additive variables, i.e. the variables defining the type of measure and all consecutive trials. An interaction effect for these variables was included for PM and PO as this generated the lowest AIC. This figure is related to Figure 11 in the main text.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