RESEARCH ARTICLE

Odor source localization in complex visual environments by fruit flies

Nitesh Saxena[1,*], Dinesh Natesan[1,2,3,*] and Sanjay P. Sane[1,‡]

ABSTRACT

Flying insects routinely forage in complex and cluttered sensory environments. Their search for a food or a pheromone source typically begins with a whiff of odor, which triggers a flight response, eventually bringing the insect near the odor source. However, pinpointing the precise location of an odor source requires use of both visual and olfactory modalities, aided by odor plumes. Here, we investigated odor-tracking behavior in fruit flies (*Drosophila melanogaster*) presented with low- or high-contrast visual landmarks, either paired with or separate from an attractive odor cue. These experiments were conducted either in a gentle air stream which generated laminar odor plumes or in still air in which odor dissipates uniformly in all directions. Trajectories of flies revealed several novel features of their odor-tracking behavior in addition to those previously documented. First, in both moving and still air, odor-seeking flies rely on the co-occurrence of visual landmarks with olfactory cues to guide them to odorant objects. Second, flies abruptly decelerate upon encountering an odor plume, thereafter steering towards the nearest visual objects that had no inherent salience in the absence of odor. Thus, interception of an attractive odor increases their salience to nearby high-contrast visual landmarks. Third, flies adopt distinct odor-tracking strategies during flight in moving versus still air. Whereas they weave in and out of plumes towards an odor source in airflow, their approach is more incremental in still air. Both strategies are robust and flexible, and enable flies to reliably find odor sources under diverse visual and airflow environments.

KEY WORDS: *Drosophila melanogaster*, Multisensory integration, Odor plume, Olfaction, Olfactory working memory, Vision

INTRODUCTION

Freely flying insects live in a complex world that is both visually heterogeneous and odor rich. This poses steep challenges in locating specific sources of odor such as conspecific mates, food sources or oviposition sites. Often, these resources are camouflaged in their natural surroundings or lack the distinctive visual features that identify them as odor sources. For insects flying in natural conditions, this task is confounded by turbulence and abrupt changes in the direction of airflow, which means that instantaneous odor signals may not provide reliable information about odor source location (Murlis et al., 1992; Vickers, 2000). In breezy conditions,

odor signals propagate as intermittent, filamentous plumes interspersed with clean air pockets that greatly increase the range over which odor molecules travel (Murlis et al., 1992; Willis et al., 1994). For insects flying in gentler air currents, these plumes are more laminar but are disturbed by wing-induced upwind turbulence, which enhances odor sampling but also alters the spatial information about odor source location (Sane and Jacobson, 2006).

Airflow collimates odor cues and provides directional cues to odor-tracking insects (e.g. Kennedy and Marsh, 1974). For instance, while plume-tracking in laminar airflow, insects fly upwind, aligning with the odor plume. Such behavior typically consists of two aerial maneuvers. First, upon encountering an odor plume, insects perform surging maneuvers, which involve flying towards the upwind odor source. However, if they lose track of the plume, they cast orthogonal to the plume axis to regain contact with odor packets. The combination of casting and surging naturally channels insects towards odor sources (Farkas and Shorey, 1972; Kennedy, 1983; Baker, 1990; Vickers and Baker, 1994; Vickers, 2000).

Under natural conditions, airflow can be quite erratic, which means that insects require supplementary information from other sensory modalities, especially vision. For instance, fruit flies rely on wide-field visual cues during odor tracking (Frye et al., 2003; Budick and Dickinson, 2006; Duistermars and Frye, 2008, 2010), and moths utilize ambient visual cues to estimate airflow direction (e.g. Kennedy and Marsh, 1974). Sensing and processing by one sensory modality is often influenced by feedback from another modality during active behaviors (Duistermars et al., 2009). In odor-tracking flies, odor cues modify optomotor responses, enhancing their chances of homing in on visual features while maintaining a consistent heading (Chow and Frye, 2008).

As they approach an odor source, insects rely on local visual landmarks to identify odor objects (Raguso and Willis, 2002). Indeed, visual landmarks become attractive to insects only if odor is present (e.g. fruit flies: van Breugel and Dickinson, 2014; mosquitoes: van Breugel et al., 2015). When visual cues are decoupled from olfactory cues, the ability of the hawkmoth, *Manduca sexta*, to approach and probe an artificial flower is considerably attenuated compared with cases in which the two cues are simultaneously presented (Goyret et al., 2007). If visual cues are indistinct or ambiguous, flies may increase their reliance on odor cues to find sources of odor (e.g. Tephritid apple fly, *Rhagoletis pomonella*: Aluja and Prokopy, 1993). For flies under tethered conditions, local visual landmarks by themselves appear insufficient to guide them toward odor plumes, and they may require panoramic visual cues to navigate to the odor source (Duistermars and Frye, 2008).

These studies demonstrate the importance of combining olfactory, airflow and visual cues in guiding insects towards odor sources, but do not reveal how they identify the precise location of an odor source within complex visual environments. They also do not address which strategies are adopted by insects to identify odor sources when ambient airflow is absent. Here, we conducted

[1]National Centre for Biological Sciences (NCBS), Tata Institute of Fundamental Research, GKVK campus, Bellary Road, Bangalore 560065, India. [2]Department of Computational Science and Technology, School of Computer Science and Communication, KTH Royal Institute of Technology, Stockholm 10044, Sweden. [3]Manipal University, Manipal 576104, India.
*These authors contributed equally to this work

‡Author for correspondence (sane@ncbs.res.in)

S.P.S., 0000-0002-8274-1181

experiments to understand how insects parse between odor and visual cues to find an odor source in the presence and absence of ambient airflow. Specifically, these studies were conducted using *Drosophila melanogaster* Meigen 1830 Canton-S strain (fruit flies/vinegar flies) as our model organism, for its ability to find odor objects in diverse visual and airflow environments (Budick and Dickinson, 2006; Duistermars and Frye, 2008; Frye and Dickinson, 2004; Frye et al., 2003; Stewart et al., 2010; van Breugel and Dickinson, 2014). We spatially staggered visual and odor cues, which compelled flies to find an odor source with or without airflow in simple or complex arrays of visual landmarks. We then reconstructed and analyzed their 3D flight trajectories to determine how these stimuli influenced their flight prior to landing.

MATERIALS AND METHODS

We used 2–3 day old flies from an in-house culture maintained at 24–27°C on a 12 h:12 h light:dark cycle. Prior to the experimental trials, flies were starved overnight for ~12 h to motivate foraging, and provided with moist paper to prevent dehydration. We conducted experiments during the flies' photoperiod to ensure robust flight activity.

Visual cues

It was not possible to precisely control visual contrast for freely flying flies, because the object–background view changes from one instant to another depending on the flies' heading relative to the object. Instead, we presented two objects that coarsely constituted low- or high-contrast visual landmarks, as described below.
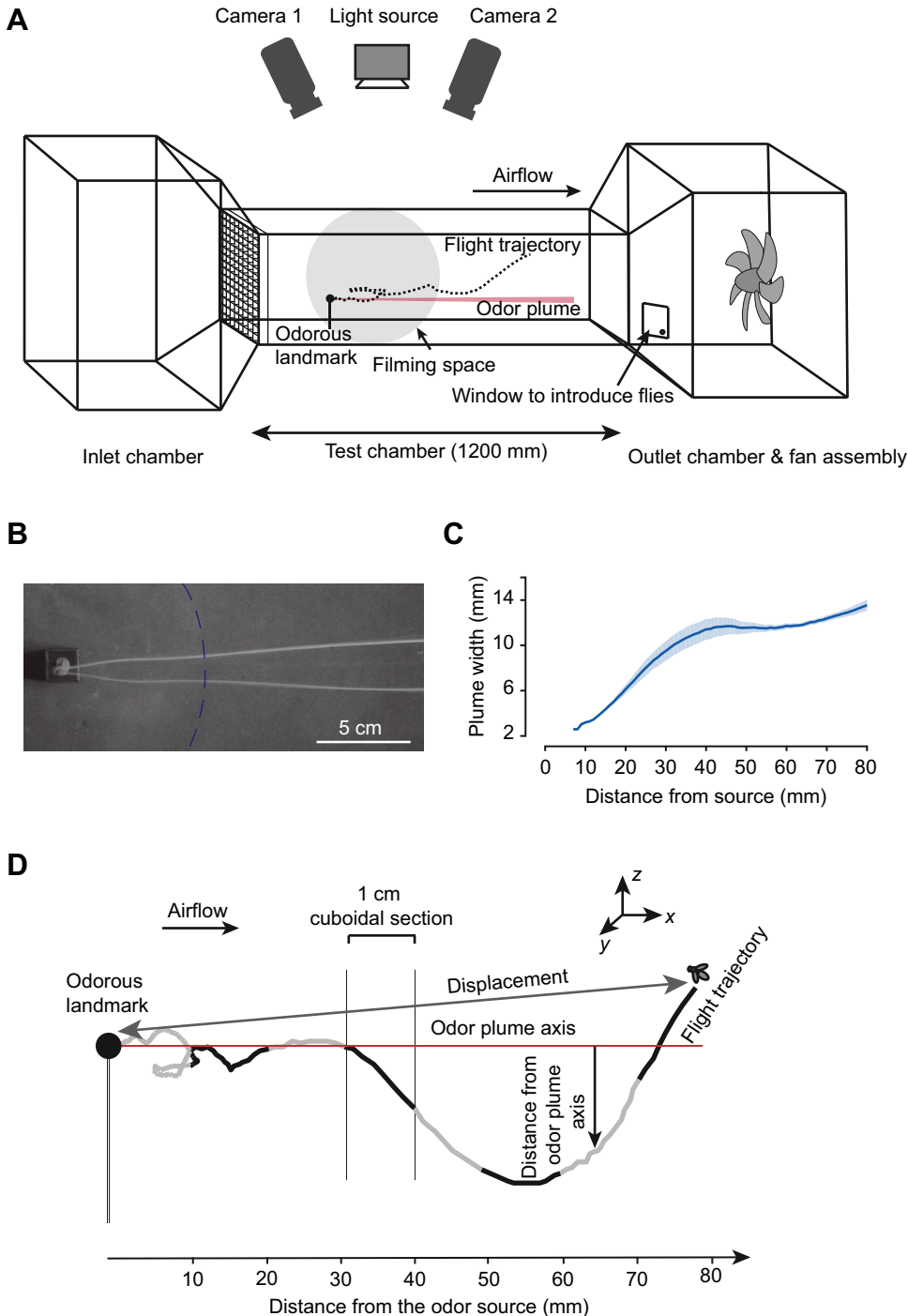

Fig. 1. Experimental setup and flight variables. (A) Flies tracked the odor plume inside a customized wind tunnel of test chamber dimensions 1200 mm×280 mm×280 mm. The flies tracking the odor plume (red) were filmed at 100 frames s^{-1} using two cameras mounted above the wind tunnel (filmed region shown by shaded gray circle) and their 3D flight trajectories were reconstructed from these images. (B) Raw image of a laminar smoke plume from a low-contrast landmark source at an airflow of 0.1 m s^{-1} (top view). Dashed line shows the 80 mm radial cut-off, which is our region of interest. (C) Change in plume width versus distance from the source. The dark blue line shows the mean plume width and the light blue band shows the s.e.m. (N=4). (D) Schematic diagram of a fly's approach to an odor source. The trajectories are indicated by black and gray lines, each depicting flight along 10 mm stretches. The odor plume axis (red line) indicates the alignment of the odor plume, determined using smoke visualization. We calculated speed, flight duration, tortuosity and hover duration to quantify the flight behavior in a spherical region of 80 mm radius centered on the odor source (see Materials and methods).

