# The 'Educage': an automated platform for studying auditory perceptual learning in mice

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**Abstract**

Perceptual learning (PL) is a form of implicit learning that requires lengthy training in order to improve perceptual abilities. Without reliable automation, this can require arduous manual labor which can impede experimental progress. We developed an automated platform, the Educage, that enables PL of groups of mice training simultaneously in a home-cage environment. The system is low-cost, runs automatically for long durations, and allows a large degree of flexibility in experimental design. We used the Educage to train mice on a go/no-go auditory discrimination task. Mice were trained to discriminate between pure tone sequences or frequency modulated vocalizations. We found that gradually increasing cognitive load by manipulating task difficulty was necessary to improve learning outcomes. Task difficulty was also associated with increased decision times and decreased discrimination performances of individual animals. The Educage is a new tool for studying, among others, perceptual learning in mice.

## Introduction

Understanding animal and human cognition is one of the major goals in the natural and social sciences. Cognitive phenomena are best studied through behavior. However, even simple behaviors are variable and complex because they depend on a rich set of past experiences, the present state of the animal and the animal's predictions about the future; all of which affect learning, decision making and consequently, behavior (Hanks and Summerfield 2017, Juavinett, Erlich, and Churchland 2018, Shettleworth 2001). In order to make meaningful interpretations of behavioral data, researchers must rely on statistically robust data quantification, which in turn requires the collection of large behavioral datasets (Gomez-Marin and Mainen 2016, Krakauer et al. 2017). Human and monkey psychophysics have been at the forefront of quantitative approaches to behavior. However, the use of humans or monkeys has limitations like the low number of subjects per study (monkeys) and the limited ability to manipulate the brain (humans). The extensive use of other animal models in biological research call for an expansion of the behavioral toolkits to allow quantitative approaches of behavioral analysis, including massive, parallel and automated behavioral data collection. Mice, for example, are a powerful model system for studying the molecular and cellular basis of cognitive phenomena in mammals (Dymecki and Kim 2007, Huang 2014, Tye and Deisseroth 2012), yet, robust quantitative tools of complex behavioral protocols for mice are scarce.

Given the complexity of most behaviors, a successful strategy in rodent research has been to rely on highly simplified versions of specific tasks. For example, simplified

learning tasks like contextual and cued fear conditioning have been used successfully to understand the role of different brain structures (e.g. amygdala and prefrontal cortex) in learning and memory (Herry and Johansen 2014). But simplified behavioral paradigms test very limited attributes of learning, whereas more complex behavior could be vital for studying more advanced forms of learning. In addition, mouse behavior has been tailored to the limitations of the experiments, which are often stressful like learning under restrained conditions. Automated behavioral platforms that are able to monitor behavior quantitatively in home-cage settings for long durations, and in freely engaging animals seem to be a good solution for rigorously studying complex behavioral paradigms, especially when extensive training is needed. Despite this need, there are only a limited number of commercial options designed for high throughput/extensive training paradigms and custom-made systems are difficult to replicate and implement in other settings (Abraham et al. 2004, Aoki et al. 2017, de Hoz and Nelken 2014, Francis and Kanold 2017, Murphy et al. 2016, Scott et al. 2015, Vannoni et al. 2014).

Automatic behavioral platforms are particularly valuable when studying paradigms that involve prolonged training. One such behavior is perceptual learning (PL) which requires lengthy training in order to induce improvement in perceptual abilities (Ghose 2004, Gibson 1969, Goldstone 1998, Sagi and Tanne 1994). Examples of PL span multiple systems but are commonly studied in the context of sensory discrimination. Common examples include the Vernier acuity task (Poggio, Fahle, and Edelman 1992, Westheimer and McKee 1978), the motion discrimination task (Ball and Sekuler 1982) and the two tone discrimination task (Karmarkar and Buonomano 2003, Kurt and Ehret

2010). Extensive research on PL has been carried out in humans and in several sensory modalities (Hawkey, Amitay, and Moore 2004, Watanabe and Sasaki 2015). However, in order to better understand the neuronal mechanisms underlying PL it is important to translate PL tasks into procedures that other model animals, like mice, can perform. Here we present an affordable, reliable, flexible, and automatic behavioral platform designed for multiple behavioral paradigms in mice, including PL. Using audition as a model, we test and discuss various aspects of learning, perceptual abilities, and behavioral performance in mice.

## Materials and Methods

### *Behavioral setup*

The 'Educage' is composed of a small chamber (10x10x10 cm), opening on one end into a standard animal home cage where mice can enter and exit freely (Fig. 1A). On the other end, the chamber contains the hardware that drives the system, hardware for identifying mice and measuring behavioral performance. Specifically, at the port entrance there is a coil radio antenna (ANTC40 connected to a LID665 stationary decoder; Dorset) followed by infra-red diodes used to identify mice individually and monitor their presence in the port. This port is the only access to water for the mice. Water is delivered via a solenoid valve (VDW; SMC) allowing precise control of its volume on each visit. Water is delivered via a water spout, which is also a lickometer (based on 1 microampere current). An additional solenoid valve is positioned next to the water spout in order to deliver a mild air puff as a negative reinforcement if necessary. For sound stimulation, we positioned a speaker (ES1; TDT), at the center of the top wall of the chamber. Sound was delivered to the speaker at 125 kHz sampling rate via a driver and a programmable attenuator (ED1, PA5; TDT). For high speed data acquisition, reliable control, on-board sound delivery, and future flexibility, the system was designed via a field programmable gate array (FPGA) module and a real-time operating system (RTOS) module (CompactRIO controller; National Instruments). A custom made A/D converter was connected to the CompactRIO controller, mediated signal from infra-red diodes and lickometer and controlled the valves. A custom code was written in Labview to allow an executable user-friendly interface with numerous options for user-input and

flexibility for designing custom protocols. A detailed parts list and software can be downloaded from Github (https://github.com/MizrahiTeam/Educage ).

### *Animals*

A total of 58 7-8 week-old female mice were used. We used both C57BL/6 mice as well as transgenic mice (PV–Cre; Ai9 double-heterozygous; B6.129P2-Pv and Gt(ROSA)26Sortm9(CAG-tdTomato)Hze lines, Jax). No differences were found between these strains. Prior to the experiment, each mouse was implanted, under light and a very short period of Isoflurane anesthesia, with Radio Frequency Identification (RFID; Trovan) chip under the skin at the back of its head. Food was provided *ad libitum*. Water was accessible only in the Educage, but was never restricted as mice could engage the water port anytime. Mice were never deprived of food nor water. All experiments were approved by the Hebrew University's IACUC.

### *Training paradigm*

At the beginning of each experiment, RFID-tagged mice were placed in a homecage that is connected to an Educage. Before training, we removed the water bottle from the home cage, mice were free to explore the Educage and drink the water at the behavioral port. Every entry to the behavioral port was identified by the RFID antenna and the infra-red beam after which a trial was initiated. Before learning, any entry to the port immediately resulted in a

drop of water, but no sound was played. Following 24hrs of such exploration, mice were introduced for the first time to the 'target' stimulus – a series of six 10 kHz pure tones (100 ms duration, 300 ms interval; 3 ms on and off linear ramps; 62 dB SPL) played every time the mouse crossed the IR beam. The trials were separated by 2 seconds of inter-trial interval in which a new trial could not be initiated. To be rewarded with water, mice were required to lick the spout during the 900 ms response window. This stage of operant conditioning lasted for 2-3 days after which all mice performed at >70% hit rates.

Then, we switched the system to the first level of discrimination when mice learned to identify a non-target stimulus (a 7.1 kHz pure tone series) from the target stimulus (i.e. tones separated by 49%/octave). On each trial, one of two possible stimuli was played for 2.1 seconds – either a target tone or a non-target tone. Mice were free to lick the water spout anytime, but for the purpose of evaluating mouse decisions we defined a response window and counted the licking responses only within 900 ms after the sound terminated. Target tones were played at 70% probability and non-target tones at 30% probability, in a pseudorandom sequence. A 'lick' response to a target was rewarded with a water drop (15 microliters) and considered as a 'Hit' trial. A 'no lick' response to a target sound was considered as a 'Miss' trial. A lick response to the non-target was considered a 'False Alarm' ('FA') trial, which was negatively reinforced by a mild air puff (60 PSI; 600ms) followed by a 9 second 'timeout'. A 'no lick' response to the non-target was considered a correct rejection ('CR') and was not rewarded.

Once mice learned the easy discrimination, we switched the system to the second level of discrimination. We increased task difficulty by changing the non-target tone to 8.333

kHz, thus decreasing the inter tone distance to 26%/octave. The following level of difficulty was 14%/octave and then down to 3%/octave. This last transition was often done in a gradual manner (»10%/octave»8%/octave» 6%/octave»3%/octave). Some animals were trained to their JND and then back to an easier level. For some mice we played 'catch trials' during the first and second sessions of the 14%/octave discrimination stages. In catch trials, different tones spanning the frequency range of the whole training band (7.1-10 kHz) were presented to the animals in low probability (6% of the total number of sounds). Catch trial were neither negatively nor positively reinforced.

