A high-throughput behavioral paradigm for *Drosophila olfaction* - The *Flywalk*

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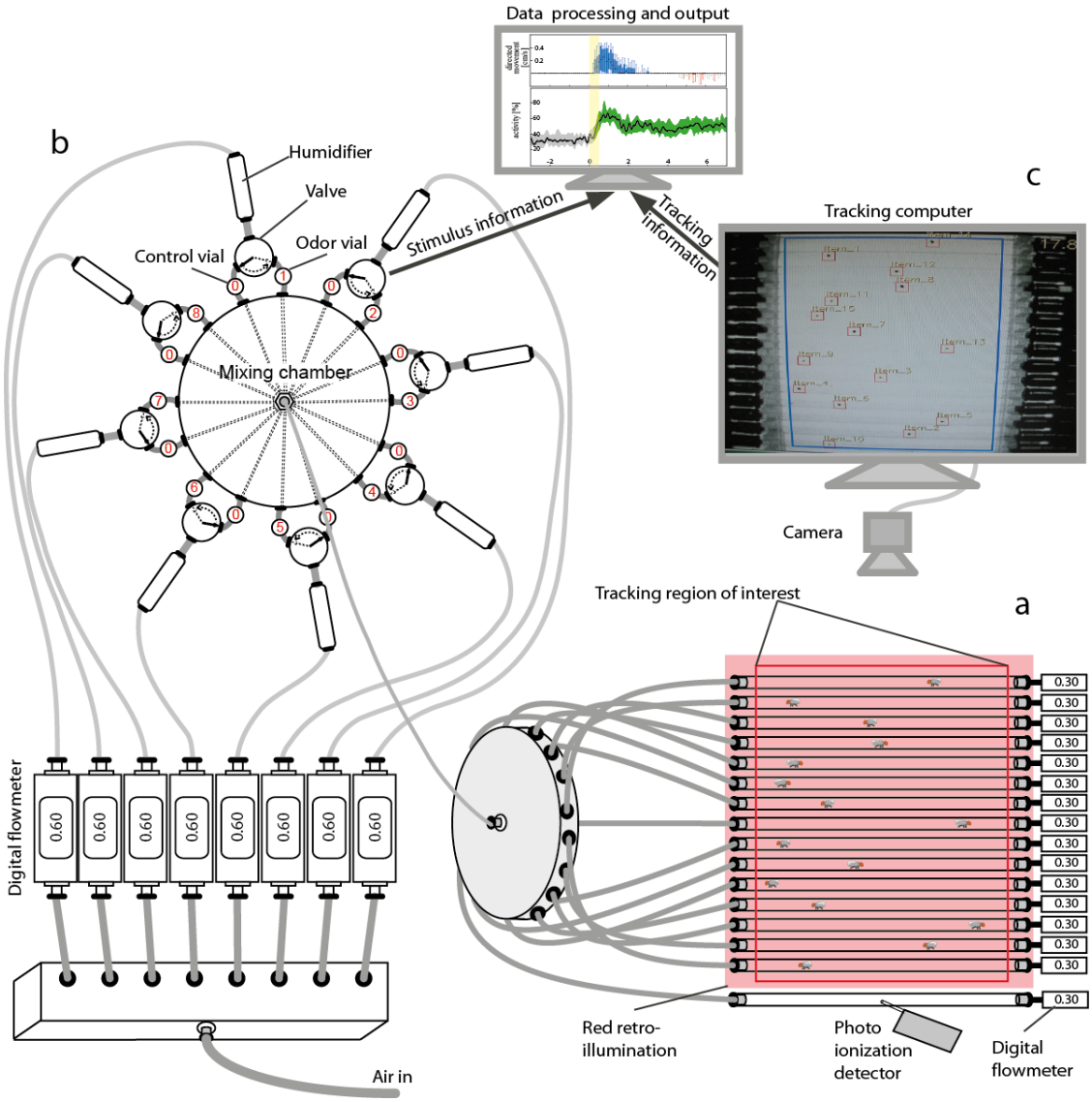
Supplementary Information

