Distinct activity-gated pathways mediate attraction and aversion to CO$_2$ in *Drosophila*

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Carbon dioxide is produced by many organic processes and is a convenient volatile cue for insects$^1$ that are searching for blood hosts$^2$, flowers$^3$, communal nests$^4$, fruit$^5$ and wildfires$^6$. Although *Drosophila melanogaster* feed on yeast that produce CO$_2$ and ethanol during fermentation, laboratory experiments$^7$–$^{12}$ suggest that walking flies avoid CO$_2$. Here we resolve this paradox by showing that both flying and walking *Drosophila* find CO$_2$ attractive, but only when they are in an active state associated with foraging. Their aversion to CO$_2$ at low-activity levels may be an adaptation to avoid parasites that seek CO$_2$, or to avoid succumbing to respiratory acidosis in the presence of high concentrations of CO$_2$ that exist in nature$^{13,14}$. In contrast to CO$_2$, flies are attracted to ethanol in all behavioural states, and invest twice the time searching near ethanol compared to CO$_2$. These behavioural differences reflect the fact that ethanol is a unique signature of yeast fermentation, whereas CO$_2$ is generated by many natural processes. Using genetic tools, we determined that the evolutionarily conserved ionotropic co-receptor IR25a is required for CO$_2$ attraction, and that the receptors necessary for CO$_2$ avoidance are not involved in this attraction. Our study lays the foundation for future research to determine the neural circuits that underlie both state- and odorant-dependent decision-making in *Drosophila*.

*D. melanogaster* feed, mate and deposit eggs on rotting fruit. Between 10 and 14 days later, the next generation of flies must locate a fresh ferment. Because of the high volatility of CO$_2$, the emission of CO$_2$ is greatest near the start of fermentation$^5$, whereas ethanol emission increases more slowly (Extended Data Fig. 1a). Other odours associated with fermentation (for example, acetic acid and ethyl acetate) form later, when bacteria break down ethanol. In trap assays, *Drosophila* show a preference for two-day-old apple juice ferments compared to older solutions (Extended Data Fig. 1b, c), which suggests that they might be attracted to CO$_2$. Although it is difficult to estimate concentrations of CO$_2$ in wild ferments, we measured the CO$_2$ concentration in bottles commonly used to rear flies to be 0.5–1% (Extended Data Fig. 1d–g).

This evidence that CO$_2$ might attract *Drosophila* contradicts previous studies conducted using small chambers$^7$–$^{12}$. To study how flies respond to odours under more-ethological conditions, we recorded trajectories that passed through one of the coloured volumes shown in c (gold, cyan or green) after also passing through a control volume (white or gold). Approaches to landing pad, gold-from-white; landings, cyan-from-gold; approaches to dark spot, green-from-white. Number of trajectories per condition: 44–1,288 (control), 228–1,815 (odour). Experiments were performed at two concentrations: 15 ml min$^{-1}$ (left) and 60 ml min$^{-1}$ (right). Letters above data indicate statistically significant groups (two-tailed Mann–Whitney *U*-test at $P < 0.05$ with eight-way Bonferroni corrections). In all panels, shading indicates the bootstrapped 95% confidence interval around the mean.

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Fig. 1 | *Drosophila* are attracted to ethanol and CO$_2$ in flight. a, Diagram of wind tunnel. b, Photograph of the wind tunnel and odour-emitting landing platform. c, d, Heat maps indicating relative occupancy of flies in the presence of either CO$_2$ or ethanol. Cohorts of 12 flies were introduced into the wind tunnel and their behaviour recorded over 16 h. Throughout the experiment, 100 ml min$^{-1}$ of clean air emerged from both odour ports. For 30 min every hour, 60 ml min$^{-1}$ of either CO$_2$ or clean air bubbled through 100% ethanol was added to one odour port. Control data come from segments with clean air. Number of cohorts: 9 (CO$_2$), 6 (ethanol). Number of trajectories: 59,970–101,539 per panel. e, Percentage of trajectories that passed through one of the coloured volumes shown in c (gold, cyan or green) after also passing through a control volume (white or gold). Approaches to landing pad, gold-from-white; landings, cyan-from-gold; approaches to dark spot, green-from-white. Number of trajectories per condition: 44–1,288 (control), 228–1,815 (odour). Experiments were performed at two concentrations: 15 ml min$^{-1}$ (left) and 60 ml min$^{-1}$ (right). Letters above data indicate statistically significant groups (two-tailed Mann–Whitney *U*-test at $P < 0.05$ with eight-way Bonferroni corrections). In all panels, shading indicates the bootstrapped 95% confidence interval around the mean.
the flight trajectories of flies in a wind tunnel that contained a landing platform, which was programmed to periodically release plumes of CO₂ or ethanol (Fig. 1a, b). Both odours elicited approaches, landings and explorations of a conspicuous visual feature (Fig. 1c, d), which is consistent with previous experiments with flies and mosquitoes.15,16 Flies were more likely to approach the platform or dark spot in the presence of ethanol compared to CO₂, but were equally likely to land in response to either odour (Fig. 1e).

