Sniff-synchronized, gradient-guided olfactory search by freely moving mice

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Abstract For many organisms, searching for relevant targets such as food or mates entails active, strategic sampling of the environment. Finding odorous targets may be the most ancient search problem that motile organisms evolved to solve. While chemosensory navigation has been well characterized in micro-organisms and invertebrates, spatial olfaction in vertebrates is poorly understood. We have established an olfactory search assay in which freely-moving mice navigate noisy concentration gradients of airborne odor. Mice solve this task using concentration gradient cues and do not require stereo olfaction for performance. During task performance, respiration and nose movement are synchronized with tens of milliseconds precision. This synchrony is present during trials and largely absent during inter-trial intervals, suggesting that sniff-synchronized nose movement is a strategic behavioral state rather than simply a constant accompaniment to fast breathing. To investigate the spatiotemporal structure of these active sensing movements, we used machine learning methods to parse motion trajectories into elementary movement motifs. Motifs fall into two clusters, which correspond to investigation and approach states. Investigation motifs lock precisely to sniffing, such that the individual motifs preferentially occur at specific phases of the sniff cycle. This work clarifies sensorimotor strategies for mouse olfactory search and guides ongoing work into the underlying neural mechanisms.

Introduction

Sensory observations are often made in concert with movements (*Ahissar and Assa 2016; Gibson 1966*). During active search behavior, animals make sampling movements in order to extract relevant sensory information from the environment (*Gibson 1962; Schroeder et al. 2010*). Sampling behavior is flexible, and can be customized for the prob-

lem the animal is trying to solve (*Kleinfeld et al. 2006; Yarus 1967*). In the brain, sensory and motor systems interact extensively (*Andersen and Mountcastle 1983; Duhamel et al. 1992; McGinley et al. 2015; Musall et al. 2019; Niell and Stryker 2010; Poulet and Hedwig 2006; Sommer and Wurtz 2002; Stringer et al. 2019*), which reflects the importance of interpreting self-induced stimulus dynamics (*von Holst and Mittelstaedt 1950; Sommer and Wurtz 2008; Sperry 1950; Webb 2004*). Here, we show how mice sample the environment while navigating a noisy odor gradient.

Navigating by chemical cues may be one of the most ancient problems motile organisms evolved to solve, and it remains crucial in the lives of almost all modern species. Unicellular organisms and some invertebrates navigate chemical gradients by chemotaxis (*Bargmann 2006; Berg 2000; Lockery 2011*). In essence, their movement programs can be described as having two states: they move straight when the concentration is increasing and reorient their movements when the concentration is decreasing. Whereas chemical gradients are stable and informative at the spatial scale of these organisms, for many larger or flying organisms, odor gradient cues do not provide useful positional information (*Baker et al. 2018; Crimaldi et al. 2002; Murlis et al. 1992*). At this larger spatial scale, turbulent airflow moves odor molecules in dynamic spatiotemporal patterns, disrupting concentration gradients and nullifying classical chemotaxis strategies. Instead, olfactory cues often gate movements that depend on other sensory modalities. Here too, these organisms' behavioral structure can be described as transitions between two states: detection of odor promotes upwind movement while the absence of odor promotes crosswind casting movement (*van Breugel and Dickinson 2014; Kennedy and Marsh 1974; Vickers and Baker 1994*). In this behavioral program, known as odor-gated anemotaxis, odor cues gate behavioral responses to positional information provided by another modality. In both chemotaxis and odor-gated anemotaxis, search tasks can be described with a two-state search model.

In comparison to invertebrates, our understanding of olfactory search behavior in vertebrates is more rudimentary, even in commonly-studied rodent models. In these animals, access to the olfactory environment is gated by respiration, which is in turn responsive to incoming olfactory stimulation (*Kepecs et al. 2006; Wachowiak 2011*). Novel odors evoke rapid sniffing, during which respiration synchronizes with whisker, nose, and head movements on a cycle-by-cycle basis (*Kurnikova et al. 2017; Moore et al. 2013; Ranade et al. 2013*). Thus, during active mammalian olfaction, sensory and motor systems interact in a closed loop via the environment, as is true for other sensory modalities such as vision or somatosensation (*Ahissar and Assa 2016; Gibson 1966*). The cyclical sampling movements coordinated by respiration further synchronize with activity in widespread brain regions (*Karalis and Sirota 2018; Kay 2005; Macrides et al. 1982; Vanderwolf 1992; Yanovsky et al. 2014; Zelano et al. 2016*) similarly to correlates of locomotor, pupillary, and facial movements observed throughout the brain (*McGinley et al. 2015; Musall et al. 2019; Niell and Stryker 2010; Stringer et al. 2019*). Respiratory central pattern generators may coordinate sampling movements to synchronize sensory dynamics across modalities with internal brain rhythms (*Kleinfeld et al. 2014*).

Previous work has shown that rodents follow odor trails, where the concentration gradient is steep and stable, with rapid sniffing accompanied by side-to-side head movements (*Jones and Urban 2018; Khan et al. 2012*). In these conditions, serial sniffing and stereo olfactory cues guide movements of the nose. Likewise, moles used concentra-

tion comparisons across space and time to locate a food source in a sealed experimental chamber in which a lack of airflow allowed for even diffusion of a chemical gradient (*Catania 2013*). In this study, when input to the nares was reversed, moles navigated towards odor sources at a distance, but demonstrated significant deficits at identifying odor location when near the source. Behavioral modeling in mice further supports that inter-naris concentration comparison plays a more important role in search near the source (*Liu et al. 2020*). Thus, both serial sniffing and stereo cues can guide olfactory search behavior. The sensory computations and movement strategies employed during navigation of an airborne odor plume are less clear. In previous experiments where rodents searched in airborne odor plumes, mice developed a memory-based strategy of serially sampling each possible reward location for the presence of odor, turning search tasks into detection tasks. (*Bhattacharyya and Singh Bhalla 2015; Gire et al. 2016*). Thus, it remains unclear whether mammals can follow noisy concentration gradients under turbulent conditions.

To better understand the sensory computations and sampling strategies for olfactory search, we designed a two-choice behavioral assay where mice use olfactory cues to locate an odor source while we monitor sniffing and movements of the head, nose, and body. We found that mice use a concentration gradient-guided search strategy to navigate olfactory environments that contain turbulent flow. We found that these navigational behaviors are robust to perturbations including introduction of a novel odorant, varying the concentration gradient, and naris occlusion. Given the fundamental importance of sniffing to olfactory function, we hypothesized that mice would selectively sample the environment such that nose movement would be tightly coupled to respiration. Consistent with this hypothesis, we found that mice synchronize rhythmic three-dimensional head movements with the sniff cycle during search. These sniff-synchronized movement rhythms are prominent during trials, and largely absent during the inter-trial interval, suggesting that sniff synchronous movement is a pro-active strategy rather than a reactive reflex. To find structure in this search strategy, we used unsupervised computational methods to parse movement trajectories into discrete motifs. These movement motifs are organized into two distinguishable behavioral states corresponding to investigation and approach, reminiscent of the two-state olfactory search programs described in smaller organisms. Further, these motifs lock to the sniff cycle with precision at a tens of milliseconds scale. Our findings reveal the microstructure of olfactory search behavior in mice, identifying sensory computations and movement strategies that are shared across a broad range of species.

Results

Olfactory search in noisy gradients of airborne odor

We developed a two-alternative choice task in which freely-moving mice report odor source location for water rewards (Methods and Fig. 1A). To capture the search behavior, we measured respiration using nasal thermistors (*McAfee et al. 2016*) and video-tracked the animal's body, head, and nose position in real time at 80 frames/s (Fig. 1B,E; Fig. S1). The mouse initiates a trial by inserting its nose in a port (Fig 1C; "Initiation"), which activates odor release from two ports at the opposite end of the arena. The mouse reports the location of higher odor concentration by walking toward it (Fig. 1C; "Search").

Figure 1. Behavioral assay for freely-moving olfactory search. A) Diagram of experimental chamber where mice are tracked by an overhead camera while performing olfactory search. B) Top. Nose & head position are tracked using red paint at the top of the head. Sniffing is monitored via an intranasally implanted thermistor. Bottom. Example of sniffing overlaid on a trace of nose position across a single trial. C) Diagram of trial structure. Initiation. Mice initiate a trial via an initiation poke (grey oval). Search. Odor is then released from both odor ports (grey rectangles) at different concentrations. Outcome. Mice that cross the decision line (red) on the side delivering the higher concentration as tracked by the overhead camera receive a reward at the corresponding water port (blue ovals). D) Color maps of average odor concentration across 15 two-second trials captured by a 7x5 grid of sequential photoionization detector recordings. Rows represent side of stimulus presentation (left or right). Odor concentration beyond the decision line were not measured. E) Comparison of sniff recordings taken with an intranasally implanted thermistor and intranasally implanted pressure cannula. These are implanted on the same mouse in different nostrils. Top. Example trace of simultaneous pressure cannula (blue) and thermistor (red) recordings with inhalation points (as detected in all future analyses) overlaid on the traces in their respective colors. Bottom Left. Histogram of peak latencies (pressure inhalation onset – thermistor inhalation onset). 14/301 inhalations (4.7%) were excluded as incorrect sniff detections. These were determined as incorrect, because they fell more than 2 standard deviations outside the mean in peak latency (mean = 1.61585 ms, SD = $\pm$ 14.93223 ms).

In previous studies, rodents performing olfactory search tasks developed memory-guided foraging strategies. In essence, animals run directly to potential odor sources and sample each in turn, thus converting the search tasks to detection tasks (*Bhattacharyya and Singh Bhalla 2015; Gire et al. 2016*). To prevent mice from adopting sample-and-detect strategies, our task forces mice to commit to a decision at a distance from the actual source. Using real-time video-tracking (*Lopes et al. 2015*), we enforced a virtual “decision line”, such that the trial outcome is determined by the mouse’s location when it crosses this decision line (Fig 1C; “Outcome”). For stimuli, we deliver odor from two separate flow-dilution olfactometers, giving independent control over odor concentration on the two sides. To test olfactory search over a range of difficulties, we presented four odor patterns, defined by the ratio of odor concentration released from the two sides (100:0, 80:20, 60:40, 0:0). We measured the spatiotemporal distribution of odor using a photoionization detector (PID) in a 5x7 grid of sampling locations (Fig 1D; Fig S2). Pinene was used for the majority of experiments, because it is a neutral-valence odorant that is sensitively detected by the PID. As designed, varying the concentration ratios produced across-trial averaged gradients of different magnitudes. Airflow in the arena is turbulent, imposing temporal fluctuations on the odor gradient (Supp Video 2). Thus, our assay tests an animal’s ability to navigate noisy odor gradients.