For the vocalizations task we used playback of pups' wriggling calls (WC) as the target stimulus. These vocalizations were recorded with a one-quarter inch microphone (Brüel & Kjær) from P4–P5 pups (*n* = 3), that were sampled at 500 kHz (Digidata 1322A; Molecular Devices) and identified manually offline. For the non-target stimulus, we used temporal manipulations of the WC. For the first level of learning, mice were trained to discriminate between a WC and a fully reversed version of this call. The other levels of discrimination were based on a gradual change of the frequency modulation (FM) of each syllable in the call while leaving the temporal structure of the call intact. To manipulate the syllable's FM we used a dynamic linear FM ramp. This operation multiplies each sampling interval within the syllable by a dynamic speeding factor, therefore, changing the syllable according to the relative distance from its start to its end. This operation generates a new waveform by interpolating the original waveform. For example, for a 0.6 speeding factor, the beginning of each syllable was slower by a factor of 0.4 while the end of each syllable accelerated by a

factor of 0.4. The range of speeding factors used here was 0.4-1.4. A value of 0.4 is away from the WC, 0.9 similar to the WC and 1 exactly the same as the WC. A value >1 again turns away from the original WC but to the other direction (i.e. faster, and then slower). The basic task design for the non-target sound was as follows: Reverse» 0.6 »0.81» 0.9. In addition, we measured responses to catch trial stimuli spanning upstream and downstream of the modulation range used for training by testing responses to 0.4 to 1.4 speeding factors.

***Data analysis***

To evaluate behavioral performance we calculated, for different time bins 'hit' and 'FA' rates, which are the probability to lick in response to the target and non-target tones, respectively. In order to compensate for the individual bias, we used measures from signal detection theory- bias and d-prime (d'). Bias was calculated as the average of the normal inverse cumulative distribution of the 'hit' and 'FA' rates. Discriminability was evaluated using d' which is defined as the difference between the normal inverse cumulative distribution of the 'hit' and 'FA' rates (Nevin 1969). d' for each discrimination stage calculated based on trials from the last 33% of the indicated stage. Psychometric curves were extracted based on performances in response to catch trials. By fitting a sigmoidal function to these curves we calculated decision boundaries as the inflection point of each curve.

Detection time (DT) was calculated for each mouse individually, using two different methods. In the first method, DT was evaluated by determining the time in which lick patterns in the correct reject versus the hit trials diverged (i.e. the time when

significance levels crossed $P<0.001$ in a two-sample t-test). In the second method, DT was determined by the time mice retracted their heads from the behavioral port in the correct rejection trials.

## Results

### *The Educage*

We developed a behavioral platform named the 'Educage'. The Educage is an automated behavioral training apparatus designed for simultaneous use with several mice (here, we limited this number to 8). We focused on training mice on an auditory discrimination task, but other modalities are possible to integrate in the system as well. The fully automated nature of the Educage minimizes human interference or handling, as mice continue to live in their home cage and are free to engage the behavioral port at their own will.

The Educage is composed of a small behavioral chamber (10x10X10 cm), opening on one end into a standard animal home cage where mice can enter and exit freely (Fig. 1A). On the other end, the chamber contains the hardware that drives the system, hardware for identifying mice and measuring behavioral performance. Specifically, at the port entrance we positioned a coil radio antenna followed by infra-red diodes used to identify mice individually and monitor their presence in the port. This port is the only access to water for the mice. Water is delivered via a solenoid valve that allows precise control of its volume on each visit and through a water spout, which is also a lickometer. Licking of the lick spout is detected and precisely registered using a transistor based circuit-closing detector. An additional solenoid valve is positioned next to the water spout in order to deliver a mild air puff as a punishment, if necessary. For sound stimulation, we positioned a speaker at the center of the top wall of the chamber. For high speed data acquisition, system control, on-board sound delivery, and future flexibility, the system was designed via a field programmable gate array

module and a real-time operating system module (see Methods). A custom user-friendly executable interface was written in LabView with numerous options for user-input and flexibility for designing custom protocols. A schematic and a photograph of the system are shown in figure 1A.

Prior to the experiment, each mouse is implanted with a Radio Frequency Identification (RFID) chip on the back of its head. RFID chips allow identification of mice individually, which is then used by the system to control the precise stimulus delivery and track individual behavioral performance. In our experiments, we trained mice on a go/no-go auditory discrimination task. Once a mouse entered the behavioral port it is first identified individually by the antenna. Then, by breaking an infra-red beam, a trial is initiated. The trial design which we used here is as follows. In each trial, one of two possible stimuli are played for 2.1 seconds – either a target tone or a non-target tone sequence. Mice are free to lick the water spout anytime, but for the purpose of evaluating mouse decisions we defined a response window and counted the licking responses only within 900 ms after the sound terminated (Fig. 1B,D; green). Target tones were played at 70% probability and non-target tones at 30% probability, in a pseudorandom sequence. A 'lick' response to a target was rewarded with water (15 microliter) and considered as a 'Hit' trial. A 'no lick' response to a target sound was considered as a 'Miss' trial. A lick response to the non-target was considered a 'False Alarm' ('FA') trial, which was punished by a mild air puff (60 PSI; 600 ms) followed by a 9 second 'timeout'. A 'no lick' response to the non-target was considered a correct rejection ('CR') and was not rewarded (Fig. 1A, right). Notably, the user can change any of these parameters to fit different trials structures and/or behavioral paradigms.

In the behavioral protocol presented here, mice were free to decide when and how much to train. On average, mice performed 327 ± 71 trials per day. As expected, mice were mainly active at dark hours, during which they performed 74%±8 of their total number of trials (Fig. 1C).

***Auditory discrimination task***

Before training with auditory stimuli, mice were free to explore the Educage and receive water rewards at the behavioral port. Any entry to the port immediately resulted in a drop of water, but no sound was played. Mice are initially hesitant to nose poke but quickly learned to find the water source. After 24 hrs, mice normally licked the spout in >75% of their visits. Following 24hrs of exploration, mice were introduced for the first time to 'go' trials with the 'target' stimulus – a series of 10 kHz pure tones played every time the mouse crossed the IR beam. For the trial to be considered a 'hit' and the mouse to obtain water reward, it was required to lick during the 900 ms response window (Fig. 1B; 1D-hit trials). If mice did not lick during the response window, water was not delivered and the trial was scored as a 'miss' (Fig. 1D – miss trials). This stage of operant conditioning contained only 'hit' and 'miss' trials and lasted for 2-3 days or until mice achieved a stable hit rate of more than70%. Mice thus learned to associate the 10 kHz pure tone with the water reward in an operant conditioning task.

The system was then switched to the first level of discrimination in which the non-target stimuli were presented during 'no go' trials (Fig. 1D- middle, red trials). In this level,

mice learned to identify the non-target stimulus (a 7.1 kHz pure tone series) from the already known target stimulus (i.e. the 10 kHz pure tone series). These two stimuli are separated by 49% of an octave and are considered a relatively easy discrimination task. The first punishment (air puff and time out; Fig. 1D, arrow) caused an immediate reduction in licking rates for both target and non-target tones (Fig. 1D). As training progressed, licking rates were recovered for the target tone and remained low for the non-target tone (Fig. 1D; bottom panel, note the very few licks on non-target trials).

To evaluate behavioral performance we calculated hit and 'FA' rates, which are the probability to lick in response to the target and non-target tones, respectively. Behavioral performance varied widely between mice as demonstrated for three different mice in figure 2A. Some mice reached high performance (i.e. high 'hit' rates and low 'FA' rates) within dozens of trials (Fig. 2A - triangles). Some mice were more prone to licking, resulting in high 'hit' and 'FA' rates (Fig. 2A - circles) while others were more conservative in their responses and tended to withhold licking (Fig. 2A - squares). To account for these individual biases, we used measures from signal detection theory- bias and d-prime (d'). The bias is the extent to which one of the responses is more likely to occur, calculated as the average of the normal inverse cumulative distribution of the 'hit' and 'FA' rates. Negative and positive bias describe conservative and liberal licking behavior, respectively. The biases of the three mice shown in figure 2A are 0.6, 0.13, and 0.44 for circles, squares and triangles, respectively. The range of bias values across all mice was -0.3 to 0.8 and the average was 0.17±0.27 (n= 39 mice). In comparison, the bias of water deprived mice ranges from 0.47 to 0.64 (data not shown). This comparison of bias values is used to argue that the majority of our mice were motivated to

drink but not stressed as water deprived mice. Discriminability was evaluated using d' which is invariant to bias (Fig. 2B). d' is defined as the difference between the normal inverse cumulative distribution of the 'hit' and 'FA' rates (Nevin 1969). All mice (n=39) eventually learned to discriminate the 7.1 from the 10 kHz. On average, it took mice 54±38 trials to cross our criterion of learning which was set arbitrarily at d'=1 (Fig. 2C; dotted line). Mice improved with practice during the first few hundred trials. Some mice learned very fast, reaching d' above 1 already in the first 20 trials (e.g. Fig. 2C, triangles). The slowest mice took hundreds of trials to reach the criterion (e.g. Fig. 2C, circles). On average, mice continued to improve at a rate of 0.013 d'/10 trials until they reached a performance plateau (average d'=2.35±0.64). Interestingly, discriminability at the end of this stage was negatively correlated with the individual learning rates (Fig. 2D; p=0.004; the explained variance of discriminability by the individual learning rates was larger than 25%). This correlation suggests that this first learning stage already contains information about the future discrimination performance of individual mice.