Experimental setup

The test arena consisted of 15 parallel glass tubes (Ø 0.8 cm; length, 18 cm; silanized to minimize olfactory contamination). Within each tube a single fly moved freely all along the length of the tube, but was precluded from escaping by meshes at both tube ends. The positions of the 15 flies were tracked by automatic tracking software (see below). In order to increase the contrast for the tracking camera, the tubes were placed on an electroluminescent foil (Reichel Elektronik, Germany). A red filter foil was placed between light foil and tubes allowing only light with wavelengths > 630 nm, which is invisible to *Drosophila*, to pass. Therefore, the laminar air flow carrying spaced odor pulses was the only external cue for the flies. The air flow and the odor pulses were produced by an odor delivery system (Olsson et al., 2011). This system provides a continuous flow of humidified air that can be loaded with pulses of up to eight different odors well-defined in stimulus concentration, onset and duration. Splitting the single outlet of the stimulus device into 15 parallel tubes and aligning the 15 air flows by digital flowmeters downstream of each tube ensured that identical and synchronized olfactory stimuli were moving through the tubes. For stimulus characterization by use of a photo ionization device, see section below.



Supplementary Fig. S1. Experimental setup. (**a**) In 15 parallel glass tubes individual flies are exposed to controlled air flow (speed, 18 cm/s; temperature, 25° C; humidity, 70%). Digital flowmeters downstream of each hermetically sealed tube ensured identical air flow in all tubes. Air flow is provided by a stimulus delivery system (**b**) which also generates odor stimuli well-defined in timing and concentration (Olsson et al.2010). Including a solvent control the stimulus delivery system generates up to eight different odor qualities. The stimulus delivery system communicates the stimulus information to a tracking system (**c**). Relative to the stimulus onset the tracking system records the positions of the fifteen flies before, during and

after stimulation. The timing of tracking system and stimulus delivery system allows the quantification of odor-evoked movements of individual flies. Retroillumination (>630 nm) of the tubes is provided by electroluminescent foil (filled red rectangle) to eliminate visual input for the flies. Flies positioned outside of region of interest (ROI, open red rectangle) are not tracked. A photo ionization detector (PID) connected to an additional tube allows to monitor the stimulus characteristic simultaneously.

All components of the stimulus device and the split disk between device and tubes were composed of Teflon (tubing), steel (stainless or nickel-plated; valves, flow meters and connectors) or polyether ether ketone (PEEK; blending and odorant chambers, split-up disk). These materials were chosen to minimize contamination of the system or air stream by component parts or chemical residue. In particular, PEEK (Parker TexLoc, USA) was chosen for its overall strength and chemical resistance to both organic and aqueous solvents. It also exhibits thermal resistance to temperatures reaching 200°C, which allowed for heated cleaning of the system to remove odorant or solvent residuals between experimental sessions.
The stimulus device was controlled with custom written software using Labview 8.5 (National Instruments, Austin, TX). Details regarding stimulus identity, onset time and duration were communicated via TTL pulses to the tracking computer.

Stimulus characterization

The tracking system outputs data whose evaluation is based on the exact calibration of the stimulus timing, i.e. when and how fast an odor pulse is traveling through the tube. In order to characterize the odor pulse we conducted a series of studies involving a mini photo-ionization device (PID,

Model 200A, Aurora Scientific Inc., Canada). This system detects non-air volatiles with a high temporal resolution (330 Hz). In order to ensure that measurements taken with the PID are comparable with the sensitivity of a fly we simultaneously recorded the responses of a fly antenna (electroantennogram EAG) and the response of the PID to pulses of ethyl acetate and benzaldehyde (Fig. 2a). We found that the onset of the PID signal correlates with the onset of the EAG. The time difference between the onsets of the EAG and PID signals do not differ from zero for both tested odorants (Fig. 2b). Therefore, for further calibrations we used the onset of the PID signal, as this reflects the responsiveness of the fly's antenna. We next measured at three positions in the tube the odor pulse as it travelled through. These measurements were repeated with six different air flows (Fig. 2c). The linear slopes reveal the speed at which the pulse is travelling through the tube at a given air flow. The intercept with the x-axis depicts the delay, i.e. the time the stimulus needs to travel from the vial to the upstream end of the tube. These two parameters, wind speed and delay, are fed into the Matlab routine which calculates the meeting time between the pulse and the fly. For all tested air flows we found high correlation between the time and the position of the PID measurements, i.e. the pulse is travelling with a linear speed through the tube. However, as flies cease any movement at wind speeds higher than 70 cm/s (Yorozu et al. 2009) we decided to use a low air flow (i.e. 0.3 l/min) which results in a wind speed of 18 cm/s. Therefore we used a wind speed which is far below this critical value.