Mice learn the olfactory search task rapidly and robustly. We trained mice in the following sequence (Fig. 2A): First, naïve, water-restricted mice obtained water rewards from all ports in an alternating sequence (“water sampling”; Fig. S3A). In the next phase of training, we added odor stimulation such that odor delivery alternated in the same sequence as reward, so that the mice would learn to associate odor with reward ports (“odor association”; Fig S3B). Following these initial training steps, mice were introduced to the olfactory search paradigm. Odor was pseudo-randomly released from either the left or right odor source (“100:0”), signaling water availability at the corresponding reward port. Almost all mice performed above chance in the first session (binomial test, $p < 0.05$ for 24 out of 25 mice, $75 \pm 9.2\%$ correct, mean $\pm$ s.d.; Fig. 2B). Within 4 sessions, most animals exceeded 80% performance (19 out of 26). Following 100:0, mice were introduced to the 80:20 condition with mean performance across mice in the first session reaching 60% (Fig. S3D). Most subjects improved to exceed 70% performance over the next 7 sessions (17 out of 24). The mice that did not were excluded from subsequent experiments.

Next, we tested whether mice trained to search pinene plumes would generalize their search behavior to a novel odorant. We chose vanillin as the novel odorant, because, unlike pinene, vanillin does not activate the trigeminal fibers of the nose (*Cometto-Muniz and Abraham 2010; Doty et al. 1978; Hummel et al. 2009*). Thus we could test whether trigeminal chemosensation is necessary for performance in our task. We found no differences in performance between vanillin and pinene sessions for these mice (Wilcoxon rank-sum test, $p = 0.827$, Fig. 4A; $n = 3$). These data suggest that this search behavior generalizes across odors and does not rely on the trigeminal system.

Figure 1. Legend continued: E) Bottom Right. Peak latencies, defined as the difference between pressure inhalation onset and thermistor inhalation onset, plotted against instantaneous sniff frequency.

Figure 2. Mice use concentration gradient cues in turbulent flow to perform search. A) Initial training steps. Water Sampling. In this task, mice alternate in sequence between the initiation, left, and right nose pokes to receive water rewards. Odor Association. Next, mice run the alternation sequence as above with without water rewards released from the initiation poke, making its only utility to initiate a trial. Further, odor is released on the same side of water availability to create an association between odor and reward. Odor Search. Here, mice initiate trials by poking the initiation poke. Odor is then randomly released from the left or right odor port. Correct localization (see Fig. 1C, decision line) results in a water reward and incorrect is deterred by an increased inter-trial interval (ITI). B) Performance curve across sessions for the Odor Search (100:0) training step ($n = 26$). C-F) Session statistics for four different experiments. Each colored line is the average of an individual mouse across all sessions, black points are means across mice, and whiskers are ± 1 standard deviation across mice. Top. Percent of correct trials. Middle. Average trial duration. Bottom. Average path tortuosity (total path length of nose trajectory/shortest possible path length). C) Odor omission. The 80:20 concentration ratio (Fig. S1) and odor omission (0:0) conditions randomly interleaved across a session. Data shown includes all sessions for each mouse ($n = 19$). D) Variable ΔC , Constant $|C|$. Three concentration ratio conditions (100:0, 80:20, 60:40) randomly interleaved across a session. Data shown includes all sessions for each mouse ($n = 15$). E) Constant ΔC , Variable $|C|$. Concentration ratio conditions 90:30 and 30:10 randomly interleaved across a session ($n = 5$). Data shown for first session only. F) Naris occlusion. 80:20 sessions for mice with no naris stitch, a sham stitch that did not occlude the nostril, and a naris stitch that occluded one nostril ($n = 13$). Data shown includes all naris occlusion sessions, even if the mouse did not perform under every experimental condition.

Mice can use gradient cues in turbulent flow

We reasoned that mice would solve this task using odor gradient cues. To vary odor gradients between trials, we trained mice in sessions with interleaved concentration ratios (100:0, 80:20, 60:40) across the trials of a session. In addition to these concentration ratios, odor omission probe trials (0:0) were randomly interleaved into all experimental sessions. During these trials, airflow was identical to 80:20 trials, but air was directed through an empty vial rather than a vial containing odorant solution. These odor omission trials served a two-fold purpose: they acted as controls to ensure behavior was indeed odor-guided and they allowed us to observe how absence of odor impacts search behavior. On these probe trials, mice performed at chance (binomial test, $p=0.9989$), with longer trial durations (Wilcoxon rank-sum test, $p<0.05$) and more tortuous trajectories (Wilcoxon rank-sum test, $p<0.05$) than on non-probe trials (Fig. 2C; $n = 19$, all data from 80:20 condition with probe trials). Performance drops with the concentration ratio (ΔC), consistent with our reasoning that mice would use odor gradient cues in this task (pairwise Wilcoxon rank-sum tests, $p<0.05$; Fig. 2D; $n = 15$). Varying the concentration ratio from 80:20 to 60:40 did not affect trial duration or path tortuosity, defined as actual path length divided by direct path length (pairwise Wilcoxon rank-sum tests, $p>0.05$; Fig. 2D). However, trial duration and path tortuosity were slightly, but statistically significantly longer in the 100:0 condition (pairwise Wilcoxon rank-sum tests, $p<0.05$).

Given that these results were obtained using a single absolute concentration ($|C|$) across ratios, mice could be solving our task with two distinct categories of sensory computation. One possibility is that information about source location is extracted from the odor gradient. An alternative strategy would be to make an odor intensity judgement that gates a response to positional information from non-olfactory cues, such as wind direction, visual landmarks, or self-motion. This computation would be reminiscent of the odor-gated visual and mechanosensory behaviors observed in insects (*Álvarez Salvado et al. 2018*; *van Breugel and Dickinson 2014*; *Kennedy and Marsh 1974*). To distinguish between these possible strategies, we tested mice in sessions interleaving the air dilution ratios 90:30 and 30:10. 30 is the correct answer in one condition and incorrect in the other, so that mice cannot use an intensity judgement strategy to perform well in both ratio conditions. In both conditions, mice performed equally well in the first session of training (Wilcoxon rank-sum test, $p=0.465$; Fig. 2E; $n = 5$). This equal performance is true within the first 20 trials of the session (Wilcoxon rank-sum test, $p=0.296$; Fig. S4B). These results indicate that odor gradients guide olfactory search under these conditions.

We next asked how the mice are sensing the concentration gradient. Many mammals can use stereo-olfaction: comparing odor concentration samples between the nares (*Catania 2013*; *Parthasarathy and Bhalla 2013*; *Porter et al. 2007*; *Rabell et al. 2017*; *Rajan et al. 2006*). To test the role of stereo comparisons in our olfactory search task, we performed naris occlusion experiments. Mice were tested in three conditions on alternating days: naris occlusion, sham occlusion, and no procedure. We found that naris occlusion did not significantly impact performance or path tortuosity (pairwise Wilcoxon rank-sum tests, $p>0.05$). When compared with the no-stitch condition, the naris stitch condition resulted in a slight, but statistically significant, increase in trial duration (pairwise Wilcoxon rank-sum test, $p<0.05$). This is not true when the stitch condition is compared with the sham condition (pairwise Wilcoxon rank-sum test, $p>0.05$) indicating this may be a result of undergoing a surgical procedure. These overall results indicate that

Figure 3. Distributions of sniffs and nose positions during search task. A) Above. Sniff raster plot for three sessions. Each black point is an inhalation, each row is a trial aligned to trial initiation (dashed line). Rows are sorted by trial length. Below. Mean instantaneous sniff rate across all trials for all mice aligned to time from trial initiation. Thin lines are individual mice, the thick line is the mean across mice, and shaded region is ± 1 standard deviation. B) Histogram of inhalation duration time across all mice ($n = 11$). Thick lines and shaded regions are mean and ± 1 standard deviation, thin lines are individual mice. Green: within-trial sniffs, Pink: inter-trial interval sniffs. C) Histogram of sniff duration time across all mice ($n = 11$). D) The nose traces of each trial across a single session, colored by chosen side. E) Location of all inhalations across a single session, colored by chosen side. F) Two dimensional histogram of occupancy (fraction of frames spent in each 0.5 mm² bin). Colormap represents grand mean across mice ($n = 19$). G) Grand mean sniff rate colormap across mice ($n = 11$).

stereo comparison is not necessary in this task (Fig. 2F; $n = 13$), and that temporal comparisons across sniffs (Catania 2013; Parabucki et al. 2019) play a larger role under our task conditions.

Sniff rate and occupancy are consistent across trials and gradient conditions

To investigate active sampling over the time course of trials, we tracked the animals' sniffing, position, and posture during behavioral sessions. The overall sniff pattern was consistent across trials with an inhalation just before trial initiation followed by a long exhalation or pause at the beginning of the trial (Fig. 3A). Next, the mice emitted a rapid burst of sniffs, then sniffed more slowly as they approached the target (Fig. 3A). In this active behavioral state, inhalation and sniff durations were shorter during trials than during inter-trial intervals ($p \ll 0.01$ for all mice; Kolmogorov-Smirnov test; Fig. 3B,C), and strikingly shorter than those observed in head-fixed rodents (e.g., **Bolding and Franks 2017; Shusterman et al. 2011; Wesson et al. 2009**). During this sniffing behavior, the mice moved their nose through tortuous trajectories that were not stereotyped from trial to trial (Fig. 3D,E). Although individual mice showed position biases (Fig. S5), these biases were not systematic across mice, so that the across-mouse mean occupancy distribution was evenly distributed across the two sides of the arena (Fig. 3F; $n = 19$). Consistent with this sniffing and movement pattern, the sniff rate was highest near the initiation port, and slower on the approach to target (Fig. 3G). These measures of active sampling were not statistically distinguishable across gradient or naris occlusion conditions, but changed significantly on odor omission probe trials, with more fast sniffing and head turns overall.