Low-contrast landmark

Low-contrast landmarks consisted of a transparent glass capillary (length 100 mm, diameter 1 mm) placed within a small Plexiglas® holder tipped with a white cotton ball.

High-contrast landmark

On the above-described glass capillary, we threaded a black spherical bead (diameter 6 mm) that subtended an angle of ~5 deg on the fly retina at ~80 mm from the bead (our region of interest), thus constituting a high-contrast local visual landmark against the white background.

Odor cues

Odor cues consisted of 10 µl of apple cider vinegar (5% vinegar syrup, Zeta Food Products, Stockholm, Sweden), placed on a black bead (high-contrast odorous landmark) or cotton tip of the capillary (low-contrast odorous landmark).

Wind tunnel and filming apparatus

We used a custom-made, calibrated low-flow wind tunnel to generate laminar airflow. Flies were released in the test chamber (1200 mm×280 mm×280 mm), within which odor and visual cues were placed (Fig. 1A). For experiments in the presence of airflow, the laminar airflow speed within the test section was set to 0.1 m s^{-1}, in the range of naturally occurring values (Budick and Dickinson, 2006). Air speed was measured using a hot-wire anemometer (Kurz490-IS Portable Anemometer, Monterey, CA, USA; also see Sane and Jacobson, 2006). For still-air experiments, the fan was switched off and both ends of the wind tunnel were sealed using Plexiglas® sheets to reduce ambient flow to lower values than could be measured by the anemometer. The odorous landmark was placed within the still chamber and odor was allowed to diffuse for ~20 min, after which flies were released in the wind tunnel. Contrast between the fly and background was enhanced by lining the wind tunnel walls and base with white paper, backlit by four 50 W halogen lamps. A 150 W metal halide lamp on top of the wind tunnel provided sufficient illumination for filming. A red filter placed on the 150 W lamp cut off wavelengths below 610 nm. Because *Drosophila* are insensitive to wavelengths above 600 nm (Heisenberg and Wolf, 1984), this filter ensured minimal impact of illumination on flight behavior. Average illumination in the chamber was ~350 lx (Center 337 light meter, Center Technology Corporation, Taipei, Taiwan).

We filmed flight trajectories at 100 frames s^{-1} using two high-speed cameras (Phantom v7.3/Miro eX4, Vision Research, Wayne, NJ, USA) placed above the wind tunnel. Filmed volume was approximately 16% of the entire wind tunnel volume. In every trial, 5–6 flies were released into the wind tunnel to reduce waiting time for landing events. Trials were terminated when a fly landed on any landmark. In rare cases, if multiple flies approached the odor

Table 1. Summary of the experiments

Airflow	Experiment	Treatment	% Correct landings	N	Chi-squared test (P-value)
Presence of airflow	1	●	–	20	–
		◉ (red circle)	–	22	–
		◎ (red circle)	–	24	–
	2	1 cm	40	25	0.32
		2 cm	72	25	0.03
		5 cm	91.3	23	0.07×10^{-3}
	3	1 cm	95.2	21	0.03×10^{-3}
		2 cm	100	20	0.08×10^{-4}
		5 cm	100	20	0.08×10^{-4}
	4	1 cm	41.7	24	0.15×10^{-2}
		3 cm	82.6	23	0.03×10^{-4}
		3 cm	82.8	29	0.11×10^{-6}
Absence of airflow	5	◎	–	21	–
	6	1 cm	60	20	0.37
		2 cm	75	20	0.02
		5 cm	84.2	19	0.29×10^{-2}
	7	5 cm	57	21	0.51
		5 cm	95.2	21	0.34×10^{-4}

Small open circles denote low-contrast visual landmark and larger solid circles denote high-contrast visual landmark. Concentric red circle around landmark represents the presence of odor. The first 12 rows represent experiments in airflow (and odor plume), whereas the bottom six rows represent experiments in still air. Correct landings are defined as landings on the odorous landmark. Sample sizes (N) per treatment and the P-value of the chi-squared test for each experiment are shown in the fifth and sixth columns. The chi-squared test compares the observed landing frequency with the expected frequency due to random landings on available landmarks (P<0.05 indicates non-random landings).

source simultaneously, such trajectories were excluded to avoid confounding effects of competitive interactions. Before starting another trial, we flushed out flies from the previous trial to ensure that only innate responses of naive flies were recorded. One flight trajectory was filmed in each trial and 3D trajectories within 80 mm of the odor source were analyzed.

Treatments

Each specific odor and visual cue arrangement constituted a treatment. Based on pilot experiments with high-contrast landmarks in the presence or absence of odor, we chose a minimum sample size of 19 flies per treatment to ensure adequate statistical power for all treatments. All treatments and corresponding sample sizes are given in Table 1.

Experimental design

We conducted seven experiments in which naive flies identified an odor source in the presence of visual landmarks. In the first set of experiments, flies flew in the presence of a constant 0.1 m s^{-1} airflow (experiments 1–4), whereas in the second set, they flew in still air (experiments 5–7). We systematically varied the arrangement of visual landmarks around the odorous landmark. Each experiment contained multiple treatments, with a fixed combination of odor, visual and

airflow conditions, as described below. Table 1 summarizes the sample sizes and landing preferences of flies in the treatments.

Presence of airflow

Experiment 1: responses to individual odor and visual cues, and their combination

Flies were flown under three conditions: (1) a single high-contrast non-odorous landmark, to observe innate responses of flies towards a visual cue in the absence of odor cues; (2) a single high-contrast odorous landmark, to observe responses towards combined visual and odor cues; and (3) a single low-contrast odorous landmark, to observe responses towards odor cues with the low-contrast visual cue.

Experiment 2: responses to decoupled odor and visual cues

To decouple odor and visual cues, the high-contrast non-odorous landmark was separated from the low-contrast odorous landmark by 1, 2 and 5 cm, respectively. These three treatments were compared with results for the single low-contrast odorous landmark from experiment 1.

Experiment 3: control for experiment 2

As a control for experiment 2, we switched the position of the odorous and non-odorous cues to give a high-contrast odorous landmark and a
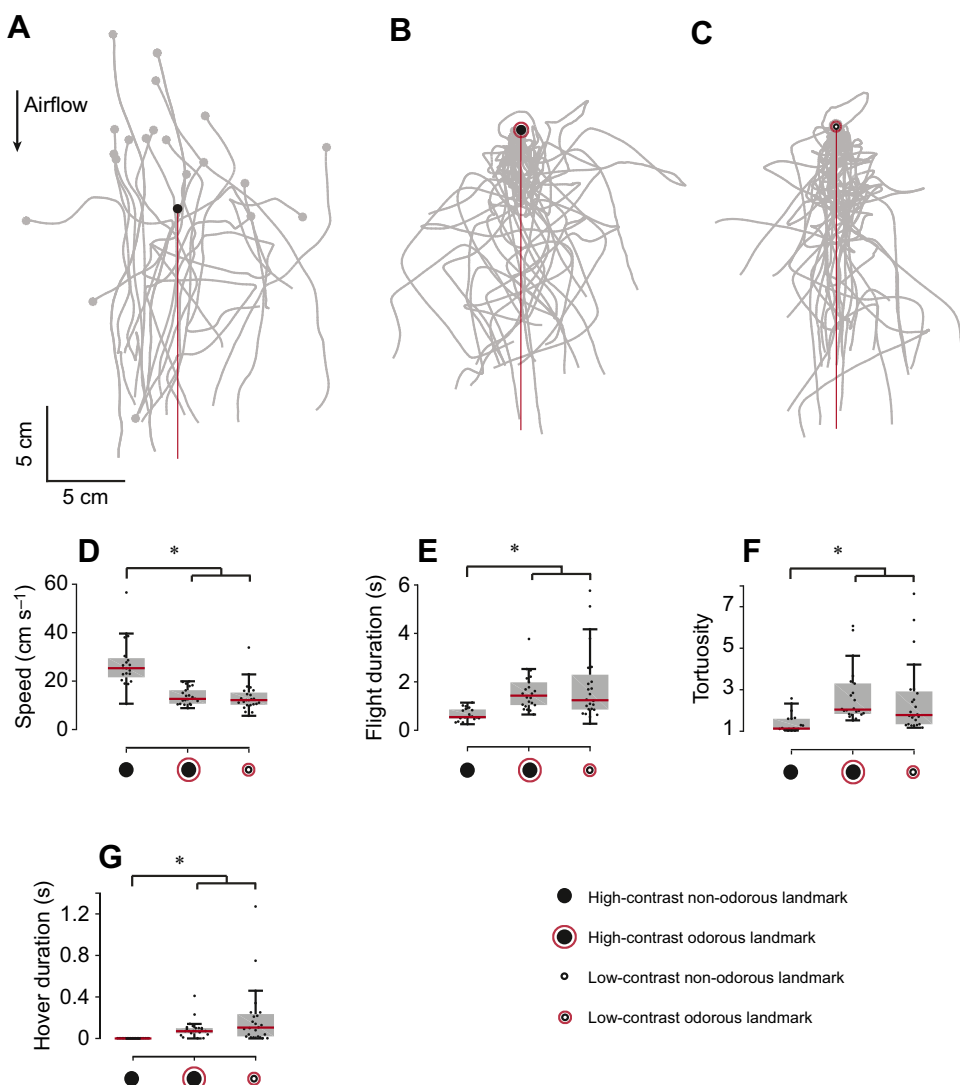