In order to monitor the shape of the pulse as it travels through the tube we recorded the entire PID signal taken at the beginning, in the middle and at the end of the tube. Therefore, we displayed the entire signal produced by the PID, i.e. the time course of the pulse (Fig. 2d). We found that the shape of the pulses (i.e. the increase and decrease of stimulus concentration) is

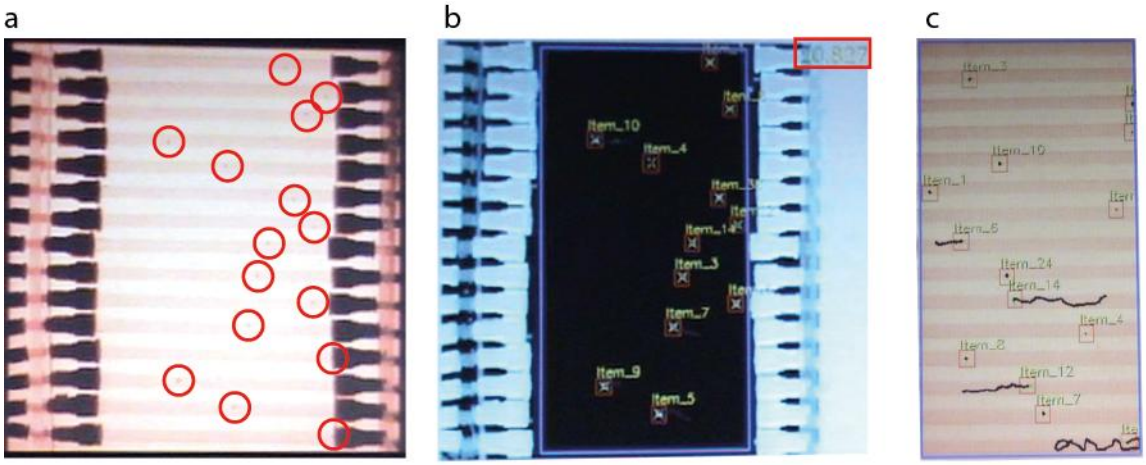
similar at the position where the pulse enters the tube (position 0 cm), in the middle (position 7 cm) and at the end of the tube (position 17 cm). Dilution of the stimulus during its movement through the tube results in a decrease of maximum concentration at position 7 cm (17 cm) of only 3% (10%) compared to the maximum value measured at position 0 cm.

Tracking

The custom-built tracking system used an overhead camera (SONY EVI, Sony Corporation, Japan) positioned approximately 1 m over the test arena (18 x 28 cm). The camera captured images at 25 Hz with a 640x480 pixel resolution. As the effective tracking frequency was slightly fluctuating depending on image processing, we interpolated the final data set to a frequency of 10 Hz. The setup rendered a spatial tracking accuracy of 0.06 cm, which is approximately 25 % of a fly's body length. The tracking software detected the individual flies as black dots in front of the red background (Supplementary Fig. S2a) and stored for each fly its XY coordinates. While the X coordinates informed about the up- and downwind movement of the fly within the tube, the Y coordinate an individual fly could reach were restricted by the tube side boundaries, and, hence, were used to discriminate between flies in neighboring tubes. We evaluated the performance of the tracking system by manually analyzing frame by frame of a 30 minutes video and comparing the outcome with that of an automatic analysis. The tracking system never exchanged identities of two animals. We found a total of 8 false positive and 30 false negative identifications, i.e. the system either identified flies that were not existing, or lost flies that were still there. However within each tube false positives were tracked only during a total of 0.34 s while false negatives, were reported for only 1.9 s during

the 30 min period. Therefore the data revealed by the automatic tracking system seemed to be reliable.