***Perceptual learning***

Perceptual learning (PL) is an implicit form of learning in which the animal improves its perceptual abilities, normally in a sensory task (Gibson 1969). PL requires plenty of practice, which is essential for improvement. We trained mice in the Educage on a PL auditory task by gradually increasing task difficulty. Difficulty was increased by changing the non-target tone closer to the target tone. For simplicity, the target tone remained constant

at 10 kHz throughout the experiment. During the first discrimination stage, the non-target tone was set at 7.1 kHz ('target'-'non-target' distance=49% of an octave). For the second discrimination stage, we increased task difficulty by changing the non-target tone to 8.333 kHz, thus decreasing the inter-tone distance to 26%/octave. In subsequent stages, the inter tone distance was further decreased to 14%/octave and then down to 3%/octave. This last transition was often done in a gradual manner (»10%/octave » 8%/octave » 6%/octave » 3%/octave). A representative example from one mouse's performance in the Educage throughout a complete experiment is shown in figure 3A. This mouse rapidly learned the easy task (Fig. 3A- 49%/octave), showed lower performance rates in the more difficult tasks and could not discriminate the sounds at 10%/octave. At 3%/octave all mice failed to discriminate the non-target tone (9.8 kHz) from the target tone (10 kHz). Mice showed heterogeneous patterns of hit and 'FA' rates with different biases along the learning stages. Using the abovementioned paradigm we could evaluate the just noticeable difference (JND) for each mouse. The JND of the mouse shown in figure 3A is between 10-14%/octave. JND varied between mice but was always between 3-14%/octave (Fig. 3B). The average ±SD value of failure to discriminate was 8.6±4.7%/octave; n=39 mice). These values of JND are typical for frequency discrimination in mice (de Hoz and Nelken 2014, Ehret 1975). For learning to be regarded as PL it is often required to show improved performance. Most mice improved their performance with training (Fig. 3C). This improvement was obvious in the first easy stage (Fig. 3C, light markers) but was evident in the majority of mice in later stages as well (90%, 64% and 67% of mice for the 49%, 26% and 14% task, respectively; Fig. 3C). A second way to test improved performance is by comparing the same level of difficulty after different epochs

of training on more difficult tasks. In some of the animals (25/39), we trained mice to their JND and then changed the task back to an easier level. Specifically, mice were trained in the following sequence: 49%/octave » 26%/octave » 14%/octave » 3-10%/octave » 14%/octave. We then compared their performance on the first 14%/octave stage to the second 14%/octave stage, expecting for an improvement on the latter. Indeed, and despite the failure at 3-10%/octave which could have had deleterious effects, the average performance on the second 14%/octave stage was higher for most mice (d'=1.68±0.5 versus d'=1.87±0.58; p=0.053; paired t-test). Together, these data support the argument that mice improve their perceptual abilities along the task.

PL is normally thought to be specific to the trained stimuli (Karni and Sagi 1991) (but see(Hawkey, Amitay, and Moore 2004)). Thus, we next tested the selectivity of the learning (n=5 mice). To do so, we played 'catch trials' during the first and second sessions of the 14%/octave discrimination stages. In catch trials, different tones spanning the frequency range of the whole training (7.1-10 kHz) were presented to the animals in low probability (6% of the total number of trials), and were neither negatively nor positively reinforced. Catch trials were used to extract psychometric curves and calculate decision boundaries that animals used in order to classify the tones as either 'target' or 'non-target'. Psychometric curves constructed from the first 14%/octave stage monotonically increased from the non-target to the target tone (Fig. 3D, light curves). By fitting a sigmoidal function to these values we infer that animals placed their decision boundary at ~11%/octave difference (Fig. 3D, light arrow). Psychometric curves extracted from the second session of 14%/octave, were significantly shifted to the left with a new decision boundary at ~8%/octave difference (Fig.

3D, dark arrow; Ranksum test on criteria: p=0.03). These data suggest that during perceptual learning mice generalize less and respond more specifically to the target tone.

In order to get insight about the strategy mice used to perform the task, we also tested a second set of catch trial stimuli spanning upstream and downstream of the frequency range used for training (i.e. 5 and 15 kHz; n=8 mice). Responses to these catch trials showed that half of the animals perceived the 15kHz tone (-50%/octave difference) as different from the target, supporting a true target detection strategy (Fig. 3E, triangles). However, the other half of the mice treated this high frequency tone as if it was the target. We interpret this result such that mice categorized the stimuli as target whenever their frequency was away from the non-target tone (and not necessarily close to the target tone). In support of this interpretation, two of the mice (Fig. 3E, squares and asterisks) also responded to the 5 kHz tone (which is far from the target but also from the non-target) at chance level. This data suggests that different mice may well use different strategies to solve the same task.

***Gradual training is necessary to improve performance***

While PL is characterized by perceptual improvements it has also been suggested that training for gradually increasing task difficulty is important for improvement (Ahissar and Hochstein 2004, Ericsson 2006, Kurt and Ehret 2010, Lawrence 1952). Thus, we next compared the abovementioned gradual training versus training that is limited to the easy task but similar in duration of training. We exploited one advantage of the Educage and

trained groups of littermate mice on these different protocols simultaneously. A group of mice was divided into two subgroups: 1) “PL” group, and 2)”Easy only” group (Fig. 4A). The PL group of mice were trained in the exact same way as described above (Fig. 3A; Fig. 4B- ‘PL’). The Easy only group trained continuously on the easy discrimination task (Fig. 4B- ‘Easy only’; 49%/octave). The two groups of mice trained together in the same cage, for similar durations and similar number of trials (17 days; 6350±707 trials). For each mouse, we compared its performance on the easy task to that of a harder task (10%/octave) at the end of training. Initially, the performance of mice in both groups on the easy task was indistinguishable (Fig. 4C, 49%/octave). Remarkably, and in marked contrast, only mice undergoing gradual training were able to perform the hard task (Fig. 4C, 10%/octave). We conclude that gradual training is necessary for auditory PL in mice.

***Detection times increase monotonically with increased task difficulty***

In the Educage, the lick pattern and the presence of the mice in the behavioral port are continuously monitored throughout the trial (Fig. 1). This information can be used to derive cognitive aspect of the performance during the task such as detection times (DTs). We first calculated DTs using lick probability (Fig. 5A). In order to receive water reward mice were required to lick the spout in a 900 ms response window starting 2.1 seconds following trial initiation (Fig. 1B). Licking prior to this defined window was not rewarded, nor punished. Thus, mice were not reinforced to stop or start licking the spout once they recognized the stimulus. Consequently, mice licked shortly after trial

initiation and regardless of stimulus identity (Fig. 1D, 5A). Evidently, despite the lack of active reinforcement during the stimulus period, trained mice stopped licking during the non-target trials shortly after stimulus onset, enabling us to evaluate DT's (Fig. 5A, dashed curves). DT was calculated for each mouse individually, by determining the time in which its lick patterns in the correct rejection versus the hit trials diverged (i.e. the time when significance levels crossed $P<0.001$ in a two-sample t-test). DT's increased monotonically by ~177 ms per each step of task difficulty (Fig. 5A – arrows; Fig. 5B; ANOVA: $F=13$ $p<0.001$). This result is expected from and complements the increase in d' values with task difficulty (Fig. 3B). In addition, these results demonstrate the increased perceptual load in harder task. Indeed, DT's and d' were negatively correlated on a per-mouse basis (Fig. 5C). Similarly, DT's can be calculated using head retraction times in correct rejection trials. Calculating DT's using this method we reached qualitatively similar conclusions (Fig. 5D-E). Taken together, these measurements provide useful information regarding the relative state of the animals during task performance and delineate a temporal boundary for the decisions being made. When the task is easy, it is usually solved within less than 500 ms. Thus, animals need only a single 100 ms tone pip (or maximum two) from the stimulus sequence to make a decision. When the task becomes more difficult, the decision is delayed. One interpretation of these data is that the confidence of the animal in the stimulus identity evolves with time and is used for decision making (Barkat, Polley, and Hensch 2011, Gold and Shadlen 2007).

***Learning of complex sounds (vocalizations)***

Despite the appeal of the simplicity of pure tones for experimental design, mice rarely encounter such stimuli, and their perceptual capabilities are used more often to distinguish natural sounds. Natural sounds are characterized by rich spectro-temporal structures with frequency and amplitude modulations over time (Mizrahi, Shalev, and Nelken 2014). Discrimination of such stimuli could be different than pure tones. Thus, we next constructed a similar task design with mouse vocalizations as the training stimuli. We used playback of pups' wriggling calls (WC) as the target stimulus. As the non-target stimulus we used temporal and frequency modulations of the WC. During the first stage of the learning, mice (n=11) learned to discriminate an easy pair of sounds - a WC versus a fully reversed version of this call (Fig. 6A top). The WC and its reverse version are different in the direction of frequency modulations in each syllable as well as in the temporal sequence of the syllables. These are thus two very different stimuli that are easily perceived apart. On average, mice learned this easy task in 195±143 trials (Fig. 6B; dotted line), yet still much slower than pure tones. After a few hundred trials (which last roughly 24hrs) mice further improved, reaching d' values that are indistinguishable from those in the pure tone task (compare Fig. 6B to 2B). We then turned to a different manipulation on the non-target stimulus in which we gradually changed the frequency modulation (FM) of each syllable in the call while leaving the temporal structure of the call intact (Fig. 6A bottom; see methods). This manipulation resulted in a set of stimuli that differ solely in the degree of FM within each syllable; a manipulation that allowed us to morph one stimulus to another by a continuous metric. The range of sound

modulation used here was indexed as a “speeding factor” with values ranging from 0.4 to 1.4. A value of 0.4 is different from the WC, 0.9 similar to the WC and 1 exactly the same as the WC. A value >1 again turns “away” from the original WC but to the other direction. The basic task design for the non-target sound was as follows: Reverse» 0.6 »0.81» 0.9. Example of one mouse trained on this task is shown in figure 6C. Similar to the gradual training paradigm of pure tones discrimination, d’ values gradually decreased as the difference between the WC and its modified version was reduced (Fig. 6C-D). In addition, in 8 mice we measured responses to catch trial stimuli spanning upstream and downstream of the modulation range used for training (i.e. 0.4 and 1.4 speeding factor). Responses became more frequently correct as the similarity to the original WC increased (i.e. 1 speeding factor), but correct responses decreased abruptly to chance levels at 1.4 (Fig. 6E).