Fig. 2. Flight behavior in the presence of odorous and non-odorous landmarks. (A–C) Flight trajectories (gray) in the presence of (A) a high-contrast non-odorous landmark (*N*=20), (B) a high-contrast odorous landmark (*N*=22) and (C) a low-contrast odorous landmark (*N*=24). The flies flew towards an odor source that was either high contrast or low contrast by tracking an odor plume along its axis (red line). Odorous landmarks are indicated by a concentric red circle around the circles depicting visual objects. (D–G) We compared these treatments for (D) average speed, (E) total flight duration, (F) tortuosity and (G) hover duration of flies as box-and-whisker plots. The height of the box indicates the range of the central 50% of data around the median (red line). The length of the whiskers represents data within 1.5 times the interquartile range. Outlier data are outside the whiskers, but were included in the analysis. Asterisks represent statistically significant comparisons (*P*<0.05, Kruskal–Wallis test, Nemenyi test) in all figures. These conventions for odorous objects, box plots and statistical tests are followed for all figures.

● High-contrast non-odorous landmark

◉ High-contrast odorous landmark

∘ Low-contrast non-odorous landmark

◎ Low-contrast odorous landmark

low-contrast non-odorous landmark separated by 1, 2 and 5 cm, respectively. These treatments were compared with results for the single high-contrast odorous landmark from experiment 1.

Experiment 4: odor source localization in visual clutter

To determine how flies identify odor sources within visually cluttered environments, we varied the number and density of landmarks around the odor source. Flies were flown under three conditions of visual clutter: (1) high-density visual clutter of seven objects – seven high-contrast landmarks each separated by 1 cm arranged in a single row; odor was contained in an off-center (fifth) landmark to avoid artifacts resulting from natural centering responses displayed by many insects when flying through confined spaces (e.g. Srinivasan et al., 1996); (2) low-density visual clutter of seven objects – seven high-contrast landmarks separated by 3 cm, with the fifth landmark containing odor; and (3) low-density visual clutter of three objects – three high-contrast landmarks in a row, each separated from its nearest neighbor by 3 cm, with the middle landmark containing odor (although the middle landmark was the odor object, it was kept off-center in the wind tunnel).

Absence of airflow

In still air, odor spreads primarily by diffusion in all directions. We addressed how flies locate an odor source in still air in experiments 5–7.

Experiment 5: odor source localization in still air

A single low-contrast odorous landmark was presented to flies in the wind-tunnel with the fan switched off and the wind tunnel sealed.

Experiment 6: odor source localization in the presence of paired odorous and non-odorous high-contrast landmarks

We measured the ability of flies to distinguish between two identical high-contrast landmarks of which only one was odorous. The landmarks were separated by 1, 2 or 5 cm.

Experiment 7: responses to decoupled odor and visual cues

To determine how visual contrast influences odor tracking in the absence of airflow, we designed two treatments. In one, a low-contrast odorous landmark was separated from a high-contrast non-odorous landmark by 5 cm. As a control, a high-contrast odorous landmark was separated from a low-contrast non-odorous landmark by 5 cm.

Quantification of airflow
Laminarity

To ascertain laminarity of the wind tunnel, we measured airflow with a hot-wire anemometer at separate points within the test section to verify that air speed at various points was identical, and held a constant value over time (see also Khurana and Sane, 2016). For an object of characteristic length L placed in a fluid with velocity V and kinematic viscosity υ, Reynolds number (Re) is given by:

$$Re = \frac{V\,L}{\upsilon}. \qquad (1)$$

When airflow is 0.1 m s^{-1}, kinematic viscosity is 1.57×10^{-5} m^2 s^{-1} (dry air at 300 K) and characteristic lengths are from 1 mm (single capillary) to 10 mm (smallest separation distance between two landmarks), the Reynolds numbers range from ~7 to 70, within the laminar regime. This was verified by seeding the flow with
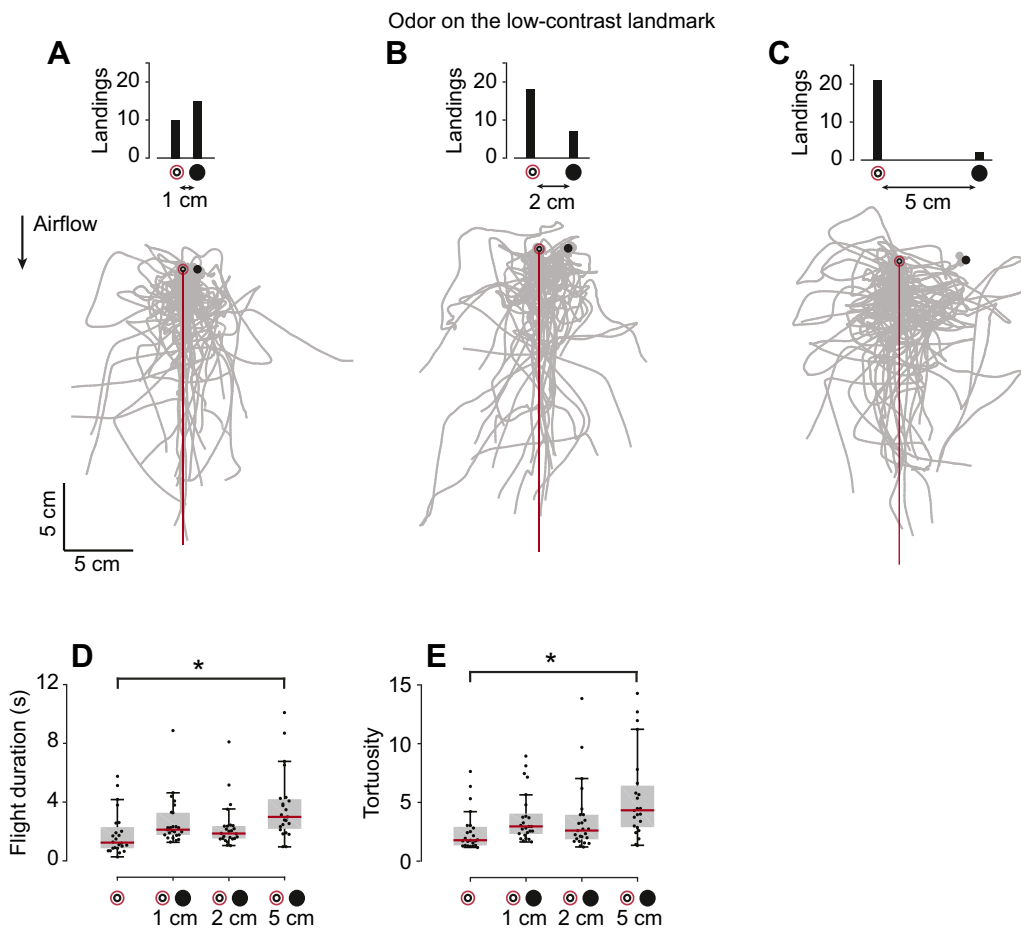

Odor on the low-contrast landmark

Fig. 3. Landing preference and flight behavior in the presence of segregated odor and visual cues. (A–C) Flight trajectories (gray) in the presence of an odorous low-contrast landmark separated from a non-odorous high-contrast landmark by (A) 1 cm (N=25), (B) 2 cm (N=25) and (C) 5 cm (N=23). Bar plots above the trajectories indicate the absolute number of landings on each landmark. The presence of a high-contrast non-odorous landmark 5 cm from the low-contrast odorous landmark significantly increased both (D) flight duration and (E) tortuosity prior to landing.

smoke to ensure that the presence of objects within the wind tunnel did not introduce additional turbulence. Using flow visualization techniques, we measured the plume width to be 1.6 cm (Fig. 1B,C; Fig. S1; see Appendix and Movie 1 for details).

Data acquisition and analysis

Two cameras simultaneously recorded the fly's trajectory, allowing reconstruction of their 3D position using software custom-written in MATLAB (Hedrick, 2008; Mathworks, Natick, MA, USA). Extracted trajectories were processed through a fourth-order low-pass filter (Butterworth) with a 30 Hz cut-off (see Appendix). The high-contrast landmark subtended an angle of ∼5 deg at ∼8 cm distance, ensuring that this angle exceeded the smallest inter-ommatidial angle (∼4.5 deg in *Drosophila*; Gonzalez-Bellido et al., 2011). We analyzed only flight trajectories within an 8 cm radius of the odor source, and calculated several flight variables of which four best captured the spatio-temporal features of their trajectories (Fig. 1D): (1) flight speed: the average speed of the fly; (2) flight duration: the total duration of flight trajectories; (3) hover duration: the total time spent by a fly at speeds less than 37.5 mm s^{-1} – this cut-off represents a value closer to true hover (less than 5% body

length traversed over a single wingbeat duration of ∼4 ms); (4) tortuosity: the ratio of the total distance traveled by a fly to its net displacement.