Mice synchronize three-dimensional kinematic rhythms with sniffing during olfactory search

To test the hypothesis that nose movement locks to respiration during olfactory search, we aligned movement dynamics with the sniff signal. Using Deeplabcut (**Nath et al. 2019**), we tracked the position of three points: tip of snout, back of head, and center of mass (Fig. 4A). From the dynamics of these three points, we extracted the kinematic parameters nose speed, head yaw velocity, and Z-velocity (Fig. 4B-D). Synchrony between movement oscillations and sniffing is apparent on a sniff-by-sniff basis (Fig. 5), consistent across mice, and selectively executed during olfactory search. On average, nose speed accelerates during exhalation, peaks at inhalation onset, and decelerates during inhalation (Fig. 5A.i). Head yaw velocity, which we define as toward or away (Fig. 4; centripetal or centrifugal) from the body-head axis, reaches peak centrifugal velocity at inhalation, decelerates and moves centripetally over the course of inhalation (Fig. 5A.ii). Although our videos are in two dimensions, we can approximate movement in depth by analyzing the distance between the tip of the snout and the back of the head (Fig. 4B). This measure confounds pitch angular motion and vertical translational motion, so we conservatively refer to this parameter as "z-velocity". Because mice point their head downward during task performance, shortening of the distance between the tip of the snout and the back of the head indicates downward movement, while increases in the distance correspond to upward movements. The z-velocity reaches peak upward velocity at inhalation onset, decelerates and goes downward during inhalation, and rises again at exhalation (Fig. 5A.iii). These modulations were absent from trial-shuffled data (1000 shuffles; Fig. S6). Cross-correlation and spectral coherence analysis further demonstrate the synchrony between nose movement and sniffing (Fig. 5B,C). These results demonstrate that kinematic rhythms lock to sniffing with tens of millisecond precision, consistent with a previous re-

Figure 4. Quantifying kinematic parameters during olfactory search. A) Schematic of kinematic parameters. Left. Two example frames from one mouse, with the three tracked points marked: tip of snout, back of head, and center of mass. B) Quantified kinematic parameters: "nose speed": displacement of the tip of the snout per frame (12.5 ms inter-frame interval). "Yaw velocity": change in angle between the line segment connecting snout and head and the line segment connecting head and center of mass. Centrifugal movement is positive, centripetal movement is negative. "Z-velocity": change in distance between tip of snout and back of head. Note that this measure confounds pitch angle and Z-axis translational movements. C) Segments of example trajectories. Left. The trajectory of the nose during one second of trial time. Green: path during inhalations. Black: path during the rest of the sniff. Right. Same for an inter-trial interval trajectory. D) Traces of sniff and kinematic parameters during the time windows shown in C. Color scheme as in C.

port demonstrating that rats make similar movements during novel odor-evoked investigative behavior (Kurnikova *et al.* 2017). Our findings show that precise cycle-by-cycle synchronization can also be a feature of goal-directed odor-guided behavior.

Mice selectively deploy this pattern of sniff-synchronized three-dimensional nose movement. For nose speed, yaw velocity, and z-velocity, sniff synchrony is significantly reduced

Figure 5. Kinematic rhythms synchronize with the sniff cycle selectively during olfactory search. i-iii) Nose speed, yaw velocity, and z-velocity respectively (see Fig. 4 for definitions). A) Top. Color-plot showing movement parameter aligned to inhalation onset for within-trial sniffs taken before crossing the decision line. Taken from one mouse, one behavioral session. Dotted line at time 0 shows inhalation onset, the second line demarcates the end of the sniff cycle, sorted by duration. Data are taken from one behavioral session. Middle. Color-plot showing each movement parameter aligned to inhalation onset for inter-trial interval sniffs taken before the first attempt at premature trial initiation. Bottom. Sniff-aligned average of each movement parameter. Thin lines represent individual mice ($n = 11$), bolded lines and shaded regions represent the grand mean $\pm$ standard deviation. Green: within-trial sniffs, Pink: inter-trial interval sniffs. B) Normalized cross correlation between movement parameter and sniff signal for the same sniffs as above. C) Spectral coherence of movement parameter and sniff signal for the same sniffs as above.

during the inter-trial interval when the mouse is returning from the reward port to initiate

the next trial, even when the mouse is sniffing rapidly. Modulations in nose speed were slightly different than trial-shuffled data, showing that sniff-synchronized movement is not totally absent during the inter-trial interval, whereas modulations in yaw velocity and z-velocity were indistinguishable from trial-shuffled data (Fig. S6). This reduction of kinematic synchrony when the mouse is not performing the task suggests that sniff-synchronized movement is not an inevitable biomechanical accompaniment to fast sniffing, but rather reflects a strategic behavioral state. Further support for this idea comes from analyzing time intervals when the mouse attempts to initiate a trial before the end of the inter-trial interval. After such premature attempts at trial initiation, the mice execute sniff-synchronized movement, despite the absence of the experimenter-applied odor stimulus (Fig. S7). Lastly, sniff synchrony changes dramatically in the time interval between crossing the virtual decision line and entering the reward port, when odor is still present yet the animal has committed to a decision (Fig. S7). Taken together, our observations indicate that sniff synchronous movement is a proactive, odor-seeking strategy rather than a reactive, odor-gated reflex.

State space modeling finds recurring motifs that are sequenced diversely across mice

In our olfactory search paradigm, the overall rhythm of nose movement synchronizes with sniffing (Fig. 4; Fig. 5), and yet the mice move through a different trajectory on every trial (Fig. 3D). Given this heterogeneity, it was not obvious to us how to best quantify common features of movement trajectories across trials and subjects. Rather than guess at suitable features, we used an unsupervised machine learning tool, modeling the movement data with an auto-regressive hidden Markov model (AR-HMM) (Murphy 2012; Poritz 1982). This model parses continuous sequential data into a discrete set of simpler movement motif sequences, similarly to “Motion Sequencing” (MoSeq) (Wiltschko et al. 2015). We fit AR-HMMs to the allocentric three-point coordinate data (front of snout, back of head, and center of mass) pooled across a subset of mice and trial conditions (See Methods and Fig 2; e.g. 80:20, 90:30, nostril stitch). Models were then tested for their ability to explain a separate set of held-out trials (see Methods). These models defined discrete movement patterns, or “motifs”, that recur throughout our dataset (e.g., Fig. 6A). We fit different AR-HMM models each constrained to find a particular number of motifs (between 6 and 100) and found that the cross-validated log-likelihood of these fits continued to rise up to 100 motifs (Fig. S8). For visualization, we will focus on a model with 16 states, which we narrow to 11, by excluding rare motifs that take up <5% of the assigned video frames (Fig. 6B; Fig. S8C,D). Models with more or fewer states gave equivalent results (Fig. S9 - Fig. S11).

The motifs extracted by this model have interpretable spatiotemporal trajectories on average (Fig. 6B; Supp Video 6), although averaging masks considerable across-instance variability (Supp Video 7). Across trials for a given mouse, motifs occurred in consistent but non-stereotyped sequences (Fig. 6C; Supp Video 5). Across mice, these sequences appeared visually similar, but most mice were uniquely identifiable from how they sequenced motifs across trials. A classifier trained to decode mouse identity from the motif sequences on a trial by trial basis was able to perform above chance for 8 out of 9 mice ($p < 0.01$; Fig. 6D). Across the different concentration ratios (Fig. 2D), movement sequences were not statistically distinguishable (Fig 6E). The only condition that gave distinguishable

Figure 6. Recurring movement motifs are sequenced by our model diversely across mice and consistently across stimuli. A) 8 example frames from one instance of a behavioral motif with tracking overlaid. B) Average motif shapes. Dots and lines show the average time course of posture for 8 frames of each of the 11 motifs ($n = 9$ mice). All instances of each motif are translated and rotated so that the head is centered and the head-body axis is oriented upward in the first frame. Subsequent frames of each instance are translated and rotated the same as the first frame. Time is indicated by color (dark to light). Background color in each panel shows the color assigned to each motif. C) Across-trial motif sequences for two behavioral sessions for one mouse. Trials are separated into trials where the mouse chose left and those in which the mouse chose right. Trials are sorted by duration. Both correct and incorrect trials are included. Color scheme as in B. D) Linear classifier analysis shows that mice can be identified from motif sequences on a trial by trial basis. Grayscale represents the fraction of trials from a given mouse (rows) that are decoded as belonging to the data of a given mouse (columns). The diagonal cells represent the accuracy with which the decoded label matched the true label, while off-diagonal cells represent trials that were mislabeled by the classifier. Probabilities along rows sum to 1. Cells marked with asterisks indicate above chance performance (label permutation test, $p < 0.01$). E) Linear classifier analysis identifies odor omission trials above chance, but does not discriminate across odor concentration ratios ($n = 9$ mice).

motif patterns were the odor omission trials (0:0), in which the mice made longer, more tortuous trajectories (Fig. 2C). Thus, although this model is sensitive enough to decode mouse identity (Fig. 6E), it does not detect stimulus-dependent modifications of sampling behavior, suggesting that the mice do not modify their sampling behavior in a gradient-dependent manner, at least in the movement parameters we measured.

Movement motifs reveal two-state organization of olfactory search

Many behaviors have hierarchical structure that is organized at multiple temporal scales. Brief movements are grouped into progressively longer modules, and are ultimately assembled into purposive behavioral programs (*Berman et al. 2016; Gallistel 1980; Tolman 1932; Weiss 1968*). Olfactory search programs in smaller organisms are often organized into two overarching states: move straight when concentration is increasing, and reorient when concentration is decreasing (*Bargmann 2006; Berg 2000; Lockery 2011; van Breugel and Dickinson 2014; Kennedy and Marsh 1974; Vickers and Baker 1994; Gomez-Marin et al. 2011*). We hypothesized that olfactory search motifs in mice are organized similarly. To reveal higher-order structure in the temporal organization of these motifs, we applied a clustering algorithm that minimizes the Euclidean distance between rows of the Markov transition matrix (*i.e.*, purely based on the conditional probabilities of motifs following them). This clustering separated motifs into two groups (Fig. 7A), with several distinct properties. These properties were present in models with more or fewer states (Fig. S9 - Fig. S11). Based on these differences (see below), we label these groups as putative “investigation” and “approach” states. First, investigation and approach motifs cluster their onset times in the trial, with investigation motifs tending to occur early in the trial, while approach motifs tend to begin later (Fig. 7B). Grouping motifs into these higher-order states shows a consistent trial sequence, with trials beginning with investigation and ending with approach (Fig. 7C,D). Importantly, entering the approach state is not a final, ballistic commitment to a given water port – switches from approach back to investigation were common (Fig. 7C,D; Supp Video 8). This pattern suggests that the mice are continuously integrating evidence about the odor gradient throughout their trajectory to the target. Second, these states correlated with distinct sniff rates and movement speeds. During investigation motifs, the mice moved more slowly and sniffed more rapidly, whereas the approach states were associated with faster movement and slower sniffing (Fig. 7E). Third, the sniff-synchronized kinematic rhythms (Fig. 4; Fig. 5) were distinct in the two states (Fig. 7F; Kolmogorov-Smirnov test, $p < 0.01$). Given the consistent sequence from investigation to approach and given that mice sniff faster during the early part of trial, these differences in kinematic parameters could reflect across-trial tendencies instead of within-trial synchrony. To test this possibility, we calculated the Kolmogorov-Smirnov statistic, which quantifies the difference between two cumulative distributions, for real and trial-shuffled data (S12). This analysis showed that nose speed and yaw velocity modulation exceeded what would be expected from across-trial tendencies (1000 shuffles, $p < 0.001$), while the z-velocity modulation did not ($p = 0.31$). Switches between the investigation and approach state mark behavioral inflection points that can be identified from trial to trial. We reason that these behavioral inflection points are a signature of key moments in the mouse’s evolving decision process. Thus, our analysis can provide a framework for temporal alignment of diverse movement trajectories with simultaneously-recorded physiological data (*Markowitz et al. 2018*).