Finally, and similar to the pure tones paradigm, we calculated DT’s. DT’s increased with task difficulty (Fig. 7A; ANOVA $F=26.7$, $p<0.001$) and were negatively correlated with d’ on a per-mouse basis ($R^2=0.21$, $p<0.01$). The absolute values of DT’s of these stimuli were substantially higher than the values observed in the pure tone discrimination task (Fig.7B). These differences may arise from the difference in the delay, inter-syllable interval and temporal modulation of each stimulus type. While pure tone sequence is a repetitive (and hence easily predictive) stimulus, vocalization is dynamic providing additional information accumulating supra-linearly over time (Brunton et al., 2013). Taken together, these behavioral results demonstrate learning of

complex sound discrimination, in the form of frequency modulations in natural vocalizations, in the Educage.

## Discussion

### *A tool for studying perceptual learning in mice*

Using the 'Educage', a custom made and cost effective automatic behavioral platform, we trained groups of mice on a PL auditory task. Training mice on PL paradigms has traditionally been performed manually over many weeks, sometimes months, of training (Kurt and Ehret 2010, Polley, Steinberg, and Merzenich 2006, Radziwon et al. 2009). Besides being time-consuming, the researcher involvement in the training could affect the stress levels of the animals (Sorge et al. 2014) and could also result in subjective errors (Longordo et al. 2011). Automating this procedure using the Educage platform allows to simultaneously train multiple animals, minimize handling, and thus increase the efficiency of the training. The simple design and operation of the 'Educage' together with its affordable hardware and user friendly software makes it a potentially valuable tool for studying learning in mice.

### *Variability in behavior*

Behavior is confounded by variability of all sorts, even in the controlled conditions of a laboratory. Variability is affected by many factors like an individuals' experience, internal state and motivation (Renart and Machens 2014). One way to handle variance is averaging across trials and/or mice. The ease of use of the Educage, especially in face of restricted resources, allows to sample more mice. Moreover, since mice are not stressed they perform hundreds of trials per day, which increases sample size considerably. The

software of the Educage provides online performance measures such as d' and bias for single mice. This allows the experimenter to monitor the results as the normal progression of learning advances. Another source of behavioral variability may stem from different decision strategies adapted by individual animals; even for a simple task like two-tone discrimination (Fig. 3E). By integrating catch trials into the design of the Educage we could assess this type of variability that is often neglected in behavioral studies. Finally, having numerous mice training simultaneously without individual human handling and for lengthy periods is a positive factor in reducing inter-animal variability and reducing stress related to social deprivation.

***Transfer along a continuum***

Mice learned to discriminate pairs of close by pure tone stimuli by applying a gradual mode of training (Fig. 4). This training design, based on the principle known as 'transfer along a continuum', has been shown to be important for efficient learning (Ahissar and Hochstein 1997, Lawrence 1952). In fact, this principle was already demonstrated in one of Pavlov's experiment, where dogs were reinforced to salivate only after seeing an ellipse but not a circle. Pavlov managed to train the dogs to differentiate ellipse from circle only by using very elongated ellipses which he then shortened gradually (reviewed in (Ahissar and Hochstein 2004)). Incremental training has also been suggested to enable greater capacity for plasticity in adult barn owls (Linkenhoker and Knudsen 2002), and may well serve as a canonical strategy for the central nervous system to allow plasticity.

***Reward and punishment for positive and negative reinforcements***

Reward and punishment motivate behavior and form complex interactions between distinct and non-overlapping neuronal pathways (Cohen and Blum 2002, Gourley and Taylor 2016, Leknes and Tracey 2008, Seymour, Singer, and Dolan 2007). The protocol we used exploits both reward (water) and punishment (air puff and time-out) outcomes to drive mice to their perceptual limit. While the use of punishment is beneficial for increased learning rates and strength (Freedberg et al. 2017, Steel et al. 2016), it could also raise problems of overstressing the animal towards hopelessness-like behavior (Maier and Seligman 1976). Indeed, when increased levels of punishment were used (or epochs of repeated 'FA' trials occurred), we occasionally encountered mice that stopped licking abruptly and started engaging in a "defensive burying behavior" by covering the behavioral port with sawdust from their home cage (De Boer and Koolhaas 2003). Indeed, punishment could have strong impact on perceptual performance and the motivation to respond (Hollon, Burgeno, and Phillips 2015, Resnik, Sobel, and Paz 2011). This tendency was also evident in our data as the length of inter-trial intervals following 'FA' trials was significantly longer than for any other outcome (Fig. 8A).

Given the ease of exploiting the Educage in different protocols, we also conducted preliminary experiments, training mice (n=8) without punishment at all. We designed this training protocol such that mice were required to execute nine consecutive licks during the target tone sequence presentation in order to receive the water reward. We set the threshold to be nine licks so mice will have to invest energy in

their response. Unlike the punished version of the task, here target and non-target stimuli were played at equal probabilities (Fig. 8B). Without punishment, the initial learning phase had to be easier (130%/octave) as compared to the punished version of the task (49%/octave). Even with 130%/octave, learning rates were slow (930 trails to cross criterion; Fig. 8C). Although mice eventually reached comparable d' values (Fig. 8D), they often failed at 14%/octave. Thus, the Educage may prove useful not only for calibrating the impact of vital behavioral modulators like punishment and reward but also to reveal their effects on different learning paradigms and cognitive abilities.

## Figure legends

**Figure 1. Auditory discrimination task in the 'Educage'. A.** Left: Schematic design of the 'Educage' system components. Right: Schematic representation of the go/no-go auditory discrimination task. Bottom: Top view photograph of the Educage system: 1. Home cage; 2. Connector tube; 3. Auditory speaker; 4. IR diodes monitoring the behavioral port; 5. Water spout and licko-meter; 6. RFID antenna; 7. Connection to D/A converter; 8. Electrically conductive grid floor (for lickometer circuit). **B.** Structure of the 3 second trial. The trial starts with a break of the infra-red (IR) beam, continues with the presentation of either the target (blue) or non-target (red) tone and is followed by a response window. 'Hit' trials are positively reinforced with a water drop while 'FA' trails are negatively reinforced with an air puff (AP) and 9 seconds timeout immediately following the trial (grey). **C.** Histogram of the hourly distribution of visits in the Educage during a 24hr cycle. Grey shade represents dark hours. N=39 mice (mean ± s.e.m). **D.** Raster plots showing the licking behavior of one representative mouse during three consecutive stages of training. Top: end of association stage; Middle: beginning of the first discrimination level; Bottom: end of the first discrimination level. Target and non-target stimuli are shown in blue and red, respectively. Only licks during the response window (green area) were considered as a response and scored accordingly as 'Hit', 'Miss', 'FA' or 'CR' (represented on the right column as blue, green, red or magenta bars, respectively).

**Figure 2. Behavioural variance. A.** Representative examples of the performance of three mice during the first stage of discrimination. The three mice represent the wide range of biases of different mice in the dataset. See text for details. **B.** Bias and d' values of 39 mice during the first stage of discrimination (p=0.3, $R^2$=0.007). **C.** d' values of the three representative mice shown in 'A' (dotted line curves) and the population average ± s.e.m (solid curve) for all mice during the first level of discrimination. Different mice took considerably different number of trials to reach the criterion (d'=1). **D.** Learning rates of individual mice are correlated with their level of discriminability at the end of the level ($R^2$=0.21, p=0.002). Each dot is data from one mouse.

**Figure 3. Auditory perceptual learning in the 'Educage'. A.** Lick responses to the target tone (solid line, "Hit rate") and non-target tone (dashed line, "FA rate") of one representative mouse during the different levels of discrimination. This mouse failed to discriminate at 10%/octave inter-tone difference. **B.** Population average (N=39 mice; mean ± s.e.m) of d' values for the different levels of discrimination (3-10% octave apart groups all mice that failed to discriminate at this range). **C.** Individual d' values at the end versus the beginning of the training level. Only the first three levels of difficulty are shown. Most mice could perceive the difference between the tones in all levels and often improved during training. **D.** Normalized psychometric curves of five mice constructed from the first (light curves) and the second (dark curves) level set at 14%/octave. Light and dark arrows indicate average decision boundaries in the first and second sessions respectively. **E.** Normalized psychometric curves of 8 mice (marked by

different markers) extracted from responses to catch trials stimuli spanning downstream ('-50'; 15 kHz) and upstream ('100'; 5 kHz) of the frequency range used for training. Arrows represent the target and the non-target tones.

**Figure 4. Gradual training is necessary for perceptual learning. A.** Schematic design of the 'Educage' housing two subgroups training simultaneously: 1) "PL" group (blank RFIDs), and 2)"Easy only" group (grid lines RFIDs). **B.** Training timelines and levels of difficulty therein for the two experimental subgroups. **C.** d' values at the beginning and end of training for the two subgroups. Each mouse is shown by a dot, at the two time points. d' values are not significantly different between groups at 49%/octave but are different at the hard discrimination level (Mann-Whitney U-test: ***$p<0.001$). The Easy only group did not cross criterion at 10%/octave.