Because flight activity near the odor source is non-uniform as a result of steady deceleration, we segmented the volume in front of the odor source into 784 cm^3 (1 cm×28 cm×28 cm) cuboids (Fig. 1D). For each treatment, we separately analyzed free-flight behavior in each spatial zone and statistically compared changes in flight variables across these segments. Calculated values of flight trajectory variables were not normally distributed (Lilliefors test for normality at $P<0.05$) and did not have equal variances (Bartlett test for equal variance at $P<0.05$). To detect whether any groups were statistically different (at $P<0.05$) from other groups, we used a non-parametric Kruskal–Wallis test. If this test indicated significant differences between groups, we used a *post hoc* Nemenyi test to pairwise compare each group to identify which specific treatments differed from each other. We used the chi-squared test to ascertain whether the observed landing distributions were directed towards the odorous landmark or whether they occurred by random chance (uniform distribution). The corresponding P-values for the chi-squared test in all treatments are shown in Table 1.
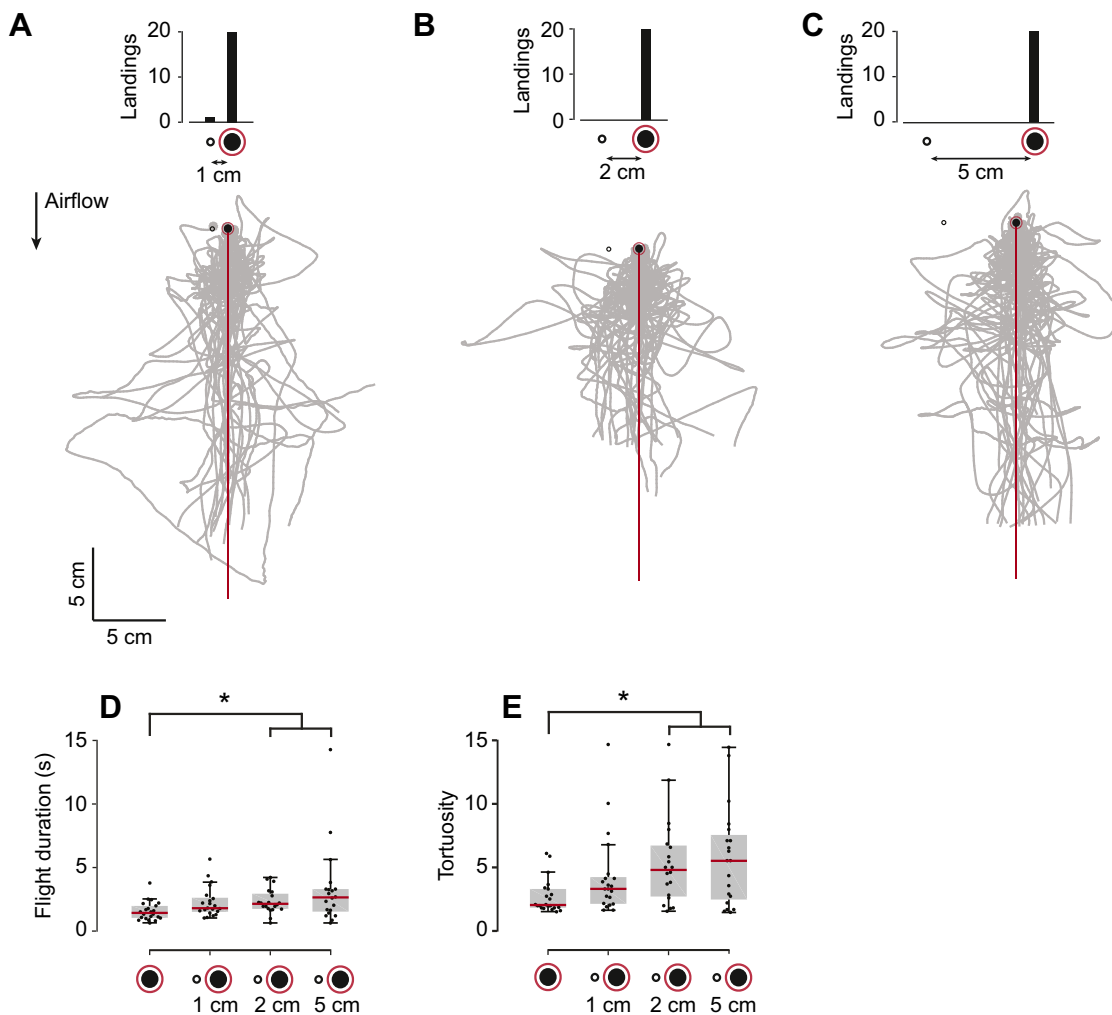

Fig. 4. Landing preference and flight behavior on a high-contrast odorous landmark separated from a low-contrast non-odorous landmark. (A–C) Flight trajectories (gray) in the presence of a high-contrast odorous landmark and a low-contrast non-odorous landmark at (A) 1 cm (*N*=21), (B) 2 cm (*N*=20) and (C) 5 cm (*N*=20) separation. Bars above plots indicate landing preferences on each landmark. (D,E) The presence of a low-contrast non-odorous landmark near the high-contrast odorous landmark significantly increased both (D) flight duration and (E) tortuosity prior to landing.

RESULTS

The presence of odor cues alters the response of flies toward visual landmarks

When presented with a high-contrast non-odorous landmark, flies maintained an upwind heading but did not approach the visual landmark (Fig. 2A; see also Maimon et al., 2008). However, the landmark became attractive to flies when it emitted an appetitive odor (Fig. 2B,C). Before landing, flies aligned themselves along the plume axis as they approached the high-contrast and low-contrast odorous landmark (Fig. S2B,C), whereas their flight towards the non-odorous landmark was not directed along any specific axis (Fig. S2A). Flies also flew at significantly slower speeds (Fig. 2D), for longer duration (Fig. 2E), and their trajectories were more tortuous (Fig. 2F) in the presence of odor. In addition, their hover duration was significantly greater near an odorous landmark than a non-odorous landmark (Fig. 2G). Thus, presence of odor increased flight activity in general. Average speed, flight duration, tortuosity and hover duration for flies approaching high- versus low-contrast odorous landmarks were not statistically different from each other (Fig. 2D–G). Thus, odor cues were necessary and sufficient for flies to seek out a visual landmark, including those of low contrast.

Flies integrate odor and visual cues prior to landing

We next presented flies with two choices for landing – a low-contrast and a high-contrast landmark – of which only one was odorous. The two landmarks were separated by 1, 2 or 5 cm in separate treatments. In the first set of experiments, odor was paired with a low-contrast landmark (Fig. 3), and in the second set, with a high-contrast landmark (Fig. 4). If presence of odor alone determines the landing site, then landings should occur only on the odorous landmark regardless of other landmarks. However, flies showed some likelihood of landing on the high-contrast non-odorous landmark rather than the low-contrast odorous landmark, with the frequency of incorrect landings gradually decreasing with increased separation between the objects (Fig. 3A–C, Table 1). In contrast, when given a choice between high-contrast odorous versus low-contrast non-odorous landmark, flies always chose the former (with a single exception, see Fig. 4A), regardless of separation between landmarks (Fig. 4A–C, Table 1). Thus, co-occurrence of an odor cue with a single high-contrast visual cue is sufficient for flies to land on that object.

The flight duration and tortuosity values for flies approaching a high-contrast non-odorous landmark placed 5 cm from a low-contrast odorous landmark were significantly greater than those for flies approaching a single low-contrast odorous landmark, but flight parameters were similar for smaller separations (Fig. 3D,E). Thus, the flies spent time investigating the high-contrast landmark when it was not odorous, and their search strategy was influenced by neighboring landmarks. The presence of a low-contrast non-odorous landmark affected trajectories of the flies when placed near a high-contrast odorous landmark, and flight duration and tortuosity were significantly greater at separations of 2 and 5 cm (Fig. 4D,E). Thus, the low-contrast landmark was visible and its presence influenced flight trajectories. Flies maintained a similar speed and hover duration when approaching the combination of an odorous low-contrast landmark and a non-odorous high-contrast landmark (Fig. S2D,E) or an odorous high-contrast landmark and a non-odorous low-contrast landmark (Fig. S2F,G), regardless of their separation.

We next pooled flight parameters for all cases in which the odorous landmark was low contrast (blue bars, Fig. 5) and compared with cases in which the odorous landmark was high contrast (red bars, Fig. 5). Flies flew consistently slower (Fig. 5A) and hovered more (Fig. 5B) for a similar duration (Fig. 5C) with the
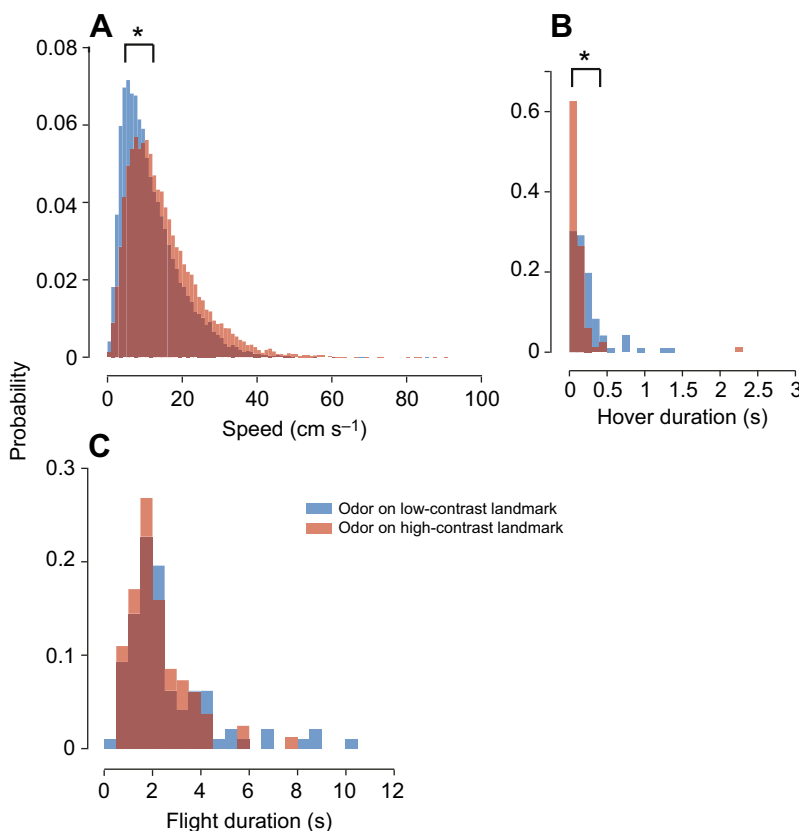
Fig. 5. Probability distributions of flight variables pooled based on visual contrast of the odorous landmark. Probability distributions for (A) speed, (B) hover duration and (C) flight duration in the presence of a low-contrast odor source and a high-contrast odor source. Flight variables for low-contrast odorous landmark treatments were pooled (experiment 2; $N=97$) and compared with pooled variables from high-contrast odorous landmark treatments (experiment 3; $N=83$). Frequency distribution was obtained by binning flight variables (speed: 1 cm s^{-1} bins; hover duration: 0.1 s bins; flight duration: 0.5 s bins). Because sample sizes in the two experiments were different, we normalized occurrences in each bin with total occurrences for each experiment to obtain probability distributions from frequencies. Statistical comparisons were conducted directly on raw flight variables. Asterisks indicate statistically significant differences in flight variables ($P<0.05$, Kruskal–Wallis test).

low-contrast odorous landmark treatment (blue) than with the high-contrast odorous landmark treatment (red).