Figure 7. Behavioral motifs can be categorized into two distinct groups, which we putatively label as investigation (blue) and approach motifs (orange). A) Transition probability matrix. Grayscale represents the log probability with which a given motif (rows) will be followed by another (columns). Clustering by minimizing Euclidean distance between rows reveals two distinct blocks of motifs. We label the top-left block as “investigation” and the bottom-right block as “approach”. B) Distribution of onset times for each motif, normalized by trial duration. Investigation motifs tend to occur early in trials, while approach motifs tend to occur later ($n = 9$ mice). C) Across-trial motif sequences for two behavioral sessions for one mouse, with motifs classified into investigation and approach. Trials are separated into trials where the mouse chose left and those in which the mouse chose right. Both correct and incorrect trials are included. Motif sequences are sourced from the same data as Fig. 6C. D) Temporal details of investigation-approach transitions with overlaid sniff signal. Data come from a subset of trials shown in Fig. 7C. In the sniff signal, green represents inhalations, black represents the rest of the sniff. E) Investigation and approach motifs differ in nose speed and sniff rate. Individual markers represent one motif from one mouse. Marker shapes correspond to the individual mice ($n = 4$). Sniff rate and nose speed are normalized within mice. F) Investigation and approach motifs differ in the kinematic rhythms (same parameters as in Figures 4 & 5). Thin lines represent individual mice ($n = 4$), thick lines and shaded regions represent the grand mean $\pm$ standard deviation. Blue: within-trial sniffs, orange: inter-trial interval sniffs. Top. Nose speed modulation, defined by a modulation index ($(\text{max speed} - \text{min speed})/(\text{max} + \text{min})$) calculated from the grand mean, is significantly greater for

Investigation motif onsets are precisely locked to sniffing

If motif transitions correspond to relevant behavioral events, their temporal structure should correlate with the temporal structure of neural activity (*Markowitz et al. 2018*). During fast sniffing, respiration matches with the rhythms of head movement (Fig. 4; Fig. 5), whisking, and nose twitches (*Kurnikova et al. 2017; Moore et al. 2013; Ranade et al. 2013*). These motor rhythms correlate with activity in numerous brain regions, including brainstem, olfactory structures, hippocampus, amygdala, and numerous neocortical regions (*Karalis and Sirota 2018; Kay 2005; Macrides et al. 1982; Vanderwolf 1992; Yanovsky et al. 2014; Zelano et al. 2016*). We hypothesized that movement motifs would lock with these behavioral and neural rhythms, so we aligned sniff signals with motif onset times. Importantly, the breath signal was not input to the model. This alignment revealed a striking organization of motif sequences relative to the sniff rhythm. For example, the onset times of motif 6 (dark blue) occurred in a precise timing relationship with sniffing (Fig. 8A). To visualize the timing relationship between onsets of all motifs and sniffing, we calculated the equivalent of a peri-stimulus time histogram for inhalation times relative to the onset time of each motif, and took the grand mean across all mice ($n = 4$; Fig. 8B). Further, to determine how motif onset times are organized relative to the sniff cycle, for each motif we calculated a histogram of motif onset in sniff phase coordinates (relative position in the sniff cycle; Fig. 8C). Sharp peaks are apparent in both histograms for investigation motifs, and less so for approach motifs (quantified below; Fig. 8B,C). Importantly, these timing relationships are consistent across mice, with some motifs tending to occur early in the sniff cycle during inhalation, and others occurring later in the sniff cycle (Fig. 8D). Thus, parsing diverse movement trajectories into sequences of recurring movement motifs reveals additional sniff-synchronized kinematic structure in a consistent manner across mice.

Are motif onsets timed with respect to inhalation times, or do they coordinate with the entire sniff cycle? In other words, is motif onset probability more modulated in time or phase? To quantify the sniff synchronization of motif onset times, we calculated a modulation index ($MI = (max - min)/(max + min)$) for each motif's across-mouse mean histogram ($n = 4$). To test whether these trial-by-trial modulation indices exceeded what would be expected from across-trial tendencies, we compared real and trial-shuffled data (Fig. S13). All investigation motifs were significantly modulated for both time and phase coordinates (filled symbols, Fig. 8E; permutation test, $p < 0.001$), with some having higher MI in time, and others in phase. One approach motif was significantly modulated in time coordinates (Fig. 8E, right-half filled symbol; $p = 0.003$), while two approach motifs were significantly modulated in phase coordinates (Fig. 8E, left-half filled symbols; $p = 0.015$ and $p < 0.001$). Comparing the modulation indices between time and phase coordinates does not reveal a consistent pattern of modulation in time vs phase – some motifs had higher MI in phase, others in time. Thus, our data are inconclusive as to how motif onsets

Figure 7. Legend continued: investigation motifs than approach motifs ($p < 0.001$, permutation test; Supp Fig 12). Middle: Yaw velocity modulation is significantly greater for investigation motifs than approach motifs ($p < 0.001$, permutation test; Supp Fig 12). Bottom: Z-velocity modulation does not significantly differ between approach motifs and investigation motifs ($p = 0.31$, permutation test).

Figure 8. Motif onsets synchronize to the sniff cycle. A) Alignment of the sniff signal to an example motif. Top. Color scheme shows sniff cycles aligned to the onsets of motif 6 (blue). Motif instances are in chronological order. Green: inhalation, black: rest of sniff. Bottom. Peristimulus time histogram of inhalation times aligned to the onset of motif 6. B) Alignment of sniff signal to onset times of all motifs across mice ($n = 4$). Colormap represents the grand means for peristimulus time histograms of inhalation times aligned to the onset of motifs. C) Alignment of motif onset times in sniff phase. Colormap represents peristimulus time histograms of motif onsets (bin width = 12.5 ms) times aligned to inhalation onset, with all sniff durations normalized to one. Dotted line shows the mean phase of the end of inhalation. D) Motifs alignment to sniff phase is consistent across mice. Thin lines represent individual mice, black points are means, and whiskers are ± 1 standard deviation ($n = 4$ mice). E) Investigation motifs are more synchronized to the sniff cycle than approach motifs. Dots represent the modulation index in time on the x coordinates and in phase on the y-coordinates. Filled dots represent motifs that are significantly modulated in both time and phase ($p < 0.01$, permutation test). Half-filled dots represent motifs that are significantly modulated in time (left half filled) or phase (right half filled).

organize relative to the sniff cycle. Nevertheless, these analyses demonstrate that kinematic inflection points synchronize with breathing during olfactory search. Given that breathing synchronizes to other motor and brain rhythms, these motifs likewise correlate to the structure of activity of many neurons. We propose that the behavioral inflection points revealed by motif transition times indicate “decisive moments” when the animal chooses its next move. Thus our analysis will be a useful tool to pinpoint behaviorally relevant activity in widespread brain regions.

Discussion

This study elucidates sensory computations and movement strategies for olfactory search by freely-moving mice. Mice learn our behavioral task in days, after which they perform approximately 150 trials daily, sometimes for months. Task performance worsens for

shallower odor gradients at a fixed absolute concentration, but is unaffected by varying absolute concentrations at a fixed concentration gradient. Taken together, these results show that mice can navigate noisy gradients formed by turbulent odor plumes. This gradient-guided search is robust to perturbations including novel odorant introduction and naris occlusion. These results give insight into sensory computations for olfactory search and constrain the possible underlying neural mechanisms.

Mice perform this task with a strategic behavioral program. During search, mice synchronize rapid three-dimensional head movements with fast sniffing. This synchrony is not a default accompaniment of fast sniffing – synchrony is absent when the mice are not searching. Movement trajectories are not stereotyped, but vary considerably across trials. To manage this complexity, we took an unsupervised computational approach to parse heterogeneous trajectories into a small number of movement motifs that recur across trials and subjects. This analysis captures common movement features across mice, but individual mice can be identified by how they sequence these motifs. Our model was not constrained to find structure at a specific timescale, and consequently identified very brief, simple motifs. To find higher-order temporal structure in the data, we clustered motifs by their transition probabilities, which revealed two clear categories, putatively corresponding to investigation and approach. Investigation motifs tend to be executed early in the trial, and entail slower movement, faster sniffing, and more sniff synchrony than approach motifs. Even so, approach motifs are not ballistic commitments to an answer – switches from approach to investigation occurred on many trials. Lastly, the onset times of motifs were precisely locked to sniffing, with investigation motifs starting at characteristic phases of the sniff cycle. Taken together, the discrete categorization of movement motifs and their precise alignment with the sniff cycle provide a robust framework for the temporal alignment of behavior and neural activity.

Olfactory navigation can be either guided or gated by odor (*Baker et al. 2018*). Some organisms operate in a regime where diffusion forms smooth chemical gradients, in which classical chemotaxis strategies can be effective (*Bargmann 2006; Berg 2000; Gomez-Marín and Louis 2012; Lockery 2011*). In contrast, other organisms, such as flying insects, often operate in a highly turbulent regime where concentration gradients are not reliably informative (*Crimaldi et al. 2002; Murlis et al. 1992; Riffell et al. 2008*). By design, mice in our task operate in an intermediate regime, where turbulent odor plumes close to the ground form noisy gradients (*Gire et al. 2016; Riffell et al. 2008*). By varying the absolute concentration and the concentration difference between the two sides, we tested whether performance in this regime is guided or gated by odor. Because behavior varies with the gradient and not the absolute concentration (Fig. 2C-E), we have shown that mice are guided by gradient cues in this regime.