**Figure 5. Detection times increase monotonically with task difficulty. A.** Average licking pattern of a representative mouse for 'hit' trials (solid lines) and 'correct-rejection' trials (dashed lines) in four consecutive levels during training (49%-10%/octave). Black arrows represent the calculated detection times when lick patterns in the correct reject versus the hit trials diverged (i.e. $P<0.001$; two-sample t-test). **B.** population averages of detection times for the different discrimination levels (N=12 mice; mean ± s.e.m). **C.** d' of individual mice are negatively correlated with their detection times ($R^2=0.18$, $p=0.002$). **D.** Population average of head retraction times in CR trials for the different

discrimination levels. N=12 mice (mean ± s.e.m). Shades denote the level of difficulty (from 49% to 10% octave apart). **E.** DT's calculated using lick probability and head retraction times in CR trials are correlated ($R^2$=0.15, p=0.005). Shades denote the level of difficulty.

**Figure 6. Perceptual learning of vocalizations. A.** Spectrograms of the wriggling call (WC) used as the 'target' stimulus (top panel) and three of the 'non-target' stimuli (reversed WC, and FM modulated WCs; bottom panels). **B.** d' values (average± s.e.m; n=11 mice) for the first stage of discrimination (WC vs Reverse). **C.** 'Hit' and 'FA'- rates of one representative mouse along the different discrimination levels. Performance dropped when it trained on discriminating WC from a FM-modulated WC with a speeding factor of 0.9. Titles correspond to the non-target stimuli (speeding factors are denoted by numbers). **D.** Average d' values for all mice along the four discrimination levels (N=11 mice; mean ± s.e.m). **E.** Normalized psychometric curves of 7 mice (marked by different markers) extracted from responses to catch trials stimuli spanning downstream (1.4) and upstream (0.4) of the FM modulation range used for training. Arrows represent the target and the non-target stimuli.

**Figure 7. Detecting vocalizations takes longer than pure tones. A.** Population average of detection times for the different discrimination levels of vocalizations. Harder tasks

take longer to discriminate. N=11 mice (mean ± s.e.m). **B.** Detection times for pure tone discrimination versus vocalization discrimination, for the easy (26%/octave and Reverse) and the more difficult levels (14%/octave and 0.81 speeding factor) during learning. DT's are significantly different between the groups in both learning conditions (Mann-Whitney U-test: *p<0.05; **p<0.01;***p<0.001).

**Figure 8. Effects of punishment. A.** Left: Histograms of inter-trial intervals (ITI) following 'Hit' (solid blue), 'Miss' (dashed blue), 'FA' (solid red) and 'CR' (dashed red) trials. Data is calculated during the 14%/octave discrimination level of the punished version of the task. Right: Histograms of ITI from training without a punishment. **B.** Left: Structure of the trial in the training without punishment paradigm. The trial starts with a break of the infra-red (IR) beam, continues with the presentation of either the target (blue) or non-target (red) tone in equal probabilities and terminates by either an execution of nine consecutive licks or by head retraction. Right: Raster plots showing the licking behavior of one representative mouse during the first and last 100 trials in the first discrimination level of the training without a punishment. **C.** Learning rates without punishment. Population average d' values (mean± s.e.m) during the first stage of discrimination (130%/octave). **D.** Population average d' values for the different discrimination levels during training without a punishment. N=8 mice (mean ± s.e.m).