The positions of the flies were stored from 15 s before each stimulus onset until 15 s after the stimulus. However data analyses concentrated only on the 3 s before until 7 s after the stimulus reached the flies. As flies positioned close to the upstream end of the tube perceive the stimulus ca. 1 s earlier than flies positioned close to the downstream end, for each fly and stimulus the onset of the stimulus was calculated individually based on the fly's position within the tube and the movement of the stimulus.

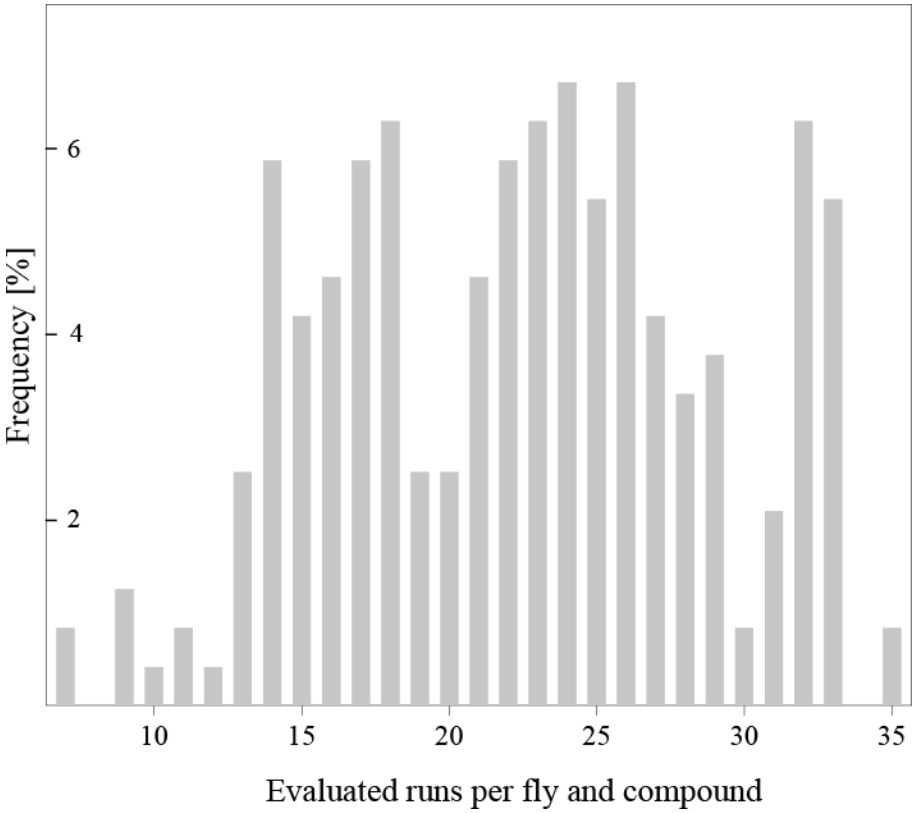


Supplementary Figure S2. Image input stream and processing by the tracking system. (**a**) Snapshot image of the arena containing 15 minute parallel wind-tunnels with the position of the flies manually labeled (black dots in red circles). (**b**) Snapshot of the processed input image shown in (**a**) displaying the automatically detected flies as numbered items. (**c**) Close-up image of the so called Region Of Interest (ROI) that sets the margins of the tracking area with superimposed traces of the tracked flies. Flies outside this region are ignored by the tracking system.

Experimental procedure

Experimental sessions started in the evening in order to test the animals during their highest activity (Levine et al., 2002). Temperature and humidity of the continuous air flow resembled with 25°C and 70% humidity the breeding conditions.