Our naris occlusion experiments demonstrate that performance is statistically indistinguishable with naris occlusion, suggesting that stereo olfaction does not play a major role in our task. This finding contrasts with previous studies of olfactory navigation in a different regime: following a depositional odor trail. In these studies, stereo manipulations had small but significant effects on performance, and led to changes in movement strategy (*Jones and Urban 2018; Khan et al. 2012*). Importantly, a study of olfactory search in moles showed that stereo reversal did not affect navigation at a distance from the target, but reversed turning behavior in the target's immediate vicinity (*Catania 2013*). These results suggest that stereo cues may be informative near a source, where gradients are

steep, but that stereo cues play less of a role at a greater distance from the source where gradients are more shallow. In this more distant condition, serial sniff comparisons have been hypothesized as a potential sensory computation for odor gradient following (*Catania 2013*). We propose that our task design, in which mice must commit to a side at a distance from the source, forces mice out of the stereo regime, and perhaps into the serial sniff comparison regime. Neurons sensitive to sniff-to-sniff odor concentration changes have been observed in the olfactory bulb of head-fixed mice (*Parabucki et al. 2019*), providing a potential physiological mechanism for this sensory computation.

On the other hand, physiological mechanisms revealed in head-fixed mice may not generalize to the freely-moving search condition. The external stimulus obtained by moving the nose through a noisy gradient differs dramatically from the square odor pulses delivered during head-fixed or odor-poke olfactory tasks. Further, the sniff statistics we observe in our mice are qualitatively faster than those reported in head-fixed mice under most conditions (*Bolding and Franks 2017; Shusterman et al. 2011; Wesson et al. 2009*). One exception is that mice sniff fast in response to a novel odor (*Wesson et al. 2009*). Such fast stimulation impacts the responsiveness of olfactory sensory neurons (*Verhagen et al. 2007; Esclassan et al. 2012; Ghatpande and Reisert 2011*). In addition to the temporal properties of odor transduction, short- and long-term synaptic and network plasticity mechanisms will influence the olfactory bulb's responses during fast sniffing (*Beshel et al. 2007; Zhou et al. 2020; Díaz-Quesada et al. 2018; Gupta et al. 2015; Patterson et al. 2013; Jordan et al. 2018; Mandairon and Linster 2009*). Without tapping into this stimulus regime, the understanding we can gain from head-fixed studies in olfaction will be incomplete at best. In the future, it will be necessary to complement well-controlled reductionist behavioral paradigms with less-controlled, more natural paradigms like ours.

Mice execute a strategic behavioral program when searching, synchronizing fast sniffing with three-dimensional head movements at a tens of milliseconds timescale. It has long been known that rodents investigate their environment with active sniffing and whisking behaviors (*Kepecs et al. 2006; Wachowiak 2011; Welker 1964*). More recent work has established that under some conditions sniffing locks with whisking, nose twitches, and head movement on a cycle-by-cycle basis (*Kurnikova et al. 2017; Moore et al. 2013; Ranade et al. 2013*). Sniffing also synchronizes with brain oscillations not only in olfactory regions, but also in hippocampus, amygdala, and neocortex (*Karalis and Sirota 2018; Kay 2005; Macrides et al. 1982; Vanderwolf 1992; Yanovsky et al. 2014; Zelano et al. 2016*). Respiratory central pattern generators may coordinate sampling movements to synchronize sensory dynamics across modalities with internal brain rhythms (*Kleinfeld et al. 2014*). Further, locomotor and facial movement, which are often synchronized to respiration, drive activity in numerous brain regions, including primary sensory areas (*McGinley et al. 2015; Musall et al. 2019; Niell and Stryker 2010; Stringer et al. 2019*). Why are respiration and other movements correlated with activity in seemingly unrelated sensory regions? In the real world, sensory receptors operate in closed loop with movement (*Ahissar and Assa 2016; Gibson 1966*). Consequently, sensory systems must disambiguate self-induced stimulus dynamics from changes in the environment. Further, active sampling movements can provide access to sensory information that is not otherwise available to a stationary observer (*Gibson 1962; Schroeder et al. 2010; Yarbus 1967*). Widespread movement-related signals may allow the brain to compensate for and capitalize on self-induced stimulus dynamics (*von Holst and Mittelstaedt 1950; Poulet and*

Hedwig 2006; Sommer and Wurtz 2008; Sperry 1950; Webb 2004). Our work advances understanding of how sensation and movement interact during active sensing.

Rigorously quantifying the behavior of freely-moving animals is more feasible than ever, thanks to recent developments in machine vision, deep learning, and probabilistic generative modeling (*Datta et al. 2019; Gomez-Marin et al. 2014; Mathis and Mathis 2020*), as our work shows. In particular, the motifs we have defined provide a compact description of the behavior, while still capturing the idiosyncrasies of individual mice. Importantly, these motifs can be grouped into two larger-scale behavioral states that we putatively call “investigation” and “approach”. Two-state search strategies are common across phylogeny. (*Bargmann 2006; Berg 2000; Lockery 2011; van Breugel and Dickinson 2014; Kennedy and Marsh 1974; Vickers and Baker 1994*). In smaller organisms, state switches have provided a useful behavioral readout for understanding the neural mechanisms of odor-guided behavior (*Baker et al. 2018; Bi and Sourjik 2018; Larsch et al. 2015*). In mice, switches between ‘investigation’ and ‘approach’ may be similarly revealing. The transition points between ‘investigation’ and ‘approach’ serve as a principled template against which to compare neural activity. Our work thus establishes a framework for studying neural mechanisms of active sensing in an unrestrained mammal.

Methods and Materials

Animals: Housing & Care

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oregon and are compliant with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. C57bl/6j mice (2-14 months old) from the Terrestrial Animal Care Services (TeACS) at University of Oregon (19 male, 7 female) were used for behavioral experiments. Mice were housed individually in plastic cages with bedding & running wheels provided by TeACS. Mice were fed standard rodent chow ad libitum, and were water restricted, receiving a daily allotment (1mL - 1.5mL) of acidified or chlorinated water. Animal health was monitored daily, and mice were taken off water restriction if they met the ‘sick animal’ criteria of a custom IACUC-approved health assessment.

Behavioral Assay Design

Arena & Task Structure

Mice were trained to perform a two-choice behavioral task where they must locate an odor source for a water reward. This 15x25cm behavioral arena was largely custom designed in lab (all designs available upon request). The behavioral arena contains a custom designed and 3D-printed honeycomb wall through which continuous clean air is delivered to the arena and a latticed wall opposite to the honeycomb allowing airflow to exit the arena. Two odor tubes (Cole-Parmer Instrument Company, #06605-27) are embedded inside the honeycomb wall and consistently deliver either clean or odorized air. There are three nose pokes in the arena: one trial initiation poke and two reward pokes. The initiation poke is embedded inside the latticed wall (where airflow exits) and is poked to initiate trials. The left and right reward pokes are embedded in the left and right arena walls against the honeycomb airflow delivery and are used for water reward delivery. Mice initiate odor release by entering the initiation poke. If the mouse locates

the odor source successfully (by entering the quadrant of the arena containing the correct odor port), water (6 μ L - 8 μ L) is available at the corresponding nose poke. An intertrial interval of 4 seconds is then initiated. If the mouse goes to the incorrect side, water is not made available and they must wait an increased intertrial interval of 10 seconds.

Odor Delivery

Odor is delivered to the arena using two custom designed and built olfactometers. For a single olfactometer, air and nitrogen are run through separate mass flow controllers (MFCs) (Alicat Scientific, #MC-100SCCM-RD) that can deliver 1000mL/min and 100mL/min at full capacity respectively. We can use these MFCs to control the percentage of total nitrogen flow (100mL/min) that runs through liquid odorant. Consequently, we can approximately control the amount of odor molecules in the resulting odorized air stream. Total flow is maintained at 1000mL/min (for example, if we are delivering 80mL/min of nitrogen, we will deliver 920mL/min of air). Nitrogen MFC output is directed through a manifold (NResearch Incorporated, #225T082) with embedded solenoids that direct flow to one of four possible vials. These vials contain odorant diluted in mineral oil or are empty. To odorize air, nitrogen is directed through a vial containing liquid odorant. The nitrogen aerosolizes the odorant and combines with airflow MFC output at the exit point of the manifold. If nitrogen is directed through an empty vial, un-odorized nitrogen will combine with airflow at the exit point. The resulting combined flow of air and nitrogen is then directed to a final valve (NResearch Incorporated, #SH360T042). Odorized air continuously runs to exhaust until this final valve is switched on at which point clean air is directed to exhaust and odorized air to the behavioral assay. Therefore, we can control the percentage of odorized flow (using the MFCs), the presence or absence of odorized flow (using the vials & solenoids), and the flow of odorized air to the assay (using the final valve). There are two olfactometers (one for each odor port), which are calibrated weekly to match outputs using a photoionization detector (PID).

Video tracking

We use a Pointgrey Fly Capture Chameleon 3.0 camera (FLIR Integrated Imaging Solutions Inc, #CM3-U3-13Y3C) for video tracking. We capture frames at 80Hz at 1200x720 pixel resolution. All real-time tracking is executed using a custom Bonsai program. We isolate the mouse's centroid by gray scaling a black mouse on a white background and finding the center of the largest object. We track head position by applying red paint on the mouse's implant between the ears and thresholding the real-time HSV image to identify the center of the largest red shape. We can then identify nose position by calculating the extremes of the long axis of the mouse shape and isolating the extreme in closer proximity to the head point. These three points are sent to python at 80 Hz for real-time tracking in our assay. We use this real-time tracking to determine successful odor localization; if the mouse enters the quadrant of the arena that contains the correct odor port, it has answered correctly. Bonsai is an open source computer vision software available online ([Lopes et al. 2015](#)), and our custom code is available upon request.

For more rigorous behavioral analysis, we increased our tracking accuracy by using the open source tracking software Deeplabcut ([Mathis and Mathis 2020](#)). All Deeplabcut tracking occurred offline following experimentation.

Sniff Recordings

We record sniffing using intranasally implanted thermistors (TE Sensor Solutions, #GAG22K7MCD419; see Methods: Surgical Procedures). These thermistors are attached to pins (Assmann WSW Components, #A-MCK-80030) that can be connected to an overhead commutator (Adafruit, #736) and run through a custom-built amplifier (Texas Instruments, #TLV2460, amplifier circuit design available upon request).

Software

All behavioral experiments were run using custom code in Python, Bonsai, and Arduino. Behavioral boards designed at Janelia Research Farms that use Arduino software and hardware were used to control all hardware. Bonsai was used to execute real-time tracking of animals, and Python was used to run the assay, communicate with Arduino and Bonsai, and save data during experiments. All programs used are open source, and all custom code is available upon request.

Surgical Procedures

For all surgical procedures, animals were anesthetized with 3% isoflurane; concentration of isoflurane was altered during surgery depending on response of the animal to anesthesia. Incision sites were numbed prior to incision with 20mg/mL lidocaine.