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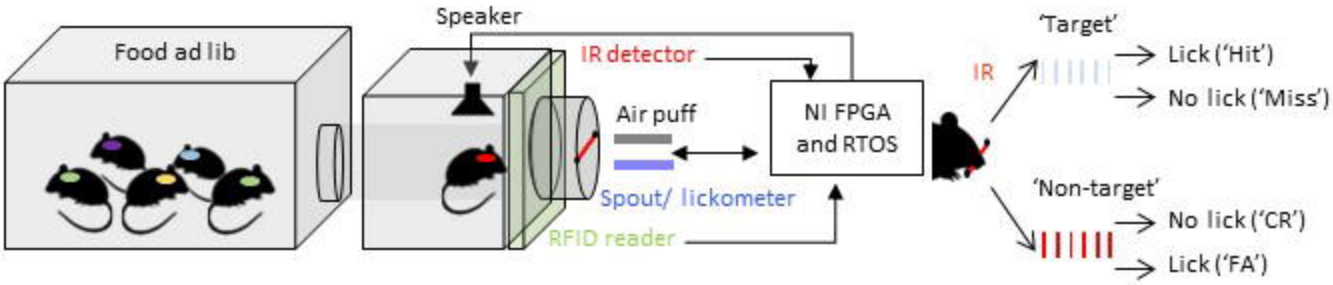
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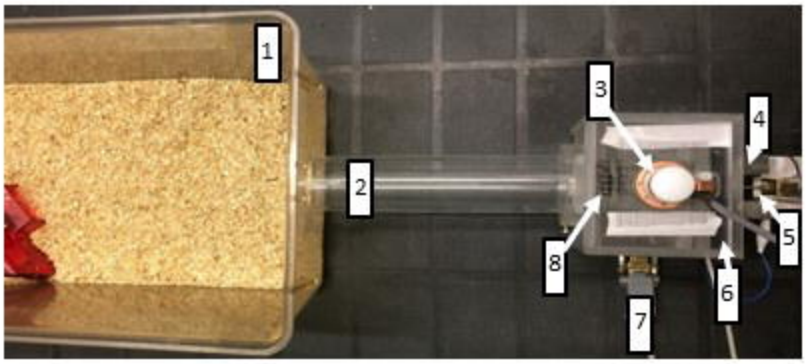
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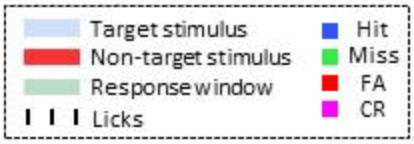
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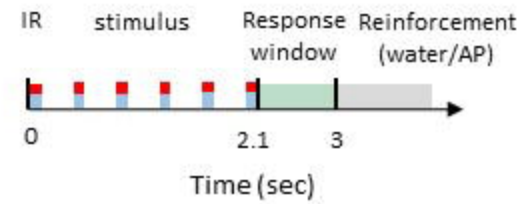
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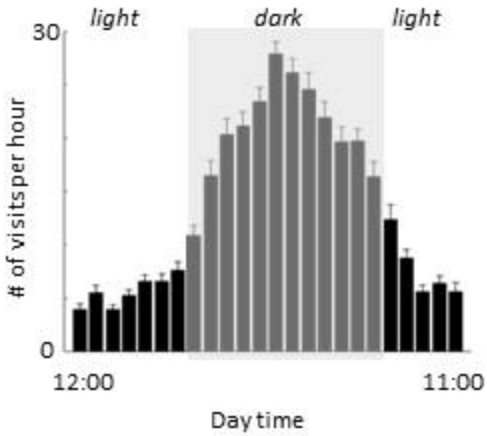
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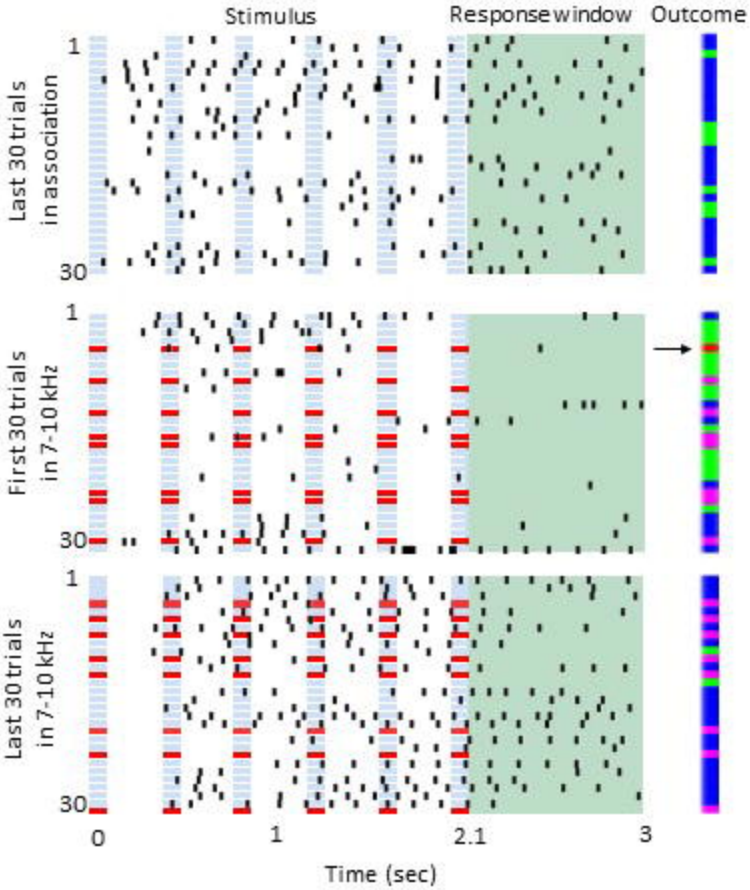
d
Stimulus
Response window
Outcome
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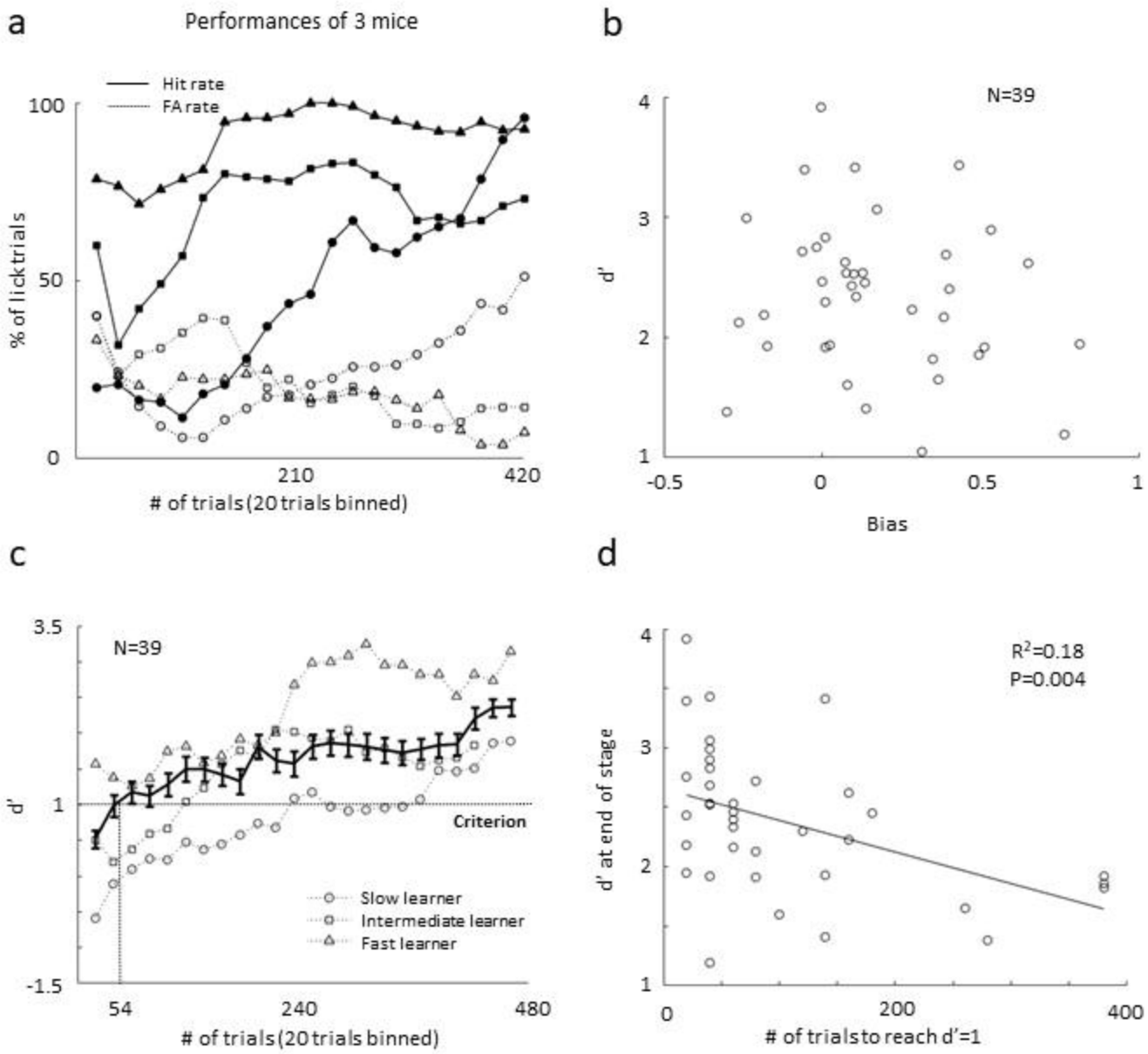
Figure 1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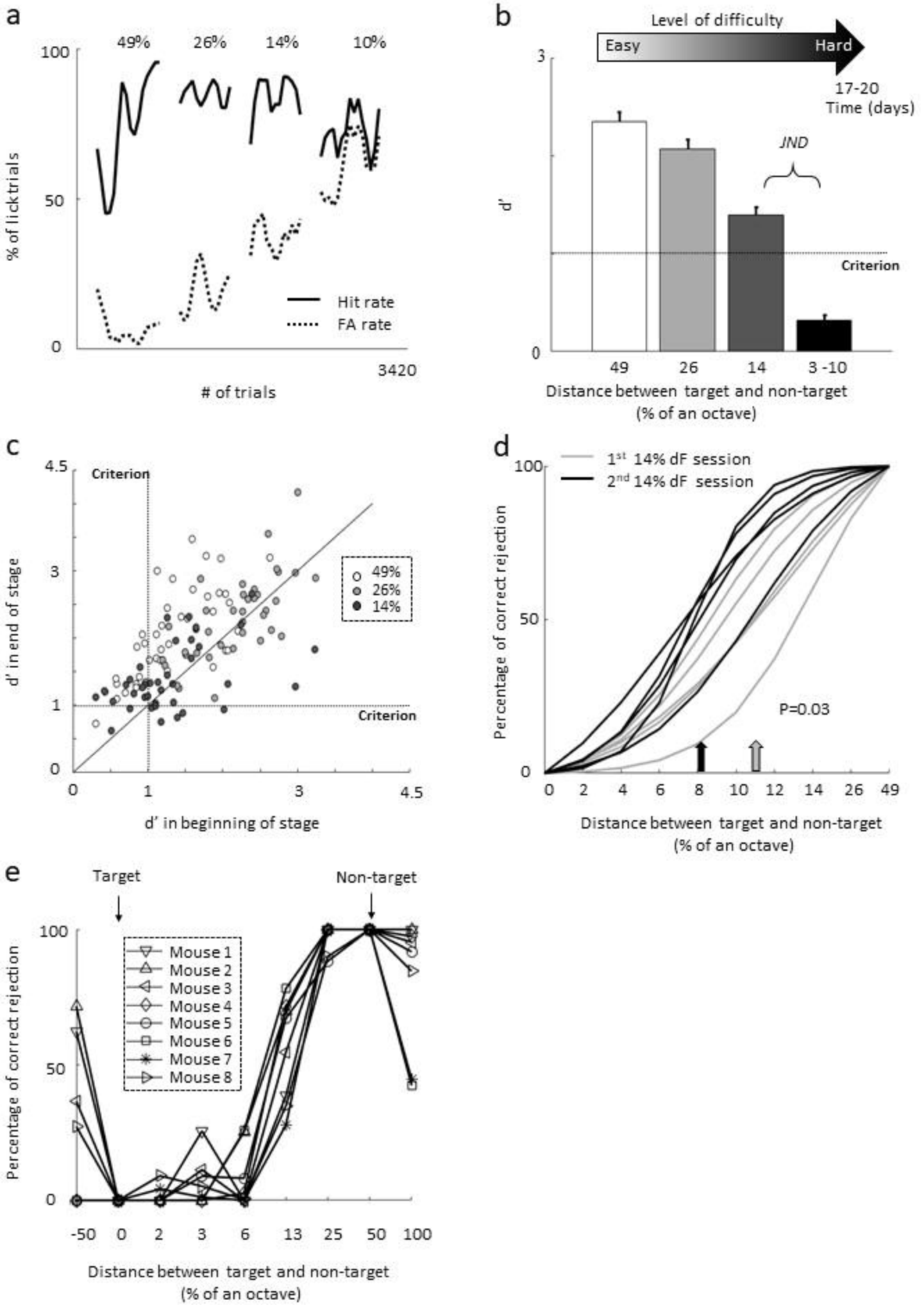
Performances of 3 mice
Hit rate
FA rate
100
50
0
% of lick trials
210
420
# of trials (20 trials binned)
b
N=39
4
3
2
1
d'
-0.5
0
0.5
1
Bias
c
N=39
3.5
1
-1.5
d'
Criterion
Slow learner
Intermediate learner
Fast learner
54
240
480
# of trials (20 trials binned)
d
R²=0.18
P=0.004
4
3
2
1
d' at end of stage
0
200
400
# of trials to reach d'=1

Figure 2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49%
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14%
10%
100
50
0
% of lick trials
Hit rate
FA rate
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# of trials
b
Level of difficulty
Easy
Hard
3
17-20
Time (days)
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Criterion
0
49
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14
3 -10
Distance between target and non-target
(% of an octave)
c
4.5
Criterion
3
49%
26%
14%
d' in end of stage
1
Criterion
0
0
1
3
4.5
d' in beginning of stage
d
1st 14% dF session
2nd 14% dF session
100
Percentage of correct rejection
50
P=0.03
0
0
2
4
6
8
10
12
14
26
49
Distance between target and non-target
(% of an octave)
e
Target
Non-target
100
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Percentage of correct rejection
50
0
-50
0
2
3
6
13
25
50
100
Distance between target and non-target
(% of an octave)