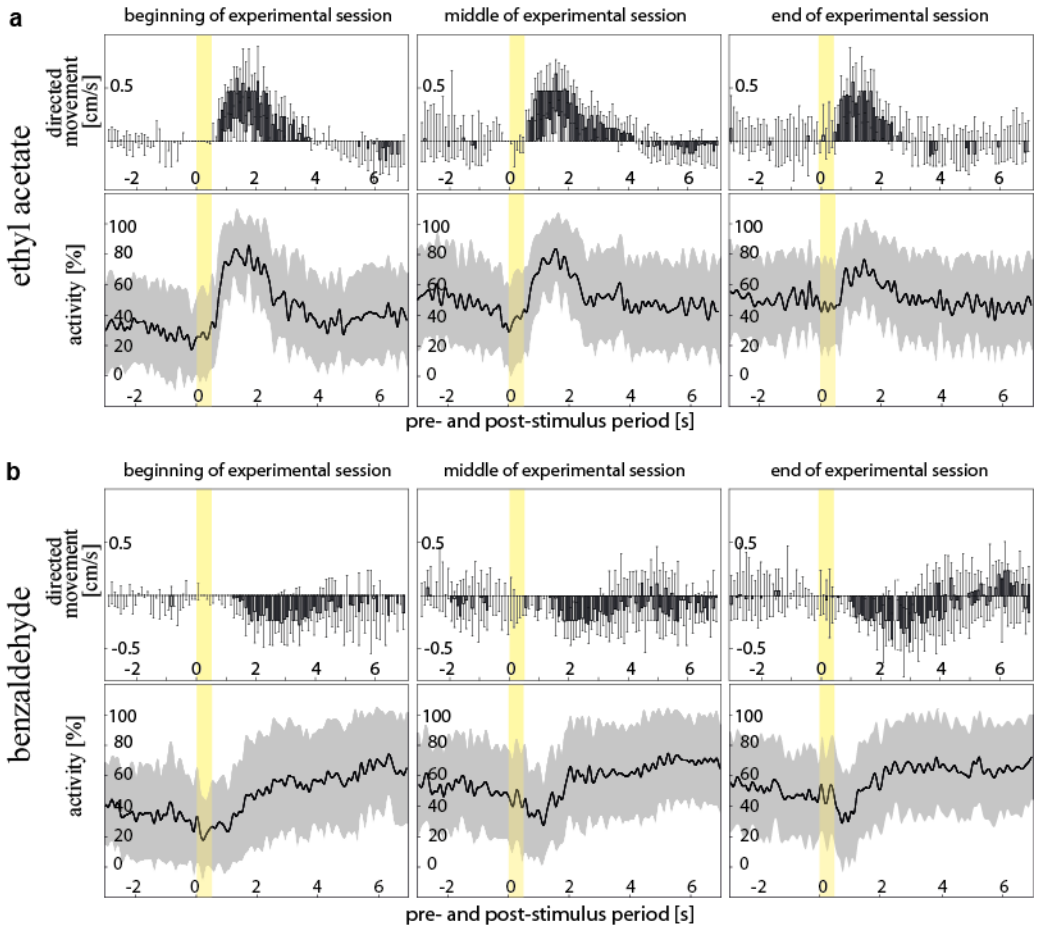
Olfactory stimulation of the flies started 30 min after they had been placed into the tubes. A typical stimulation protocol included eight different stimuli (seven odors, one solvent control) presented during 8 hours every 90 s in a pseudo-randomized order. Each fly was thus tested 40 times with each compound. However, data from flies that were positioned outside the region of interest were ignored. Therefore, a representative 8-hours experiment resulted in 7 to 35 analyzable runs per fly and compound (Supplementary Fig. S3).