Thermistor Implantation

To measure respiration during behavior, thermistors were implanted between the nasal bone and inner nasal epithelium of mice. Following an incision along the midline, a small hole was drilled through the nasal bone to expose the underlying epithelium 2mm lateral of the midline in the nasal bone. The glass bead of the thermistor was then partially inserted into the cavity between the nasal bone and the underlying epithelium. Correct implantation resulted in minimal damage to the nasal epithelium. The connector pins were fixed upright against a 3cm headbar (custom designed and 3D printed) placed directly behind the animals' ears and the thermistor wire was fixed in place using cyanoacrylate. The headbar was secured against a small skull screw (Antrin Miniature Specialties, #B002SG890I) implanted above cerebellum. A second skull screw was placed at the junction of the nasal bones to secure the anterior portion of the implant. All exposed skull and tissue were secured and sealed using cyanoacrylate. At the end of surgery, a small amount of fluorescent tempera red paint (Pro Art, #4435-2) was applied to the center of the headbar for tracking. Immediately following surgery, animals received 0.1mg/kg buprenorphine followed by 3 days of 0.03mg/kg ketoprofen. All but 9 mice were implanted prior to training. Mice that were implanted post-training were taken off water restriction at least 2 days prior to surgery and were not placed back on water restriction for at least one week following all analgesic administration.

Naris Occlusion

To test the necessity of stereo olfaction as a sampling strategy, we occluded the nostrils of C57BL/6J mice using 6-0 gauge surgical suture (SurgiPro, #MSUSP5698GMDL). Mice were given 0.03mg/kg ketoprofen and topical lidocaine on the nostril prior to induction. Suture was either pulled through the upper lip of the nostril and maxillary region to fully occlude the desired nostril or looped at the upper lip of the nostril for a sham stitch.

Commercially available VetBond was applied to protect the suture knot. To ensure full occlusion, a small water droplet was placed on the occluded nostril. The absence of bubbles or seepage indicated a successful occlusion. Occlusion was re-tested in the same manner directly before each experimental session. All stitches were removed within a week of application, and animals were stitched a total of 3 times per nostril.

Behavioral Training

All mice were trained to locate an odor source from one of two possible sources in the olfactory arena. Mice were removed from training and future experiments if they lost sniff signal or did not exceed 50 trials/perform above 60% correct in 15 sessions. The training process was divided into four primary stages.

Water Sampling

Mice were trained to alternate between the three pokes in the behavioral arena. Water (5 - 8 μ L) was made available at the nose pokes in the following order: initiation port, left reward port, initiation port, right reward port (repeat). Mice were trained in this task for 30 minutes per session until the mouse completed 70 iterations. This took mice 2-9 sessions. Data are only shown for 19 mice, because earlier iterations of the system did not save training data.

Odor Association

Mice were trained in the same sequence as water sampling. However, in odor association, water availability was removed from the initiation poke, and odor was released from whichever side water was available. Therefore, the mouse must initiate water availability by poking the initiation poke and then is further guided to the correct reward port by odor release. This task taught mice to initiate trials using the initiation poke and to associate odor with reward. However, in this step, odor is not required for reward acquisition as the task alternates left and right trials. Mice were trained in this task for 30 minutes per session until the mouse completed 70 iterations. This took mice 1-5 sessions. Data are only shown for 19 mice, because earlier iterations of the system did not save training data.

100:0

Mice were given the same task as odor association, but with odor now randomly being released from the left or right odor port following an initiation poke. 10% of these trials were randomly 0:0 condition trials. To correctly answer, animals had to enter the quadrant of the arena (as tracked by the overhead camera) where odor was being released. If they answered correctly, water was made available at the reward port on the corresponding side. If they answered incorrectly, water was not made available and the mouse received an increased intertrial interval. Mice were trained in this task for 40 minutes per session until they exceeded 80% accuracy, which took 1-4 sessions ($n = 26$).

80:20

When trials were initiated in this task, odor was released from both odor ports, but at differing concentrations. The animal had to enter the quadrant containing the odor port releasing the higher concentration. In this case, 80 means that the nitrogen MFC was set to 80mL/min on one olfactometer (see Methods: Behavioral Assay). Therefore, one odor

port would release roughly 80% of the total possible odorant concentration. If one olfactometer was set to 80, then the other olfactometer would be set to 20 in this condition. 10% of these trials were randomly 0:0 condition. Mice were trained in this task for 40 minutes per session, taking 1-9 sessions to exceed 60% performance ($n = 24$).

Behavioral Experiments

Variable ΔC , Constant $|C|$

This experiment tested how performance and sampling strategy changes with task difficulty. In this experiment, mice performed a two-choice behavioral task where they located an odor source for a water reward at varying concentration differences between the two ports. This experiment interleaved several possible conditions: 100:0 (all odor released from one port or the other), 80:20 (odor is released from both ports at different concentrations: 80% of the total possible airborne concentration & 20% of the total possible airborne concentration), and 60:40 (60% and 40%). Additionally, there was a control condition where all system settings were the same as the 80:20 condition, but nitrogen flow was directed through a clean vial so that the final flow was not odorized. 10% of the total number of trials were the 0:0 condition. Mice ran 40 minute experimental sessions and totaled 5-50 sessions ($n = 19$). These experiments were run with 1% liquid dilution of pinene.

Novel Odorant

This experiment tested how mice generalized our olfactory search task. A subset of mice were run with 1% liquid dilution of vanillin, which, unlike pinene, does not activate the trigeminal system ($n = 3$).

Constant ΔC , Variable $|C|$

This experiment tested if the animals use a thresholding strategy based on a fixed concentration threshold to solve the localization task. We ran this experiment using air dilution delivering the concentration ratios 90:30 and 30:10 interleaved randomly ($n = 5$). Mice ran 40 minute sessions and we analyzed data from the first session.

Naris Occlusion

This experiment tested the necessity of stereo olfaction in our localization task. Mice were run in the interleaved experiment (see above) initially. However, after observing no differences between concentration groups, we continued this experiment running mice in the 80:20 and 0:0 conditions only. Mice were run in one of five categories: left occlusion, left sham stitch, right occlusion, right sham stitch, and no stitch (see Methods: Surgical Procedures). Mice ran 40 minute experimental sessions and totaled 5-30 sessions ($n = 13$). Stitches were always removed after 4 days. These experiments were run using 1% pinene dilutions.

Mapping the Olfactory Environment

We used a photoionization detector (PID, Aurora Scientific Inc, #201A) to capture real time odor concentration at a grid of 7x5 sampling locations in the assay. Using vials of 50% liquid dilution of pinene, we captured 15 two-second trials per sampling point. Odor maps were generated using the average concentration detected across all trials at each location. These maps were smoothed via interpolation across space.

Data Analysis

Analyses of odormaps, sniffing, DLC tracking, and motif sequences were performed in MATLAB. Inhalation and exhalation times were extracted by finding peaks and troughs in the temperature signal after smoothing with a 25 ms moving window. Sniffs with duration less than the 5th percentile and greater than the 95th percentile were excluded from analysis. For alignment of movement with sniffing, tracking and motif sequences were shifted forward in time by 25ms (two frames), the temporal offset revealed by video calibration (Fig. S1).

Figure 1

Odormaps were visualized by smoothing the PID sampling grid with a X diameter gaussian, and colored using Cubehelix (*Green 2011*).

Figure 2

Sessions where mice performed less than 60% correct on 80:20 (90:30 for Constant ΔC , Variable $|C|$), were less than 80 trials, or had any missing folders or files were excluded. Trials longer than 10s were excluded. Percent correct was calculated by dividing the correct trials by total trials in a single session and was averaged across all sessions, all mice. Trial duration was measured between nose poke initiation and reward poke and was averaged across all trials, all sessions, all mice. Tortuosity was measured by dividing the total path length by the shortest possible path length and was averaged across all trials, all sessions, all mice.

Statistical tests were performed in python using the scipy package (*Jones et al. 2001*). A binomial test was used to test statistical significance of above chance performance. Wilcoxon rank-sum tests were used for all group comparisons with pairwise comparisons for more than two groups. Two group comparisons were tested using all trials pooled together and pairwise comparisons of three groups or more were tested across mice using individual mouse averages.

Figure 3

Occupancy and sniff rate colormaps were generated by downsampling the tracking data to a 50 X 30 grid of bins. Occupancy colormaps are a 2D histogram of the nose position data. Sniff rate histograms were generated by dividing the sniff count in each position bin by the corresponding bin in the occupancy histogram. Both histograms were gaussian-smoothed, and colored using cubehelix. Grand means are shown in Fig. 3F,G, while individual mouse occupancy heatmaps are shown in Fig. S4. Maps were colored using Cubehelix (*Green 2011*).

Figure 4 & 5

Nose speed, yaw velocity, and z velocity were calculated from the 3-point position time-series generated by DLC. For analysis, a 400 ms window centered on each inhalation time was extracted from the kinematic timeseries. Colormaps in Fig. 4 show traces surrounding individual sniffs, while colormaps were generated using Bluewhitened (Chil-dress, 2020). For within-trial sniffs, only those inhaled before the decision line were included. The inter-trial interval sniffs are taken from the time of reward port entry to the time of the first initiation port entry in the inter-trial interval. For cross-correlation and coherence analysis, we aligned the time-series of sniffing and kinematic parameters from

the entire trial, or from the interval between reward and initiation port in the inter-trial interval. Tracking glitches were excluded by discarding trials or inter-trial intervals that contained frames with nose speed above a criterion value (100 pixels per frame).

Figure 6

Average motif shapes were generated from the mean positions of the nose, head, and body points from the first 8 frames of every instance of a given motif as determined by the AR-HMM. Decoding analysis is described in the following section.

Figure 7

The transition probability matrix was clustered by minimizing Euclidean distance between rows. For analyses separating investigation and approach sniffs, sniffs were defined as investigation or approach sniffs based on the state at the inhalation time. Colors for investigation and approach were selected from the Josef Albers painting, Tautonym, (B) (*Albers 1944*).

Figure 8

Figures are generated by motif-onset triggered averages of inhalation times determined as described above. Fig. 8B,C are the grand mean of the motif onset-triggered average for each motif. Maps were colored using Cubehelix (*Green 2011*). Sniff phase (relative time in sniff) was determined by dividing the motif onset latency from inhalation by the total duration (i.e., inhalation time to inhalation time) of each sniff. Modulation index was calculated as the difference between maximum and minimum instantaneous sniff rate, divided by the sum ($max - min / max + min$).