Thus, flies rely on synchrony of visual and odor stimuli to make a landing decision; visual landmarks do not elicit landing behavior unless accompanied by odor cues and vice versa. Moreover, visual contrast of non-odorous landmarks influences landing decisions, especially near the odor plume. This means that flies would have difficulty in finding an odor source in a visually cluttered environment. We tested this prediction in the following experiments.

Visual clutter density influences landing on odor sources

We presented flies with multiple high-contrast landmarks, only one of which was odorous. This created a visual clutter of several identical landmarks from which flies had to choose the correct odor source. We tested their odor-localizing ability in high- and low-density visual clutter. The high-density visual clutter treatment contained seven identical landmarks at 1 cm separation (Fig. 6A), whereas low-density visual clutter treatment contained either seven (Fig. 6B) or three landmarks (Fig. 6C) at 3 cm separation.

Flies were more likely to land on non-odorous landmarks when visual clutter density was greater (Fig. 6A–C, Table 1), with the majority of incorrect landings occurring on landmarks immediately adjacent to the odor source. Increased separation between odorous and non-odorous landmarks elicited more elaborate search trajectories (Fig. 6A–C); flies flew significantly slower (Fig. 6D), and increased

their flight duration (Fig. 6E) and hover duration (Fig. 6F) when visual clutter density was low. Surprisingly, flies in high-density visual clutter flew at speeds statistically indistinguishable from those for the single high-contrast odorous landmark treatment (Fig. 6D) and their tortuosity was not affected by the addition of multiple landmarks (Fig. S2H). Thus, in low-density visual clutter, flies searched more and longer for the odor source, and were more likely to find the correct odorous object than in high-density clutter.

Flies decrease their speed when they encounter odor

In the above experiments, flies consistently decreased their speed upon intercepting the odor plume. How does an odor plume encounter influence their flight on an instantaneous basis? To address this question, we determined the approximate geometry of an odor plume using smoke visualization (Fig. 1B,C; Fig. S1, Movie 1; see Appendix), and examined trajectories of flies immediately before and after plume encounters within our region of interest. To avoid confounding flight-related versus landing-related speed changes, we analyzed data for only those flies that first encountered the odor plume at distances greater than 4 cm from the landing point (odor encounters in the range 4–8 cm from source). A comparison of the speed of individual flies 250 ms before and after they encounter the odor plume revealed that their speed decreased ~50–100 ms after plume encounter (Fig. 7A–C; see Fig. S3 for more trajectories). These speed changes are not part of their regular repertoire, as shown by the absence of changes in speed in the
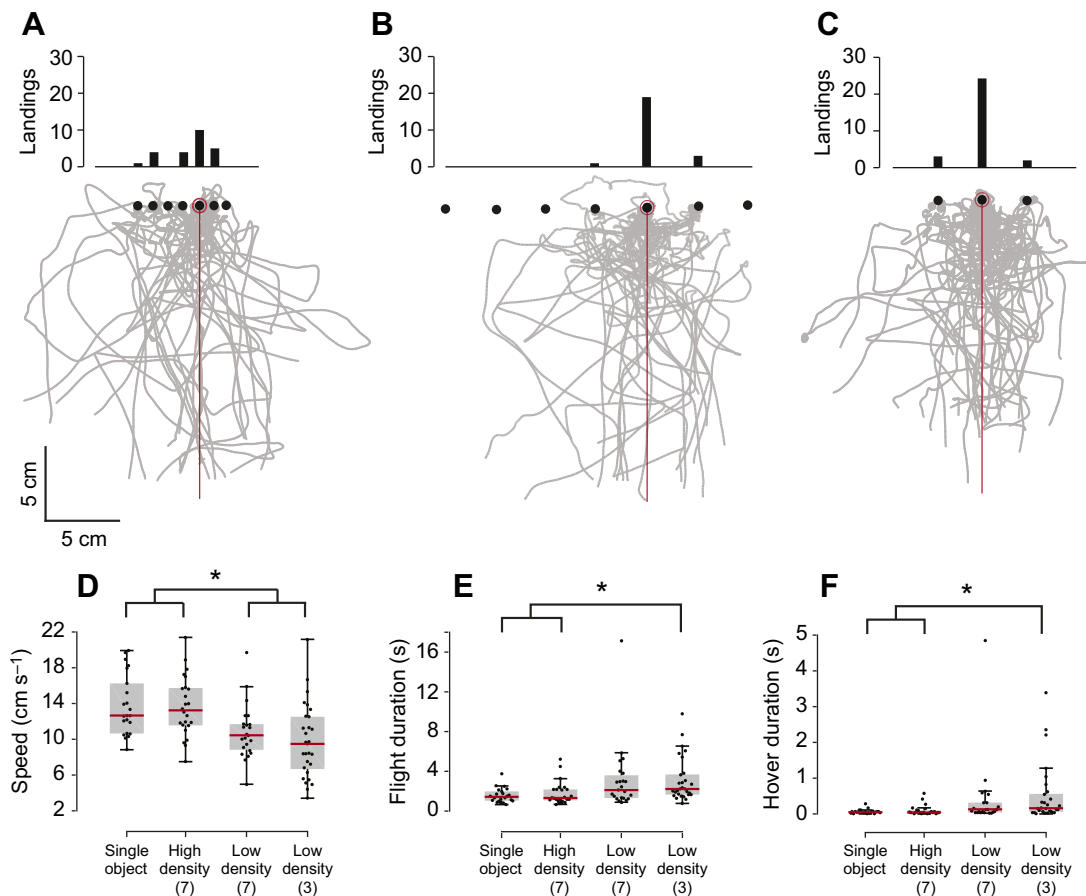

Fig. 6. Odor tracking in visual clutter. (A–C) Flight trajectories in the presence of (A) high-density visual clutter (seven landmarks at 1 cm separation, *N*=24), (B) low-density visual clutter (seven landmarks at 3 cm separation, *N*=23) and (C) low-density visual clutter (three landmarks at 3 cm separation, *N*=29). (D–F) Flies significantly decreased (D) speed and increased (E) flight duration and (F) hover duration in the presence of low-density visual clutter.
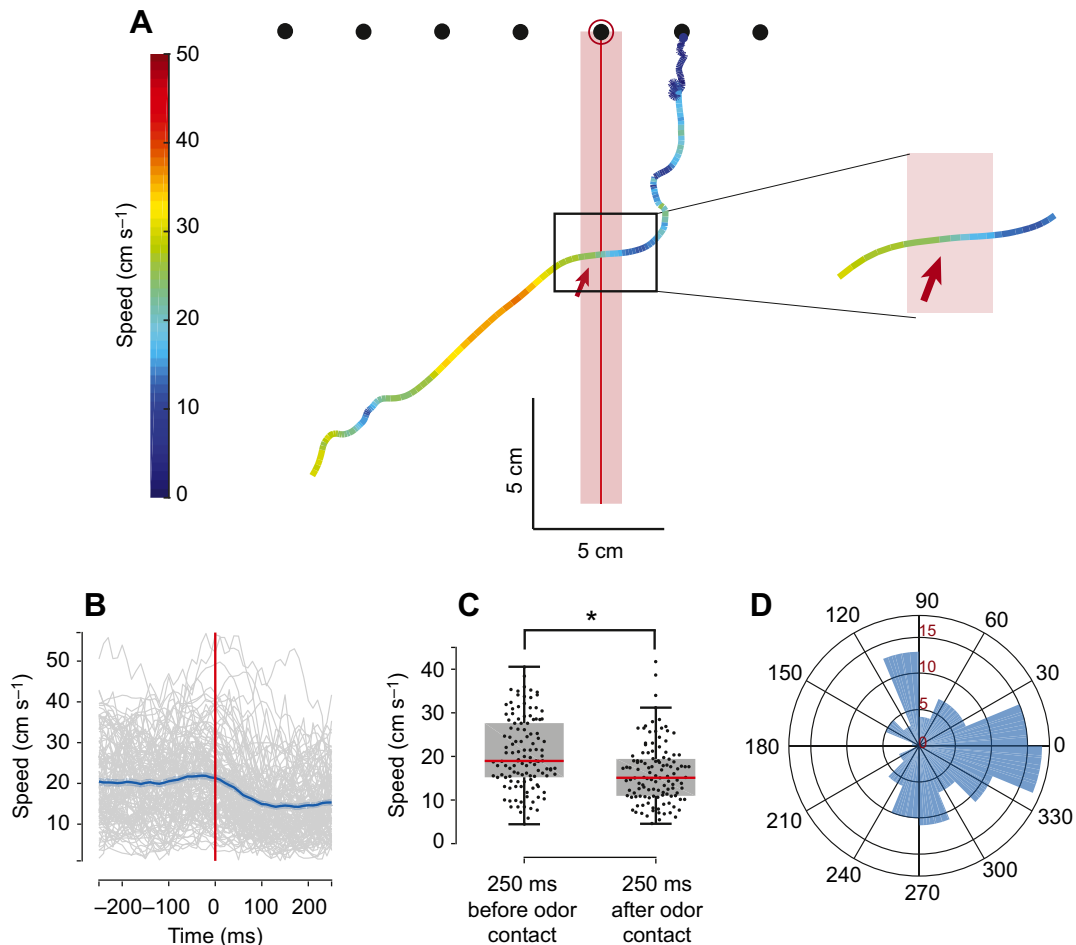
Fig. 7. Odor encounter modulates the speed of flies. (A) Sample trajectory of a fly following an odor contact with the plume (red bar of 1.6 cm width), and an enlarged view (inset). Colors represent speed (see color bar). (B) Speed at first odor contact shown in a 500 ms window centered on the likely odor contact (250 ms before and 250 ms after odor contact). N=120. Individual speed–time curves (gray) are overlaid by mean (blue) and s.e.m. (light blue) (N=120). To avoid confounding effects of speed changes due to landing responses, only flies that encountered the odor at least 4 cm before landing were used in the analysis. A decrease in flight speed was observed less than 100 ms after the first odor encounter, but not in regions before or after the first odor encounter (Fig. S4). (C) Mean speed after the first odor encounter was significantly lower than speed before the encounter. (D) Polar histogram plot showing the orientation of flies at first odor encounter within the region of interest, 4–8 cm from the odor source (angular bin 22.5 deg). Concentric circles represent frequency (red numbers). Circular distribution is non-uniform (Hodges–Ajne test for circular uniformity, P=0.00004), with a mean orientation of −10.75 deg and circular standard deviation of 84.3 deg.