Figure 3

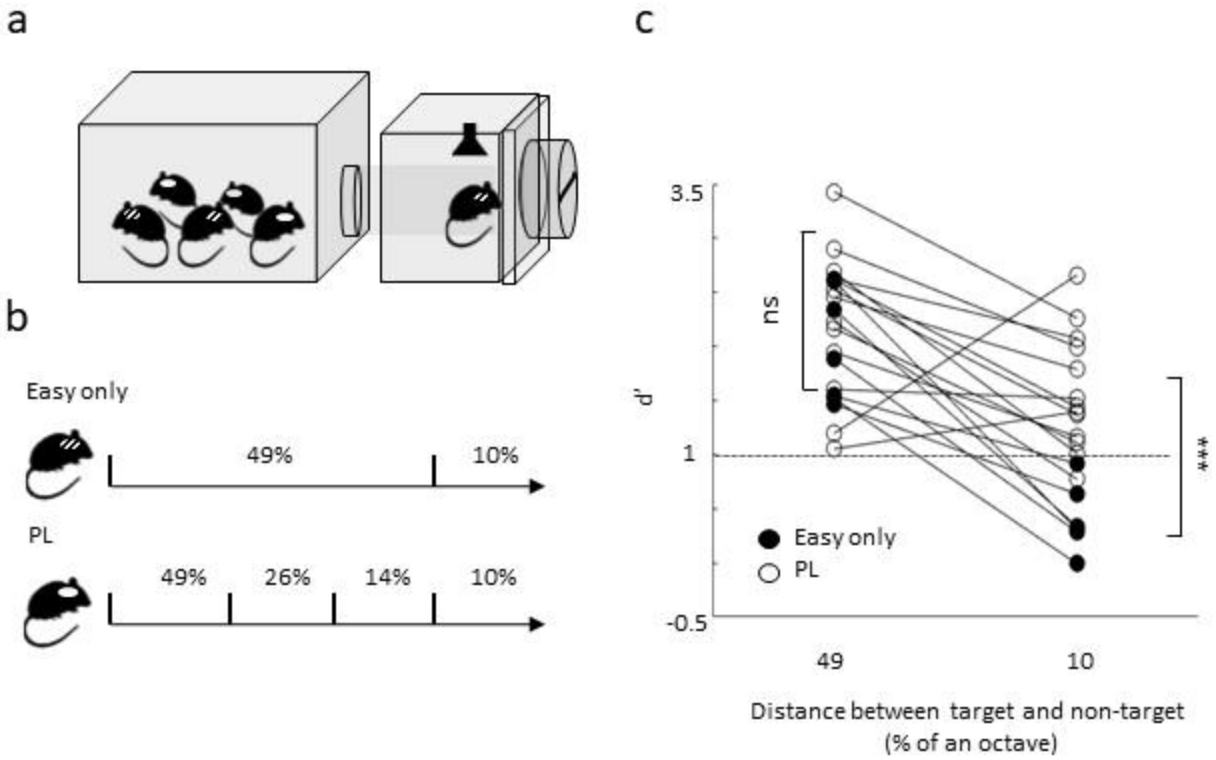
a
b
Easy only
49%
10%
PL
49%
26%
14%
10%
c
3.5
1
-0.5
d'
ns
***
Easy only
PL
49
10
Distance between target and non-target
(% of an octave)

Figure 4

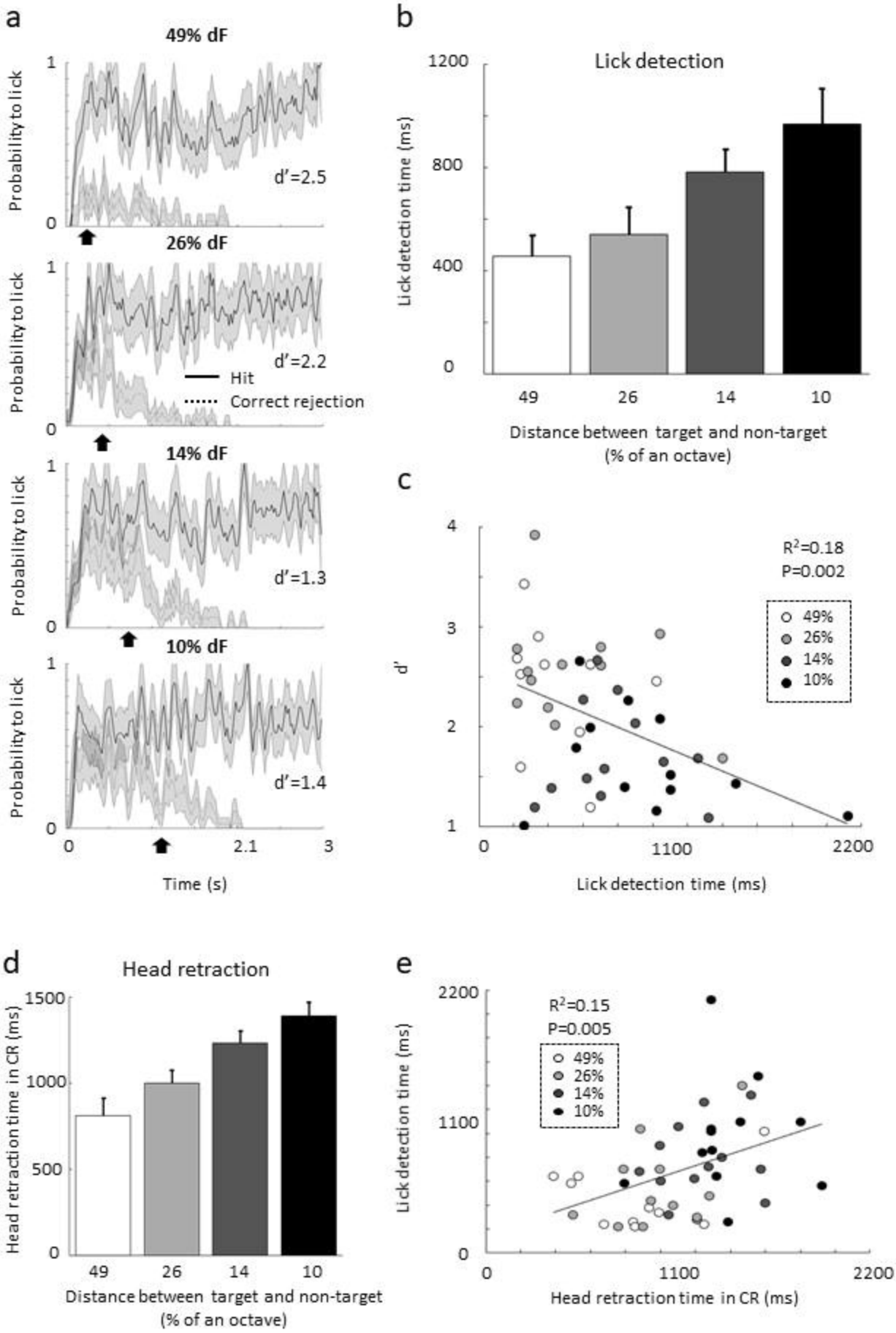
a
49% dF
Probability to lick
d'=2.5
26% dF
d'=2.2
Hit
Correct rejection
14% dF
d'=1.3
10% dF
d'=1.4
0
2.1
3
Time (s)
b
Lick detection
Lick detection time (ms)
1200
800
400
0
49
26
14
10
Distance between target and non-target
(% of an octave)
c
R²=0.18
P=0.002
49%
26%
14%
10%
d'
1100
2200
Lick detection time (ms)
d
Head retraction
Head retraction time in CR (ms)
1500
1000
500
e
R²=0.15
P=0.005
Head retraction time in CR (ms)

Figure 5

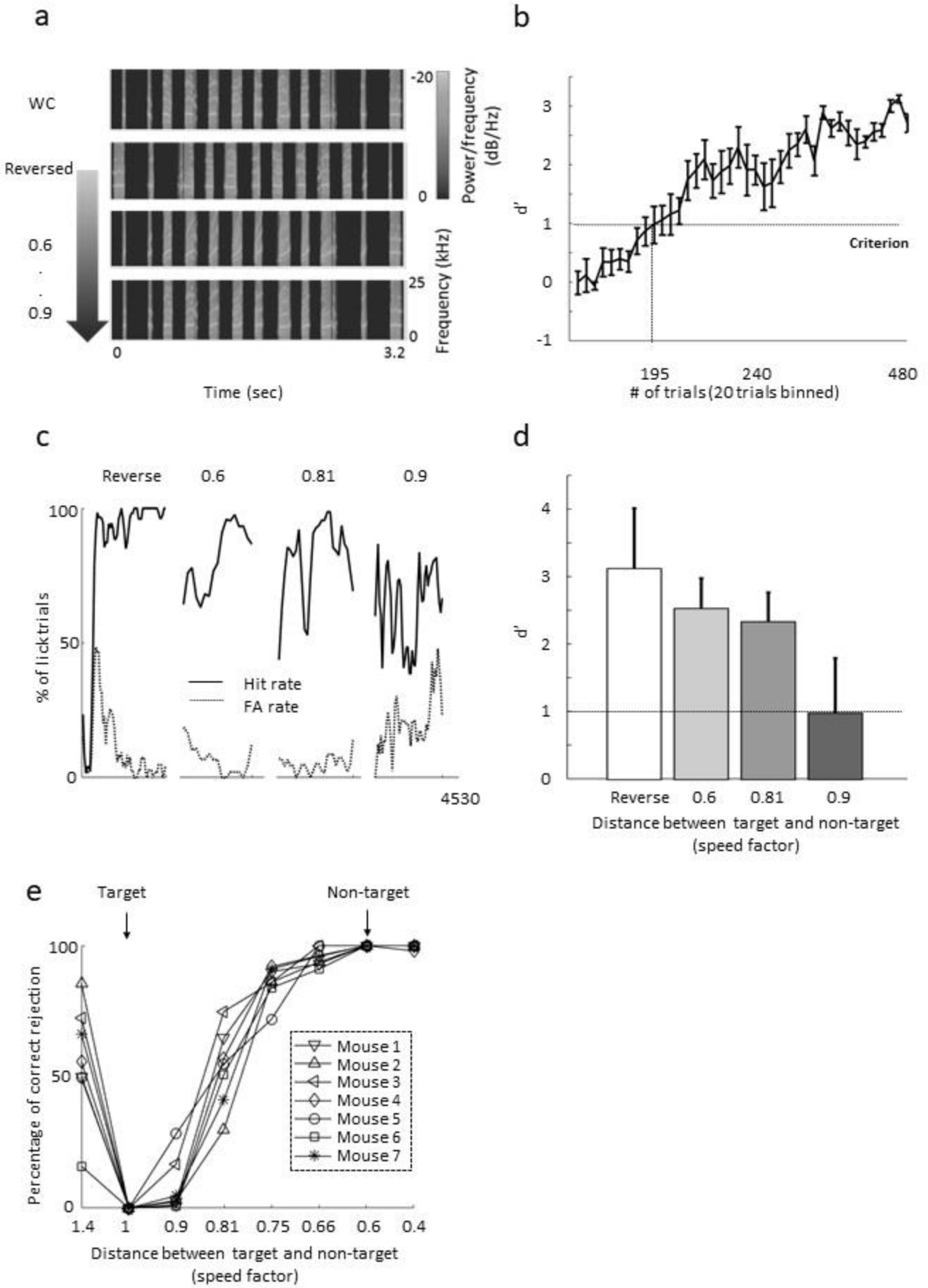
a
WC
Reversed
0.6
0.9
-20
0
Power/frequency (dB/Hz)
25
0
Frequency (kHz)
0
3.2
Time (sec)
b
d'
Criterion
195
240
480
# of trials (20 trials binned)
c
Reverse
0.6
0.81
0.9
100
50
0
% of lick trials
Hit rate
FA rate
4530
d
d'
Reverse
0.6
0.81
0.9
Distance between target and non-target
(speed factor)
e
Target
Non-target
100
50
0
Percentage of correct rejection
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
1.4
1
0.9
0.81
0.75
0.66
0.6
0.4
Distance between target and non-target
(speed factor)