Sniff synchronization

Sniff cycles were compared with kinematics to determine the extent of movement modulation at individual sampling points. Individual sniffs were cross-correlated with each kinematic signal (i.e. nose speed) at -200ms from inhalation onset to +200ms from inhalation onset. To further determine synchrony between the two signals, we measured the coherence of signal oscillation between sniff signals and individual kinematic measurements at -200ms from inhalation onset to +200ms from inhalation onset.

Auto-Regressive Hidden Markov Model

Let $\mathbf{x}_t$ denote the 6-dimensional vector of nose-head-body coordinates at video-frame t (sampled at 80 Hz), with components $(x_{nose}, y_{nose}, x_{head}, y_{head}, x_{body}, y_{body})$. We fit an auto-regressive hidden Markov model (AR-HMM) to mouse trajectory data, $\{\mathbf{x}_t^{(i)}\}$, across trials (indexed by (i)) from 13 out of 15 mice (two mice were excluded *a priori* due to low task performance). These mice performed olfactory search under the following experimental conditions: variable ΔC , constant $|C|$ (9 mice); naris occlusion (7 mice); and constant ΔC , variable $|C|$ experiments (5 mice).

The generative view: Viewed as a generative model (that generates simulated data), the AR-HMM has two “layers”: a layer of hidden discrete states (corresponding to discrete movement motifs), and an observed layer which is the continuous trajectory $\mathbf{x}_t$. We denote the temporal sequence of discrete states by z_t . In each time-step, $z_t \in \{1, 2, \dots, S\}$, i.e. it is one of an S number of states, or movement motifs. The discrete hidden states evolve in time according to a Markov chain: going from time-step t to $t + 1$, the discrete state

may change to another state according to a transition probability matrix π_{z_1, z_2} , which denotes the conditional probability of switching to z_2 having started in z_1 . The probability distribution over the initial state, $z_{t=1}$, of the Markov chain at the start of each trial was taken to be the uniform distribution.

Now suppose for time steps t_1 to t_2 (inclusive) the discrete layer remained in state z . The continuous or autoregressive part of the model dictates that, over this time interval, the continuous trajectory, $\mathbf{x}_t$, evolves according to a linear autoregressive (AR) process. The parameters of this AR process can be different in different states or motifs, z . In other words, $\mathbf{x}_t$ is governed by

$$\mathbf{x}_t = A_z \mathbf{x}_{t-1} + \mathbf{b}_z + \boldsymbol{\varepsilon}_t \quad t_1 \leq t \leq t_2 \quad (1)$$

where A_z is a 6×6 matrix and $\mathbf{b}$ is a 6×1 vector, and the noise vector $\boldsymbol{\varepsilon}_t$ is sampled from the multi-variate zero-mean gaussian distribution $\mathcal{N}(0, Q_z)$ where Q_z is a 6×6 noise covariance matrix. Moreover, the parameters A_z , $\mathbf{b}_z$, and Q_z depend on the discrete state z , and in general are different in different discrete states. The simple stochastic linear dynamics described by Eq. (1) can describe simple motions of the mouse, such as turning left/right, dashing towards a certain direction, freezing (when A_z is the identity matrix and $\mathbf{b}_z$ is zero), etc. The switches between these simple behaviors allow the model to generate complex trajectories.

The AR-HMM model is an example of a model with latent variables, which in this case are the discrete state sequence $z_t^{(i)}$ in each trial. The model, as a whole, is specified by the set of parameters $(\pi, \{A_z\}, \{\mathbf{b}_z\}, \{Q_z\})$, which we will denote by θ . For a d -dimensional trajectory ($d = 6$ here) and S states, comprises $S(S-1) + S(d^2 + d + d(d+1)/2) = S(S-1 + 3d(d+1)/2)$ parameters.

Model fits: Models with latent variables are often fit using the expectation-maximization (EM) algorithm which maximizes the likelihood of the model in terms of the parameters $\theta \equiv (\pi, \{A_z\}, \{\mathbf{b}_z\}, \{Q_z\})$ for a given set of observed data $\{\mathbf{x}_t^{(i)}\}$. In this work, we did not use the EM algorithm, but adopted a fully Bayesian approach in which both the hidden variables and the model parameters were inferred by drawing samples from their posterior distribution (Wiltschko et al. 2015). The posterior distribution combines the model likelihood and Bayesian priors imposed on its parameters, according to Bayes' rule. If we denote the joint-likelihood of observed trajectories, $\{\mathbf{x}_t^{(i)}\}$, and the latent variables, $\{z_t^{(i)}\}$, by $P(\{\mathbf{x}_t^{(i)}, z_t^{(i)}\}|\theta)$ and the prior distribution over model parameters by $P(\theta)$, then up to normalization, the joint posterior distribution of latent variables and model parameters is given by

$$P(\{z_t^{(i)}\}, \theta | \{\mathbf{x}_t^{(i)}\}) \propto P(\{\mathbf{x}_t^{(i)}, z_t^{(i)}\}|\theta)P(\theta). \quad (2)$$

For the AR-HMM model, the (logarithm of the) joint log-likelihood is given by

$$\log P(\{\mathbf{x}_t^{(i)}, z_t^{(i)}\}|\theta) = \sum_i \sum_{t=2}^{T_i} \left[\log \pi_{z_{t-1}^{(i)}, z_t^{(i)}} + \log \mathcal{N}(\mathbf{x}_t^{(i)} | A_{z_t^{(i)}} \mathbf{x}_{t-1}^{(i)} + \mathbf{b}_{z_t^{(i)}}, Q_{z_t^{(i)}}) \right] \quad (3)$$

where T_i is the length of trial i , and we use the notation $\mathcal{N}(\mathbf{x}|\boldsymbol{\mu}, Q) = e^{-\frac{1}{2}(\mathbf{x}-\boldsymbol{\mu})^T Q^{-1}(\mathbf{x}-\boldsymbol{\mu})} / \sqrt{|2\pi Q|}$ to denote the density at point $\mathbf{x}$ of a multivariate gaussian with mean vector $\boldsymbol{\mu}$ and covariance matrix Q .

We imposed loose conjugate priors on the model parameters, which were factorized over the parameters of the AR process, $(\{A_z\}, \{\mathbf{b}_z\}, \{Q_z\})$, in different discrete states z ,

and the different rows of the Markov transition matrix, π . On the rows of π , we imposed Dirichlet distribution priors with uniform distribution means, and concentration hyperparameter α , which was set to 4. We imposed matrix normal inverse-Wishart priors on the AR parameters, independently for different discrete states. Under this prior, the noise covariance Q_z has an inverse Wishart distribution with a "scale matrix" hyperparameter, which was set to the $d \times d$ ($= 6 \times 6$) identity matrix, and a "degrees-of-freedom" scalar hyperparameter set to $d + 2 = 8$. Conditional on Q_z , the remaining AR parameters, $(A_z, \mathbf{b}_z)$, have a joint multivariate normal distribution under the prior, which can be specified by the prior mean and joint prior covariance matrix of A_z and $\mathbf{b}_z$. The prior means of A_z and $\mathbf{b}_z$ were set to the $d \times d$ identity matrix and the d -dimensional zero vector, respectively, while the prior covariance matrix of the concatenation $(A_z, \mathbf{b}_z)$ was given by the tensor product of Q_z and the $(d+1) \times (d+1)$ ($= 7 \times 7$) identity matrix (equivalently, under this prior, $\mathbf{b}_z$ and different columns of A_z are independent and uncorrelated, while each of these column vectors has a prior covariance equal to the (prior) AR noise covariance, Q_z).

Bayesian model inference was carried out by sampling from (instead of maximizing) the joint posterior distribution of the model parameters and latent state variables conditioned on the observed trajectory data, Eq. (2). We did this by Gibbs sampling (an example of Markov Chain Monte Carlo; not to be confused with the Markov Chain in the AR-HMM model), which works in a manner conceptually similar to the EM algorithm: it switches between sampling $z_t^{(i)}$ in all trials, conditioned on previously sampled parameters, and then sampling the parameters θ given the previous sample of $\{z_t^{(i)}\}$. To carry out this model inference procedure, we used the Python package developed by M. J. Johnson and colleagues, publicly available at <https://github.com/mattjj/pyhsmm>.

We ran the Gibbs sampler for 300 iterations, and burned the first 200 samples, retaining 100. We used the remaining samples to obtain the posterior probabilities of hidden discrete states at each time step of each trial (by calculating the frequency of different state in that time step and trial, across the retained Gibbs samples), as well as posterior expectation of the model parameters (by calculating their averages over the retained Gibbs samples). We refer to the AR-HMM with parameters given by these latter posterior expectations as the "fit model".

Model selection: We fit AR-HMM's with different numbers of states (motifs), S , to mouse trajectory data pooled across animals. To evaluate the statistical goodness-of-fit of these fit model and select the best S (the number of states or motifs), we evaluated the log-likelihoods of fit models on trajectory data from a held-out set of trials, not used for model fitting. The corresponding plot of log-likelihoods is shown in Figure S8C. As seen, the log-likelihood keeps increasing with S , up to $S = 100$. This shows that, up to at least $S = 100$, additional motifs do have utility in capturing more variability in mouse trajectories. These variabilities may include differences in movement across mice, as well as movement variations in the same mouse but across different trials or different instances of the same movement; for example, a clockwise head turn executed with different speeds in different instances or trials. In the AR-HMM model, the AR observation distribution of a given Markov state corresponds to a very simple (linear) dynamical system which cannot capture many natural and continuous variations in movement, such as changes in movement speed. Nevertheless AR-HMM models with higher S can capture such variations with more precision, by specializing different discrete Markov states, with different AR distributions, to movement motifs of different mice, or, for example, to

capture different speeds of the same qualitative movement motif.

The goal for this modelling was to give a compact description of recurring movement features across animals and conditions, suitable for visualization and alignment. For these purposes, the goodness-of-fit did not provide a suitable criterion, because the log-likelihood plots did not peak or plateau even at very large numbers of states. Guided by visual inspection, we thus chose the model with $S = 16$, for the main figures (Fig. 6-8). Although this was a somewhat arbitrary choice, we show that the findings in Figures 6, 7, and 8 do not depend on the choice of S – models with $S = 6, 10$, or 20 gave equivalent results (Supp. Figs. S9–S11).

MAP sequences: The Gibbs sampling algorithm which we used for model inference yields (timewise marginal) Maximum A Posteriori (MAP) estimates of the latent variables $\{z_t^{(i)}\}$, as follows. Using the Gibbs samples for the latent variables we can estimate the posterior probability of the mouse being in any of the S states in any given time-step of a given trial. We made MAP sequences by picking, at any time step and trial, the state with the highest posterior probability. The inferred MAP motifs tended to have high posterior probability, which exceeded 0.8 in 66.2% of all time-steps across the 17195 trials in the modeled dataset.