250 ms duration before and after an arbitrary time point 1000 ms pre- and post-odor interception in each fly (Fig. S4A–C; Fig. S4D–F). However, their speed distribution shifted to lower speeds (Fig. S4G), resulting in a decrease in the mean speed immediately after plume encounters (Fig. 7C).

Is the speed decrease in these flies elicited by the expanding visual landmarks? To address this possibility, we measured the orientation of a subset of flies that slowed down immediately following plume entry. All flies generally head forward as a result of anemotaxis. However, if approaching visual landmarks trigger a drop in speed, we would expect the fly to be directed towards the visual landmarks with low standard deviation. Conversely, a greater standard deviation in heading during speed decrease indicates that the approaching visual landmarks exert a relatively minor influence on speed change. Our observations are consistent with the latter prediction; their heading was directed forward (Fig. 7D; mean angle −10.7 deg) and was non-uniformly distributed (Hodges–Ajne test for circular uniformity, P=0.00004; Zar, 1999), but with a circular standard deviation of 84.3 deg (sensu Mardia, 1972), with approximately 60% of the flies flying at an angle of 45 deg or

greater relative to a line joining the fly to the nearest visual landmark (also see Fig. S3).

Flies can localize odor sources in the absence of airflow
Do flies alter their search strategies in still air when directional cues are not clear? In the absence of airflow, fly trajectories were not directionally biased but were distributed uniformly around the low-contrast odor source (Fig. 8A). Such flies were significantly faster (Fig. 8B) and hovered less (Fig. 8C) than those in the presence of airflow, although some flight parameters such as flight duration and tortuosity remained unchanged (Fig. S5A,B). Their flight speed in still air (red line, Fig. 8D) was consistently greater than that of flies tracking odor plumes in airflow (blue line, Fig. 8D).

Is the ability of flies to distinguish odorous versus non-odorous landmarks impaired in still air? To address this question, we presented flies with two high-contrast landmarks, only one of which was odorous. These landmarks were separated by 1, 2 and 5 cm, respectively (Fig. 9A–C). The performance of flies in identifying the odorous landmark was worse when separation between the odorous and non-odorous landmarks was 1 cm (only 60% correct
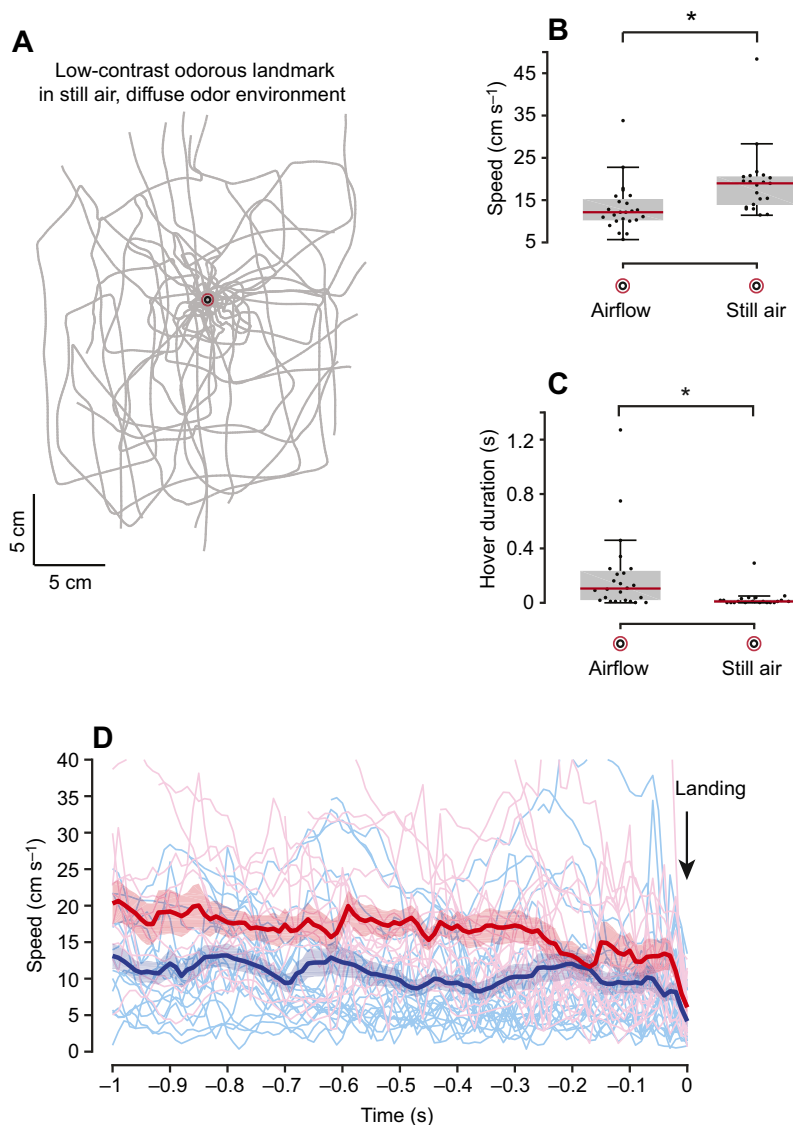
A

Low-contrast odorous landmark
in still air, diffuse odor environment

5 cm

5 cm

B

C

Fig. 8. Odor-tracking behavior in still air. (A) Trajectories of flies in the presence of an odorous low-contrast landmark in the absence of airflow (N=21; see Materials and methods for details). Flies flew at significantly greater speeds (B) and hovered less (C) when airflow was absent. (D) Speed of odor-tracking flies in the absence (red) versus presence of an airflow cue (blue) for 1 s before landing. Light-colored lines indicate speeds of individual flies and thick lines indicate their respective means, the shading around which is the s.e.m.

D

landings, Fig. 9A), but improved when separation was increased (75% and 84.2% correct landings for separation of 2 and 5 cm, respectively, Fig. 9B,C). Flies traveled for longer durations (Fig. 9D) with greater tortuosity (Fig. 9E) at 2 cm separation than at 5 cm. However, their speed and hover duration were not significantly different for any arrangement of these objects (Fig. S5C,D).

In still air, when flies had to find a low-contrast odorous landmark separated from a high-contrast non-odorous landmark by 5 cm, they landed on both objects with roughly equal probability (57% correct landings; Table 1, Fig. 10A). In contrast, they could very reliably find a high-contrast odorous landmark when it was separated from a low-contrast non-odorous landmark (95% correct landings; Fig. 10B). The flight parameters in these two cases were not significantly different from one another (Fig. 10C–F). Thus, their choice was substantially biased toward high-contrast visual objects in the absence of a plume to guide them. It is also illustrative to compare these treatments with those for a similar object arrangement in the presence of airflow (Figs 3C, 4C) and odor plume. The presence of the plume substantially enhanced the ability of flies to find the odor source, underscoring its importance in odor tracking. In both moving and

still air, flies tended to hover in front of objects just before landing (compare Fig. S5E,F with Fig. S5G,H).

Thus, synchronous odor and visual cues are also essential for odor source location in still air, for which odor spreads largely through diffusion and forms a gradient, which flies appear to successfully track.

DISCUSSION

Locating an odor source in a visually cluttered environment is a complex task which requires input from multiple senses, including the visual and olfactory modalities, which then drive motor responses (e.g. Raguso and Willis, 2002; Frye et al., 2003; Dekker and Cardé, 2005). For flying insects, this means controlling flight in three dimensions in environments that are typically turbulent (Murlis et al., 1992; Yorozu et al., 2009; Fuller et al., 2014). Because proper identification of odor sources is essential to gain access to food and mates, the question of how insects solve this problem has been of central importance to biologists over several decades (e.g. Kennedy, 1983; Raguso and Willis, 2002). What basic rules guide the flies to odor sources under visually ambiguous conditions? Previous studies outlined several
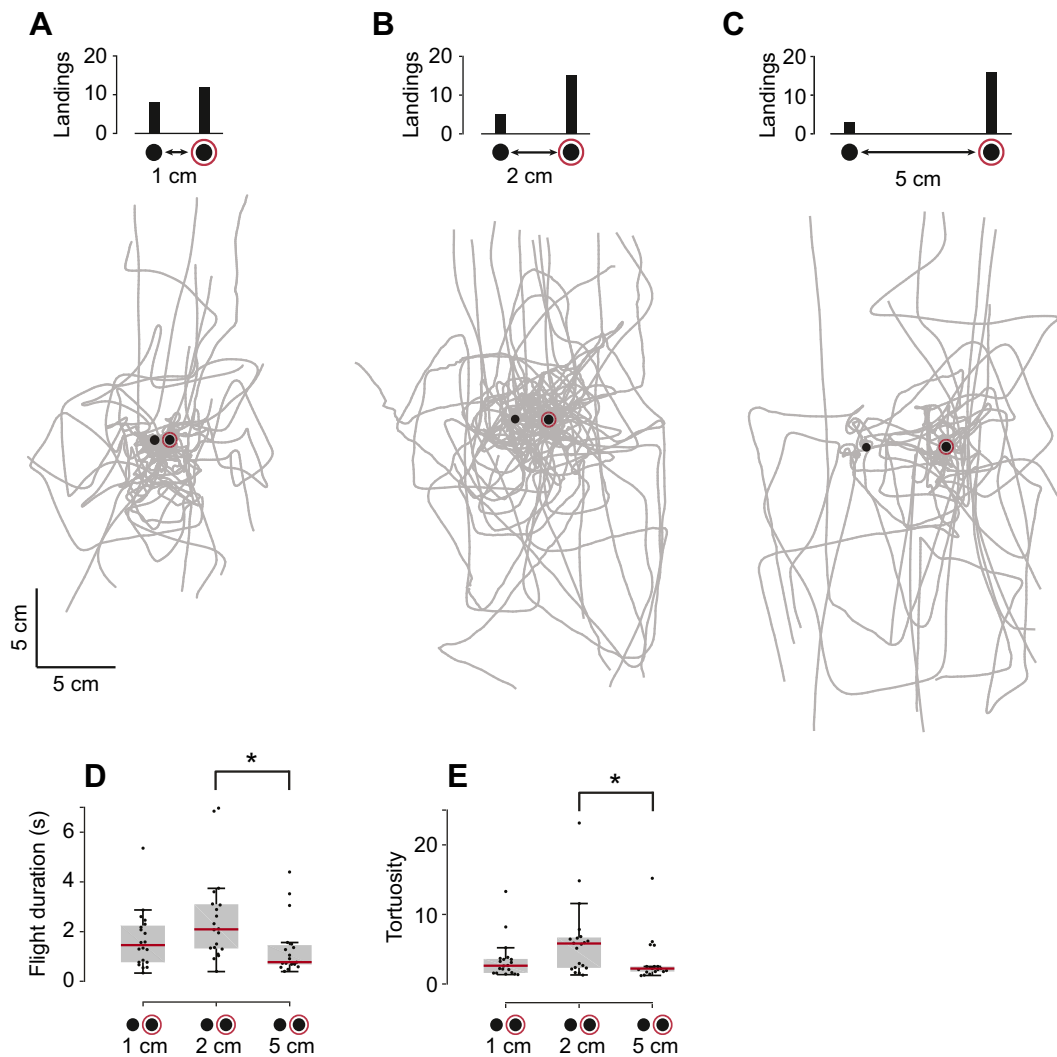
Fig. 9. Landing preference and flight behavior in the presence of paired odorous and non-odorous high-contrast landmarks, in still air. (A–C) Flight trajectories towards a high-contrast odorous landmark paired with an identical non-odorous landmark in still air. These landmarks were separated by (A) 1 cm (N=20), (B) 2 cm (N=20) and (C) 5 cm (N=19) (see Materials and methods for details). Bar plots in the upper panel show the number of landings on each object. (D,E) Flies significantly decreased their (D) flight duration and (E) tortuosity when the paired odorous and non-odorous high-contrast landmarks were separated by 5 cm.