Figure 6

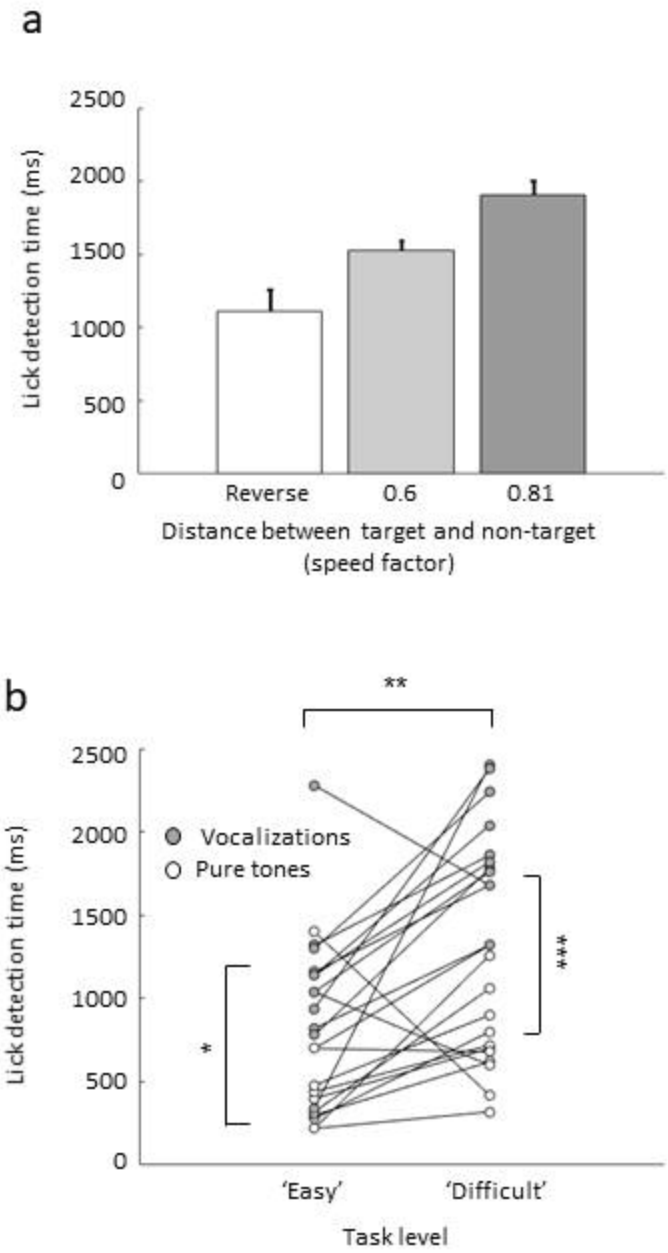
a
2500
2000
1500
1000
500
0
Lick detection time (ms)
Reverse
0.6
0.81
Distance between target and non-target
(speed factor)
b
**
2500
2000
1500
1000
500
0
Lick detection time (ms)
Vocalizations
Pure tones
*
***
'Easy'
'Difficult'
Task level

Figure 7

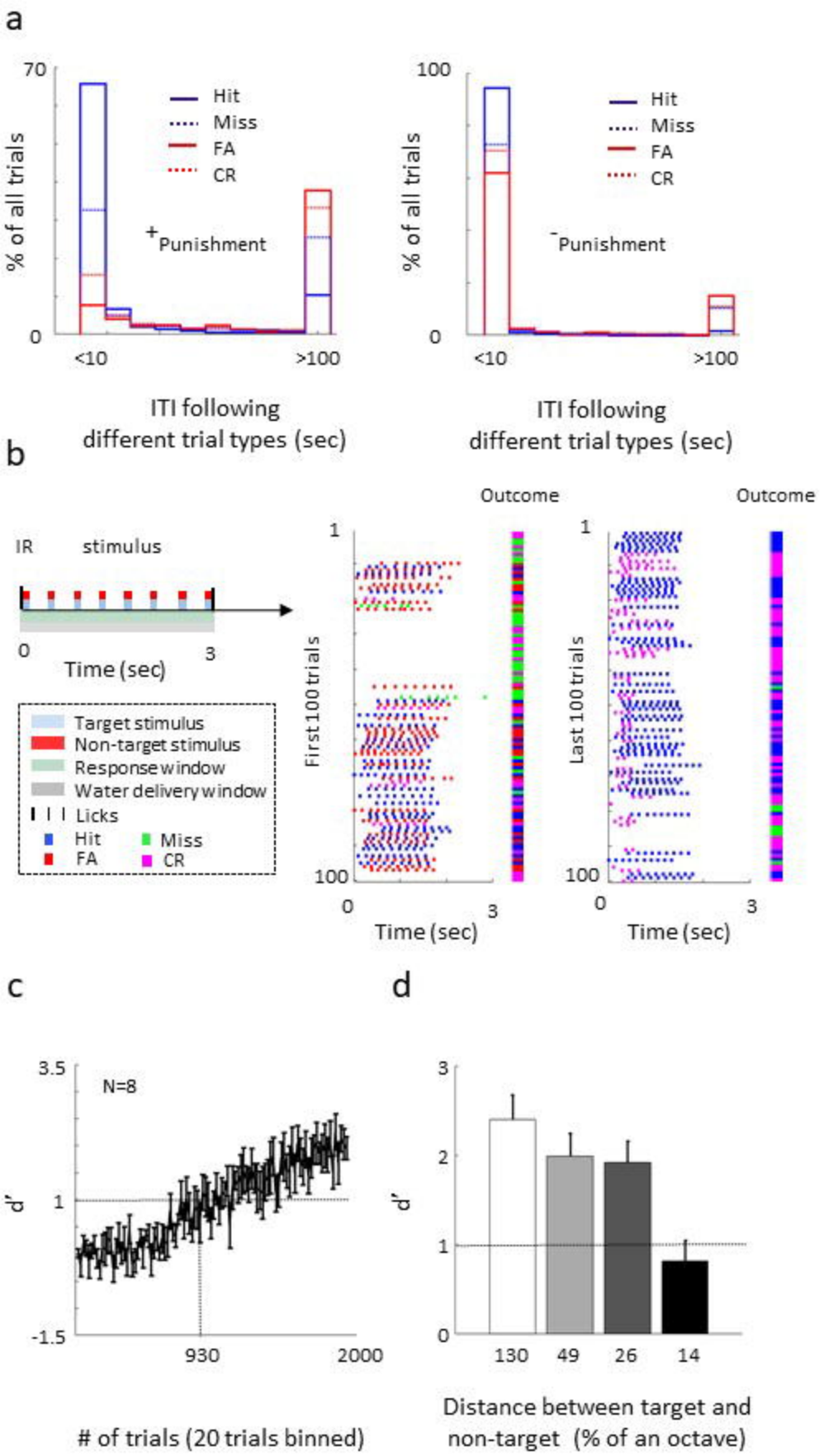
a
70
0
% of all trials
Hit
Miss
FA
CR
+Punishment
<10
>100
ITI following
different trial types (sec)
100
0
% of all trials
Hit
Miss
FA
CR
-Punishment
<10
>100
ITI following
different trial types (sec)
b
IR
stimulus
0
3
Time (sec)
Target stimulus
Non-target stimulus
Response window
Water delivery window
Licks
Hit
Miss
FA
CR
Outcome
1
First 100 trials
100
0
3
Time (sec)
Outcome
1
Last 100 trials
100
0
3
Time (sec)
c
3.5
N=8
d'
1
-1.5
930
2000
# of trials (20 trials binned)
d
3
2
d'
1
0
130
49
26
14
Distance between target and
non-target (% of an octave)

Figure 8