Supplementary Fig. S3. Frequency of evaluated runs per fly and compound. A representative experimental session results in a total of 5835 tracking

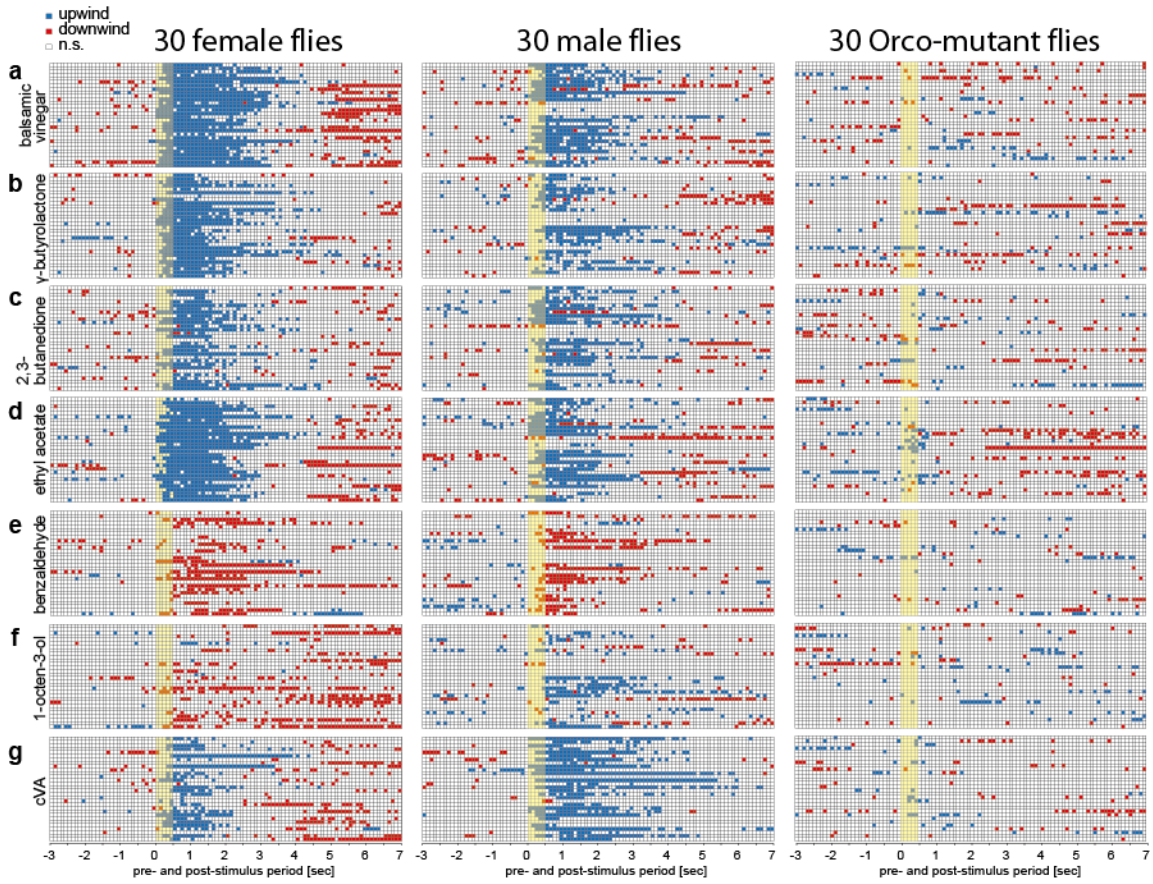
events. Flies positioned outside the region of interest were not tracked and thus, were not integrated in the evaluation. The minimal number of analyzable runs per fly and compound was seven, while maximal 35 runs per fly and compound were analyzed.

During an experimental session that lasts for up to eight hours the observed odor-induced changes in behavior are robust (Supplementary Fig. S4). We calculated for each of the female flies the median response and the average activity based on three runs at the beginning (during 1^{st} hour), in the middle (during 4^{th} hour) and at the end of the experimental session (during 8^{th} hour). While the overall activity slightly increased over time, responses to an attractive odorant (ethyl acetate) and to a repellent odorant (benzaldehyde) did not change. These findings permit that all data collected during the long-lasting experimental sessions can be included in the evaluation.