specific behaviors including optomotor anemotaxis, cast-and-surge maneuvers, odor-guided salience changes, etc., which enable insects to arrive close to an odor source (e.g. Kennedy and Marsh, 1974; Vickers, 2000; Chow and Frye, 2008). They also revealed the importance of visual cues in conjunction with odorous cues in guiding the insects to the odor source (Aluja and Prokopy, 1993; Henneman et al., 2002; Raguso and Willis, 2002; Balkenius et al., 2006; Campbell and Borden, 2006; Goyret et al., 2007; Goyret et al., 2008; van Breugel and Dickinson, 2014; van Breugel et al., 2015). Our study sought to specify how insects, having arrived near an odor source, pinpoint its precise location from among many possibilities in the decisive moments before landing.

Odor resolution is vision dependent
A key finding of this study is that when flies encounter an odor plume that indicates the presence of a potential food source, they decrease their speed with a latency of under 100 ms (Fig. 7A–C). This behavior may serve two functions: first, it provides the flies with greater sampling time to determine the spatio-temporal co-

occurrence of odor and visual feedback. Second, it increases the probability of repeated odor encounters, which would enable flies to determine the general orientation of an odor source. These observations contrast with previous studies which showed that flies increase their ground speed approximately 190±75 ms following a plume encounter (Budick and Dickinson, 2006; van Breugel and Dickinson, 2014; Bhandawat et al., 2010). However, in those studies there were no visible landmarks at the time of odor encounter, and hence landing was not imminent. In contrast, the trajectories reported here were derived from a region that was between 4 and 8 cm from the nearest visible odor source. Thus, they occur when the flies are >4 cm from the nearest visual object, and in many cases when the flies are not headed towards these visual objects, which rules out the possibility that the step change in speed occurs as a result of expanding visual stimuli. Minor differences in the methodology between the experiments [here, the odorant was apple cider vinegar and the airflow was 0.1 m s^{-1} whereas, the odorant was banana yeast extract in Budick and Dickinson (2006) and ethanol in van Breugel and Dickinson (2014) and their airflow
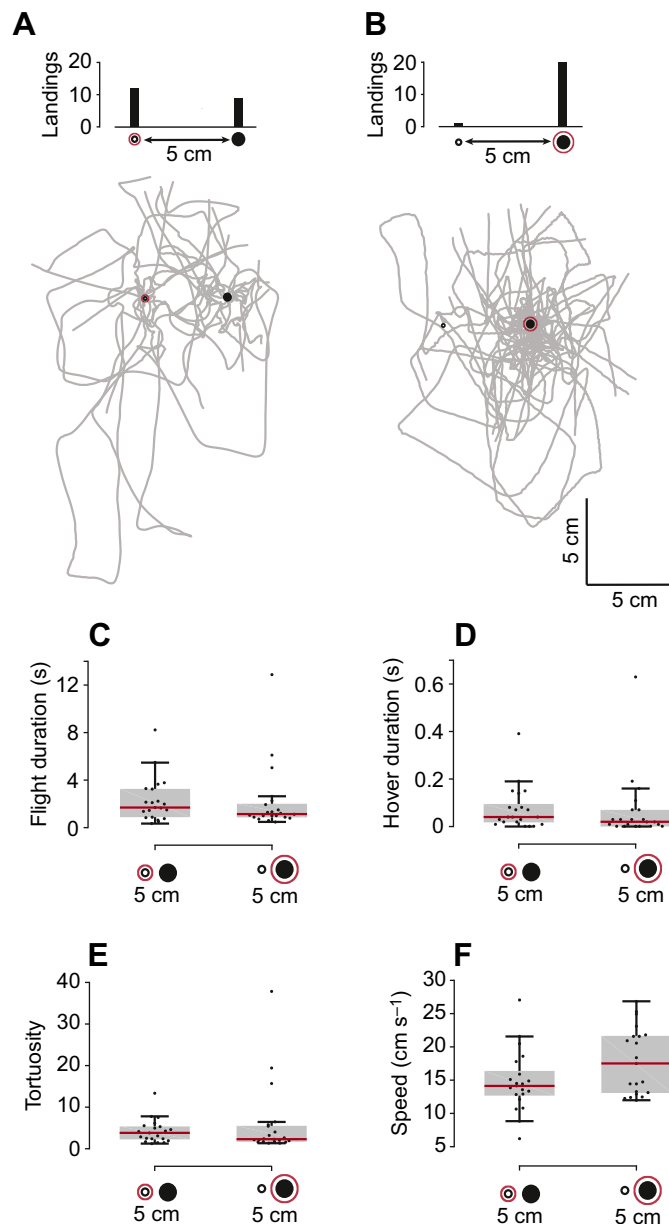
Fig. 10. Landing preferences for low- versus high-contrast landmarks in still air. (A,B) Flight trajectories (gray lines) for (A) an odorous low-contrast landmark presented in combination with a non-odorous high-contrast landmark (N=21) versus (B) an odorous high-contrast landmark presented with a non-odorous low-contrast landmark (N=21), separated by 5 cm in both the treatments. (C–F) Comparisons of (C) flight duration, (D) hover duration, (E) tortuosity and (F) speed between the above two treatments revealed no statistical differences (Kruskal–Wallis test at 95% level of significance).

value was 0.4 m s^{-1}] also do not explain the reversal of the behavior in flies after odor encounter. More likely, the odor encounter triggers a behavioral switch in flies causing them to slow down and seek visual objects, even though these have no inherent salience when odor was absent (Fig. 2A; see also Budick and Dickinson, 2006). In addition to visual landmarks, the behavior of flies after an odor encounter may also be influenced by changes in the structure of the plume envelope as it propagates downwind.

We also found an increased bias towards objects of higher visual contrast that are situated in the immediate vicinity of the odor source

(Figs 3A–C, 6A–C). This is consistent with previous experiments in *Drosophila* (van Breugel and Dickinson, 2014), mosquitoes (van Breugel et al., 2015) and the hawkmoth, *Manduca sexta* (Goyret et al., 2007). The bias towards high-contrast objects means that flies may sometimes incorrectly identify the odor source location if it does not exactly overlap with a visual landmark (Fig. 3A). However, when the two objects are sufficiently separated, flies are more successful at correctly identifying the odor source location (Fig. 3C). Thus, flies depend on the spatiotemporal co-occurrence of visual and odor cues to identify the odor source, and their odor resolution is vision dependent.

In the presence of multiple landmarks (visual clutter), flies initiate a search behavior characterized by slower speed, increased tortuosity and longer flight/hover durations (Figs 3D,E, 6D–F). This may help the fly to ascertain the co-occurrence of visual and odor cues by allowing more time to process the odor. The limited resolution of their compound eyes may impact the flies' ability to correctly pinpoint the odor source within a high-density clutter environment (Fig. 6A). Their search behavior is significantly enhanced when the location of the landmark does not match with the odor cue. In contrast, a single odorous landmark does not elicit an elaborate spatial search. Instead, flies steadily decrease their distance from the odor plume axis while approaching the target, thus closing in on the odor source, regardless of a high- (Fig. S2B) or low-contrast (Fig. S2C) landmark. These findings demonstrate the dominant influence of visual landmarks during odor searches, which are especially important in natural scenarios.

Flies use a different strategy for odor tracking in the absence of airflow

Although airflow is an important cue for odor-seeking insects (e.g. Kennedy and Marsh, 1974; Budick and Dickinson, 2006; Willis and Arbas, 1991), flies could also successfully track down an odor source in still air (Figs 8–10). In still air, odor propagation is isotropic and generates uniform concentration gradients around the odor source. These gradients may be locally disturbed by self-induced flow from flapping wings (Sane and Jacobson, 2006), possibly aiding odor detection (Loudon and Koehl, 2000). Without airflow to break the odor symmetry, flies approach the odor source equally from all directions (Fig. 8A). They fly at faster speeds (Fig. 8B) and hover less (Fig. 8C) as they steadily approach the odor source, as also reported in mosquitoes tracking CO_2 in still air (Cardé and Lacey, 2012). This alternative strategy (summarized in Fig. 11) is robust because it allows the majority of flies to successfully find the correct odor source from two visually identical objects separated by 2 cm or more (Fig. 9A–C, Table 1).