Decoding analysis

We decoded experimental conditions and animal identities from single-trial MAP motif sequences inferred using the AR-HMM model. Specifically, we trained multi-class decoders with linear decision boundaries (Linear Discriminant Analysis) to decode the above categorical variables from the single-trial empirical state transition probability matrices derived from the MAP sequence of each trial. If $\hat{z}_t^{(i)}$ is the motif MAP sequence for trial i , the empirical transition probability, $\hat{\pi}_{a,b}^{(i)}$, from state a to state b ($a, b \in \{1, \dots, K\}$), for that trial was calculated by:

$$\hat{\pi}_{a,b}^{(i)} \equiv \frac{n_{a,b}^{(i)}}{\sum_{c=1}^K n_{a,c}^{(i)}} \quad (4)$$

$$n_{a,b}^{(i)} \equiv \sum_{t=1}^{T^{(i)}-1} \mathbb{I}(\hat{z}_t = a) \mathbb{I}(\hat{z}_{t+1} = b) \quad (5)$$

where $T^{(i)}$ is the length of trial i , and $\mathbb{I}(\cdot)$ is an indicator function, returning 1 or 0 when its argument is true or false, respectively.

We used the decoder to either classify experimental condition or mouse identity, in different trials (Fig. 6D,E). For decoders trained to classify the trials' experimental condition, we used pooled data across mice. For decoders trained to classify mouse identity, we only used data from the 80-20 odor condition. Data was split into training and test dataset in a stratified 5-fold cross-validation manner, ensuring equal proportions of trials of different types in both datasets. The trial type was the combination of left vs right decision, experimental condition, and mouse identity.

To calculate the statistical significance of decoding accuracies, we performed an iterative shuffle procedure on each fold of the cross-validation. In each shuffle, the training labels which the classifier was trained to decode were shuffled randomly across trials of the training set, and the classifier's accuracy was evaluated on the unshuffled test dataset. This shuffle was performed 100 times to create a shuffle distribution of decoding

accuracies for each fold of the cross-validation. From these distributions we calculated the z-score of decoding accuracy for each class in each cross-validation fold. These z-scores were then averaged across the folds of cross-validation and used to calculate the overall p-value of the decoding accuracy obtained on the original data.

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Author Contributions

TF and MS conceived the study. MB, TF, and MS designed, built, and maintained the behavioral rig. TF, JC, BH, RA, DY, EM, NN, IC, JS, and JK trained mice. JC and TF performed naris occlusion experiments. RA, DW, and TF established and implemented Deeplabcut tracking, DW and YA designed and performed the AR-HMM analysis. TF, DW, BH, YA, and MS analyzed data and wrote the manuscript.

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Supplementary Material

Supplementary Video 1.

Odor gradients are temporally dynamic and noisy. Colormaps represent the time-course of odor colormaps for pseudo-trials assembled from individual trials at each sampling location.

Supplementary Video 2.

Example trials with sniffing. Three dots on the mouse represent the coordinates of front of snout, back of head, and center of mass extracted using Deeplabcut. Sniffing is indicated by color (blue=inhalation, pink=exhalation) and sound (higher tone=inhalation, lower tone=exhalation). Video frame rate is slowed by 4X.

Supplementary Video 3.

Movement trajectories for individual sniffs. Each video snippet corresponds to one sniff, where the frames are translated so that back of the head is centered, and rotated so that head angle is vertical, in the first frame of each sniff. Blue=inhalation, pink=exhalation. Video frame rate is slowed by 10X.

Supplementary Video 4.

Example trials with motif sequences. Three dots on the mouse represent the coordinates of front of snout, back of head, and center of mass extracted using Deeplabcut. Dots and lines are colored according to the motif to which that frame was assigned by the AR-HMM. Video frame rate is slowed by 8X.

Supplementary Video 5.

Moving occupancy histograms for motifs show their average movement dynamics. We aligned every instance of a given motif such that that instance's frames were translated to position the center of mass in frame 1 at consistent location in the image, and rotated so that the body axis points upwards in frame 1. Colormap represents regions of high occupancy with brighter, warmer colors, and lower occupancies with darker, colder colors. Video frame rate is slowed by 8X.

Supplementary Video 6.

Moving wireframes for motifs show the variability of movement dynamics for a given motif. Wireframes consist of two lines connecting coordinates of front of snout, back of head, and center of mass extracted using Deeplabcut. Each wireframe represents a single instance of every motif. We aligned frames as in supplementary video 5. Lines are colored arbitrarily to facilitate visualization of individual wireframes. Video frame rate is slowed by 8X.

Supplementary Video 7.

Example trials with investigation/approach overlaid. Three dots on the mouse represent the coordinates of front of snout, back of head, and center of mass extracted using Deeplabcut. Dots and lines are colored according to whether that frame was assigned by the AR-HMM to an investigation motif or an approach motif. Sniffing is indicated by sound (higher tone=inhalation, lower tone=exhalation). Video frame rate is slowed by 8X.

Supplementary Video 8.

Movement trajectories for individual sniffs separated into investigation and approach. Each video snippet corresponds to one sniff, where the frames are translated so that back of the head is centered, and rotated so that head angle is vertical, in the first frame of each sniff. Blue=inhilation, pink=exhalation. Video frame rate is slowed by 10X.

Figure S1. Calibrating alignment of video frames with sniff signal. A) Sinusoidal signals (5, 8, 10, and 15 Hz) were simultaneously sent to the analog input channel (used to capture sniffing) and to a phosphor-display oscilloscope (Tektronix). The display of the oscilloscope was reflected by mirrors to allow it to be video-captured inside the behavioral arena. B) The timing relationship is given by the lag between peaks in the analog input channel and the vertical peaks in the position of the oscilloscope trace. Analog input led video frames by 23.5 ± 15.7 ms (mean $\pm$ sd; approximately two frames at 80 frames/second).

Figure S2. Color maps of average odor concentration across 15 two-second trials captured by a 7x5 grid of sequential photoionization detector recordings. - Each row represents trial type (left correct or right correct). A) 80:20 odor condition (see Methods: Behavioral Training: 80:20). B) 60:40 odor condition (see Methods: Interleaved: 60:40).

Figure S3. Session statistics across trainer sessions. - Individual mice are depicted by colored lines, average across mice are black points, and whiskers are ± 1 standard deviation from the average across mice. Mice 2054-2062 did not have trainer 1 and 2 recorded (this accounts for increasing n) and mice were commonly removed from the experiment if they lost sniff signal (this accounts for the reducing n). Above. Number of trials performed or percent of correct trials. Middle. Average trial duration. Below. Average trial path tortuosity (total path length/shortest possible path length). A) Session statistics for the first trainer, water sampling ($n = 19$). B) Session statistics for the second trainer, odor association ($n = 19$). C) Session statistics for the third trainer, 100:0 or olfactory search ($n = 26$). Mice perform above 70% in first session. D) Session statistics for final training step, 80:20, that preceded experiments shown in Figure 2 ($n = 24$).

Figure S4. Mice generalize search task to novel odorants and variable $|C|$ session. – A) Performance, trial duration, and trial tortuosity (total path length/shortest possible path length) for the last session of pinene training in 80:20 and the first session of vanillin in 80:20 across mice ($n = 3$). B) Grouped by stimulus condition (90:30, 30:10, 0:0), each line represents the rolling average across mice (window=10) for the first session ($n = 5$). Shaded regions represent ± 1 standard deviation.

Figure S5. Idiosyncratic occupancy distributions across individual mice. Two dimensional histogram of occupancy (fraction of frames spent in each 0.5 mm² bin).

Figure S6. Sniff synchronization shuffle test. Sniff-aligned grand mean ($n = 11$ mice) of A) nose speed, B) yaw velocity, and C) Z-velocity for within-trial (Top) and inter-trial interval (Bottom) sniffs, overlaid on 1000 iterations of trial-shuffled grand means. Thin black lines represent individual iterations, all of which are shown.

Figure S7. Kinematic rhythms for premature initiations during the intertrial interval and between decision line and reward port during trials. Sniff-aligned average of A) nose speed, B) yaw velocity, and C) z-velocity. Thin lines represent individual mice ($n = 11$), bolded lines and shaded regions represent the grand mean $\pm$ standard deviation. Green: within-trial sniffs from the time between crossing the decision line and entering the reward port. Pink: inter-trial interval sniffs from the time between the first premature trial initiation attempt and the successful initiation of the next trial.

Figure S8. Motif statistics and examples and linear decoder results for 80:20 experiments. - A) Example nose trace across a single trial colored by motif identity. B) Linear decoder (Fig. 6: Methods: Linear decoding section) results for Variable |C| experiments ($n = 5$). C) Cross-validated log-likelihood (evaluated on trials not used for model fitting) for fit AR-HMM models with different numbers of motifs, S , shows that model log-likelihood does not peak or plateau up to $S = 100$. D) Fraction of motif usage across all mice ($n = 8$) for the model with $S = 16$. Black points are individual mice, black line is average across mice, and shaded region is ± 1 standard deviation. Colors on x axis represent motifs used in analysis (Fig. 6) and y axis are fractions of frames that motif occupies. E) The average dwell time of each motif across all mice ($n = 8$) for the model with $S = 16$. Black points are individual mice, black line is average across mice, and shaded region is ± 1 standard deviation. Colors on x axis represent motifs used in analysis (Fig. 6) and y axis are fractions of frames that motif occupies.

Figure S9. Motif shapes, sequences, transition matrices, and sniff synchronization for an AR-HMM capped at a maximum of 6 states.

Figure S10. Motif shapes, sequences, transition matrices, and sniff synchronization for an AR-HMM capped at a maximum of 10 states.

Figure S11. Motif shapes, sequences, transition matrices, and sniff synchronization for an AR-HMM capped at a maximum of 20 states.

Figure S12. Shuffle test for the difference in sniff synchronization between investigation and approach motifs for movement parameters. We quantified the difference by calculating the Kolmogorov-Smirnov statistic for the comparison between the sniff triggered averages in the two states, first for real data, and then for 1000 iterations of trial-shuffled data. Red shows the value for the real data, while the black histogram plots the distribution of Kolmogorov-Smirnov statistic for the 1000 iterations.

Figure S13. Shuffle test for sniff synchronization of motif onset for investigation and approach motifs. We calculated a modulation index ($MI = (max - min)/(max + min)$) for each motif's across-mouse mean histogram ($n = 4$), and calculated the same for 1000 trial-shuffled across-mouse mean histograms. Blue and orange lines give the value from the real data, while the black histogram shows the distribution of MI across shuffle iterations.