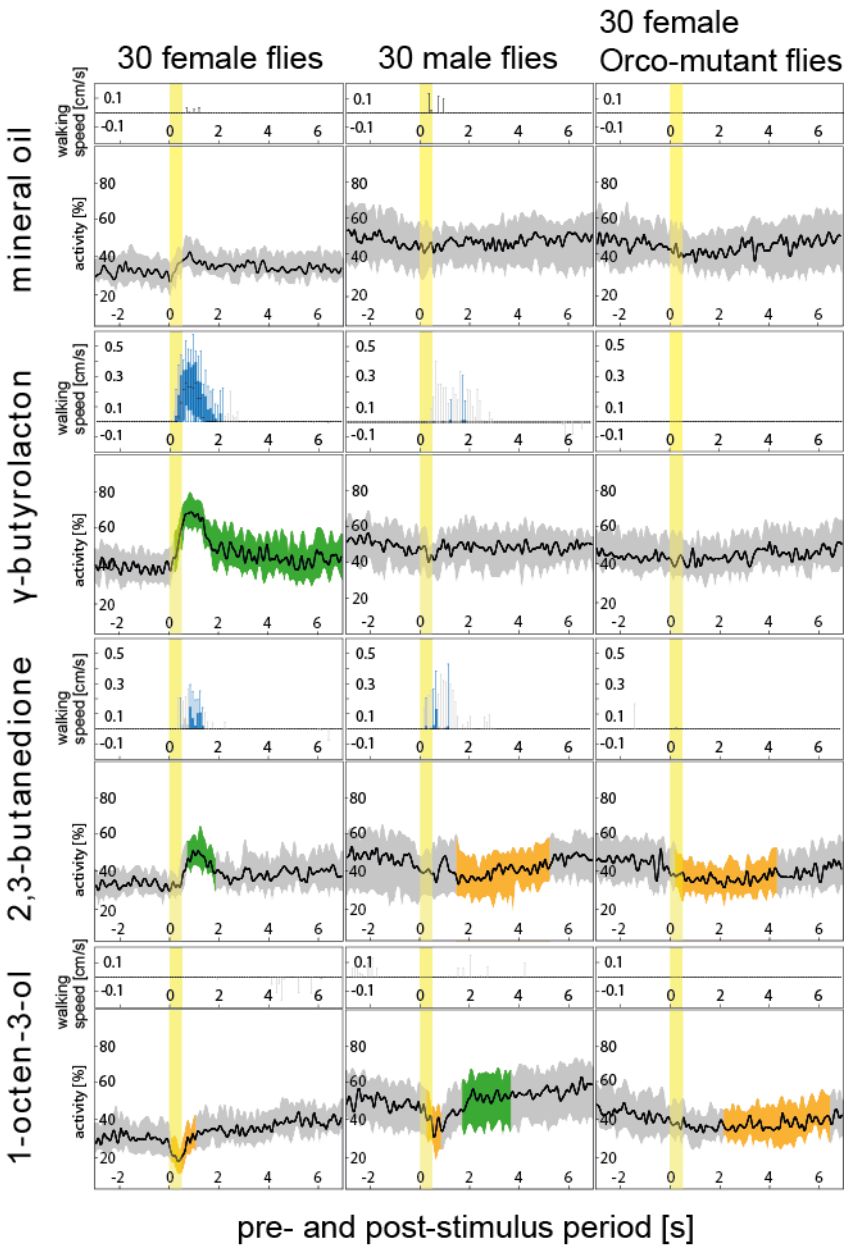


Supplementary Fig. S4. Odor-induced responses of female wildtype *Drosophila* are robust over a long-lasting experimental session. (**a**) and (**b**) Tested odors. Top graphs, boxplot representation of odor induced changes in upwind speed of 30 flies; black line, median upwind speed; box, interquartile range; whiskers, 90th and 10th percentiles. Low graphs, undirected activity of 30 flies; black line, average activity; shaded area, standard deviation. Yellow area, 500 ms odor stimulus.

Long-lasting experimental sessions resulting in on average 22 analyzable runs per fly and compound allow that behavior of individual flies can be evaluated statistically (Supplementary Fig. S5).