Do flies have an olfactory working memory?

Of the flies that tracked odor plumes, 21% landed incorrectly on an object that was not the odor source; 44% of the flies that landed incorrectly took longer than 1 s after odor encounter to land (see Fig. S6A–C for examples). Because flies ignore visual landmarks in the absence of an odor encounter (Fig. 2A; see also Budick and Dickinson, 2006; van Breugel and Dickinson, 2014), the above observation shows that the flies undergo a 'switch' in their behavior to be attracted to visual landmarks after odor encounter. Moreover, these flies continued to be attracted to visual landmarks even after leaving the odor plume, which suggests that the switch in their behavior after odor encounter is sustained over time. These observations suggest the presence of an 'olfactory working memory', which recalls previous odor encounters, and ensures that flies continue their search for odor sources even when odor cues become temporarily extinct. For spatial
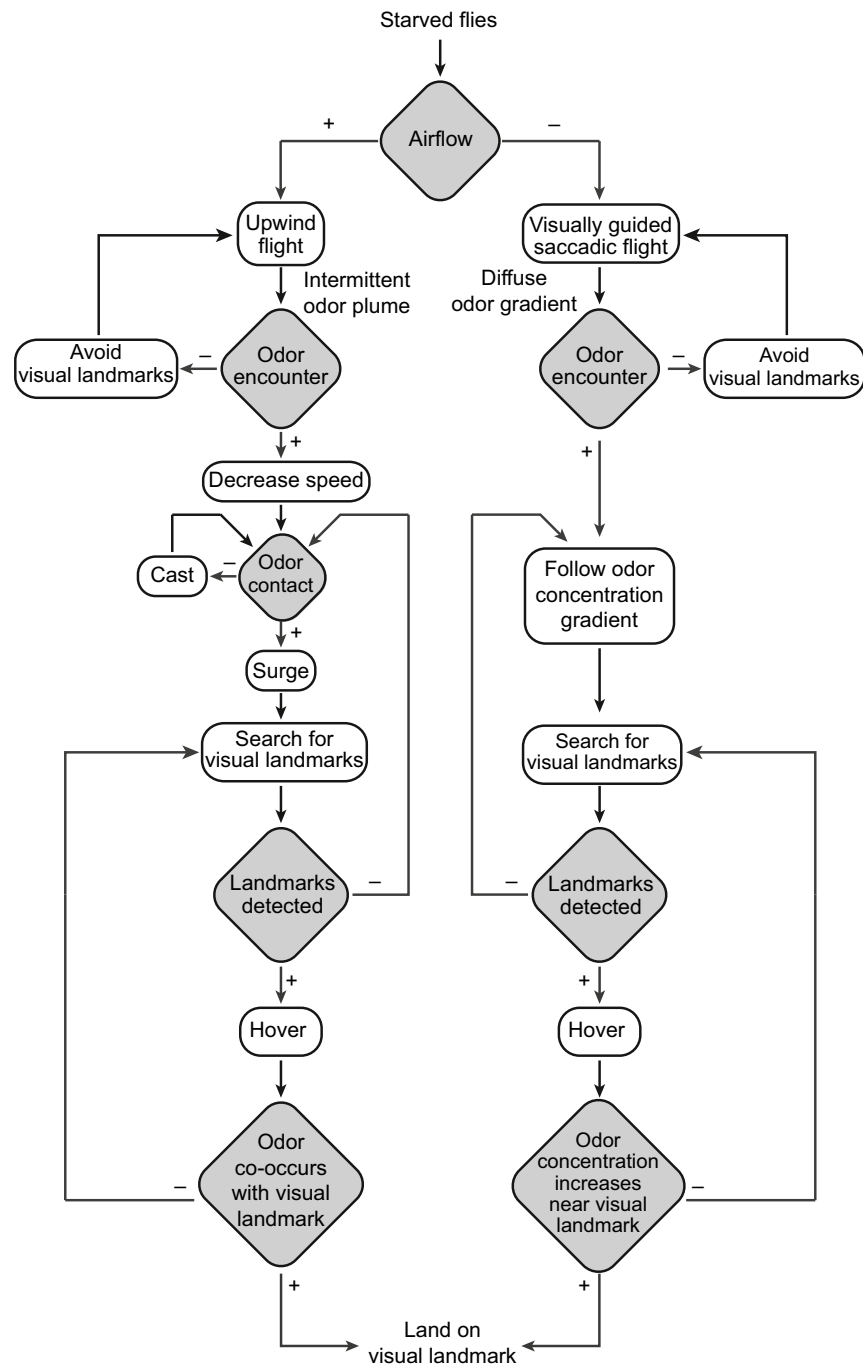
Fig. 11. Flowchart of odor-tracking strategies in flies. A flowchart synthesized from previous literature and the current study showing distinct strategies employed by flies in the presence (left) or absence (right) of airflow. + signifies the presence and − the absence of the associated cue. Gray diamonds show sensory cues and open rectangles show motor responses.

navigation tasks, the existence of a spatial working memory has been well demonstrated in the case of visual tracking, in which *Drosophila* flies moving between two vertical poles maintained their direction for several seconds after these landmarks became extinct or reappeared elsewhere (Neuser et al., 2008). A fundamental requirement for olfactory working memory is to successfully register an odor encounter, and display behavior that suggests the recall of this encounter (for examples with airflow, see Fig. S6A–C; and for examples in the absence of airflow, see Fig. S6D–F). More controlled studies are required to quantify the duration over which this memory lasts, and where in the brain it resides.

Visual and olfactory specialization in insects
From an evolutionary perspective, how do certain insects evolve to specialize on specific fruits or plants in their natural surroundings? Examples of specialists have been reported in *Drosophila*, including *D. sechellia*, which forages on a fruit (*Morinda citrifolia*; Higa and Fuyama, 1993; Jones, 2005) that is toxic to related *Drosophila* species. Similarly, *D. pachea* are found on the rotting stems of the cactus *Lophocerus schottii* (Heed and Kircher, 1965). The bias for high-contrast visual cues *vis-a-vis* odor cues suggests the testable hypothesis that specialist insects require specific olfactory and visual cues. Such preferences have been demonstrated, for instance,

in the Tephritid fly, *Rhagolettis pomonella* for apple-like stimuli (e.g. Aluja and Prokopy, 1993) in which an attractive odor stimulus makes specific landmarks in the surroundings attractive, biasing their landing decisions (Fig. 2B,C). If flies or other insects have specialized on odor objects of specific visual signatures, then we expect to see a strong bias towards objects of specific shape or color, or towards specific odor stimuli irrespective of their visual appearance. Our study demonstrates that flies make a weighted decision between odor and visual stimuli, and provides the methodology to test this hypothesis.

Conclusions

Our aim was to understand how flies precisely determine the source of an attractive odor amidst visual clutter. We show that fruit flies (*Drosophila melanogaster*) use both olfactory and visual cues while searching for odor sources in moving or still air. In moving air, odor molecules are packaged into plumes. In contrast to previous studies in which flies increased their forward speed post-plume encounter far from visible landmarks, our study shows that they abruptly decrease speed if plume contact occurs closer to visual landmarks. Thus, flies pinpoint the odor source based on the synchrony of visual and odor cues. Although odor-seeking flies ignore visual objects in the absence of odor, their behavior 'switches' upon encountering an odor plume to enhance their attraction towards the nearest high-contrast visual landmarks. Such flies continue seeking the odor source even after they are out of a plume, suggesting that the memory of an odor encounter persists for some time. In still air, an odor plume is absent and flies adopt a different strategy, which may involve flying down an olfactory gradient towards visual landmarks. Whether in the presence or the absence of airflow, a large majority (76%) of the 311 flies tracking multiple visual landmarks across 14 different treatments successfully identified the odor source, underscoring the robustness of combined strategies of plume or gradient tracking. These and previous results allow us to propose a conceptual model for two strategies of odor source identification (Fig. 11).

APPENDIX
Plume visualization

We visualized the plume by seeding the flow with smoke generated using incense. Although the heated smoke plume rises slightly compared with an unheated plume, this does not affect laminarity or plume width. The following treatments simulated odor plume conditions in all experiments: (1) capillary (i.e. low-contrast object); (2) capillary generating smoke, with a spherical bead (6 mm diameter, i.e. high-contrast object) at 1 cm; (3) capillary generating smoke, with a spherical bead at 2 cm; (4) spherical bead; and (5) spherical bead generating smoke, flanked by two beads at 1 cm.

We filmed the smoke plume at 24 frames s^{-1} using a calibrated high-resolution camera (Phantom VEO 640L, Vision Research), which directly viewed the object from above. The wind tunnel was set at 0.1 m s^{-1}. For each treatment, we filmed four trials saving a minimum of 100 frames per video, processed using Fiji software (Schindelin et al., 2012). By recursively subtracting background from each image, we obtained the averaged steady-state image of the axisymmetric plume. Undetected gaps in plume were interpolated using a piecewise Cubic Hermite spline. We filtered this image with a median filter to remove salt-and-pepper noise to obtain a binary image, which was digitized with a custom-written MATLAB code (Fig. S1A). We measured the plume width as a function of source distance by pooling the data at 1 mm resolution (Fig. S1B). The plume width saturated to become roughly cylindrical at 4–8 cm from the odor source. The neighboring spherical beads only slightly

varied the plume diameter between 1 and 1.6 cm, based on which we set 1.6 cm as the plume diameter in our calculations.

Data analysis

We calibrated our cameras using custom calibration objects, following the methodology discussed in Hedrick (2008). We then digitized the fly positions in videos to obtain the three-dimensional trajectories of flies. Both the videos and the digitized 3D coordinates are available on request. We processed the raw 3D coordinates, performed statistical tests and plotted the results using custom-written MATLAB codes. These codes are available at https://github.com/AbstractGeek/publication-supplementaries/tree/master/2017-Odor_source_localization_in_complex_visual_environments_by_fruit_flies.

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