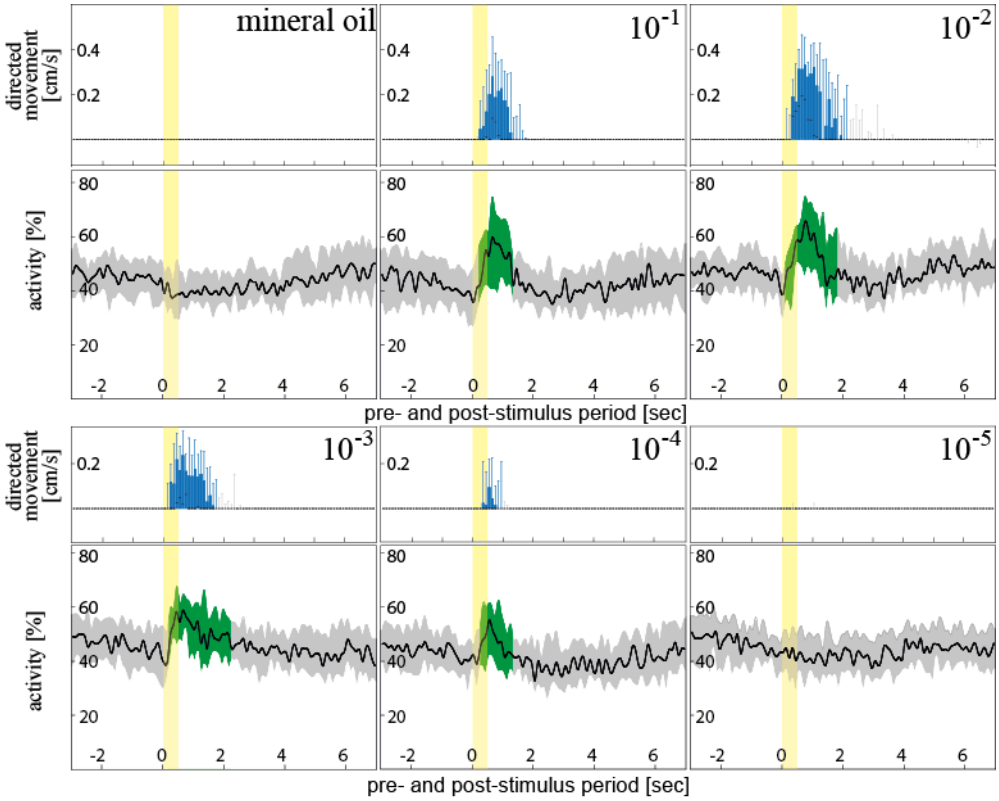
Supplementary Fig. S5. Statistically analyzed responses of 30 female and 30 male wildtype flies and 30 female Orco-mutant flies to repeated stimulations with balsamic vinegar (**a**), γ-butyrolactone (**b**), 2,3-butanedione (**c**), ethyl acetate (**d**), benzaldehyde (**e**), 1-octen-3-ol (**f**), and cVA (**g**). Each line depicts the statistically evaluated response of an individual fly to repeated stimulations. Red square, fly exhibited significantly increased downwind movement during 100-ms time frame (compared to median value during the corresponding time interval after stimulating with the solvent control); blue square, fly exhibited significant upwind movement during time frame; white square, fly did not exhibit significant up- or downwind movement. Yellow area, 500 ms odor stimulus. For statistical analysis see method section.



Supplementary Fig. S6. Odor-induced responses of female and male wildtype *Drosophila* and female Orco-mutants. Top graphs, boxplot representation of odor induced changes in upwind speed of 30 flies; black line, median upwind speed; box, interquartile range; whiskers, 90th and 10th percentiles; blue, significantly increased upwind speed within 100-ms time frame; red, significantly decreased upwind speed within 100-ms time frame. Low graphs, undirected activity of 30 flies; black line, median activity;

shaded area, interquartile range; green, significantly increased activity; orange, significantly decreased activity. Yellow area, 500 ms odor stimulus. For statistical analysis see methods part.

These responses were dose dependent (Supplementary Fig. S8).



Supplementary Fig. S7. Dose dependency of odor induced responses to ethyl acetate of female wildtype *Drosophila*. For graph explanation see Supplementary Fig. S6. Numbers in the top right corners of the graphs depict stimulus concentration.

Reference

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