To quantify the behaviour of flies after they land, we designed a platform that is suitable for automated tracking (Fig. 2a, b). At a flow rate of 60 ml min⁻¹ CO₂, the CO₂ concentration near the surface of the platform was approximately 3% (Fig. 2b, c). After landing near a source of CO₂, ethanol or apple cider vinegar, flies exhibited a local search behaviour that was similar to so-called ‘dances’17 (Fig. 2d, e, Extended Data Fig. 2a–c). Flies spent twice the amount of time exploring platforms that emitted ethanol compared to CO₂. Flies approached a source that emitted both ethanol and CO₂ more frequently than they approached vinegar, or either odour alone. Vinegar elicited smaller local searches and slightly fewer approaches compared to CO₂ consistent with the hypothesis that vinegar might indicate a less favourable, late-stage ferment. Flies spent significantly less time standing still on the platform in the presence of CO₂ compared to any other odour, with a mean walking speed > 2 mm s⁻¹ (Fig. 2e).

One previous study showed that Drosophila flies are attracted to CO₂ while flying on a tether18. Our results confirm this observation in freely flying flies; however, we also found that flies remain attracted to CO₂ after they land, which contradicts previous studies7–10,12. One potential explanation is that flies in constrained walking chambers might behave differently to those that arrived on our open wind tunnel platform after tracking the odour plume and landing. To test this hypothesis, we built an enclosed arena in which flies were unable to fly (Fig. 3a, Extended Data Fig. 3) and presented them with pulses of 5% CO₂. Groups of 10 starved flies presented with CO₂ after acclimatizing to the arena for 10 min exhibited aversion (Fig. 3b), as previously reported. However, if allowed to acclimate in the chamber for two hours, the flies exhibited attraction to CO₂ (Fig. 3c).

To study the response of these flies in more detail, we recorded the behaviour of flies for 20 h, while providing 10-min presentations of CO₂ from alternating sides of the arena every 40 min (Fig. 3d, Supplementary Videos 1, 2). To control for humidity, we continuously pumped 20 ml min⁻¹ of H₂O-saturated air through the odour ports on both sides of the chamber. The flies exhibited a clear circadian rhythm within the chamber, as indicated by their mean walking speed. At times of peak activity—near dusk and dawn—flies showed a strong initial attraction to CO₂, which decayed stereotypically during the 10-min presentation. At times of low activity—at mid-day and during the night—flies exhibited a mild aversion to CO₂. Starving flies for 24 h before the experiment changed their activity profile, resulting in a slightly elevated attraction during the night. Ethanol, by contrast, elicited sustained attraction regardless of baseline activity (Fig. 3d, Supplementary Video 3).

To probe this relationship between activity and CO₂ attraction, we increased the temperature and elevated the wind speed—manipulations that are known to elevate and depress19 activity, respectively (Fig. 3e). When we increased the bulk-flow rate to 100 ml min⁻¹, flies exhibited a peak walking speed of about 1.5 mm s⁻¹ at dusk—nearly half the speed we measured at a flow rate of 20 ml min⁻¹. Instead of showing attraction, these flies exhibited aversion to 5% CO₂, although they were still attracted to ethanol (Fig. 3e). This result helps to explain why previous studies that used higher flows (100–1,000 ml min⁻¹) to present CO₂ observed aversion.8 To further explore the effect of wind, we clipped the aristae of the flies, which destroys their primary means of detecting airflow but does not interfere with the detection of odours20. The flies without aristae exhibited the same walking speed and attraction to CO₂ at the high flow rate as was exhibited by normal flies at the low flow rate. Warming flies with intact aristae to 32°C also increased their baseline activity and recovered their attraction to CO₂ at the higher flow rate. Pooling data across all our experimental conditions, we found that flies were attracted to CO₂ when they had a baseline walking speed that was above about 2.4 mm s⁻¹ (Fig. 3f). This value is similar to the walking speed that we observed in our wind tunnel assay, which was higher for CO₂ than the other odours. To confirm that activity-dependent attraction to CO₂ is not a function of social interactions, we tested 29 single flies, which behaved similarly to the cohorts of 10 (Extended Data Fig. 4a). We also tested three concentrations of CO₂ (1.7%, 5% and 15%) and found that the 5% concentration elicited the strongest response, consistent with our wind tunnel experiments (Extended Data Fig. 4b–f, Supplementary Information).

Although the responses of flies to ethanol and CO₂ were similar at stimulus onset, attraction to ethanol was more sustained. The time course of behaviour was notably similar in the walking arena and wind tunnel (Extended Data Fig. 2d–g), which suggests that the behavioural dynamics of olfactory attraction are robust to the stimulus environment and may represent an adaptation for using information that broad (CO₂) and more specific (ethanol) odorants provide.

Previous research shows that CO₂ aversion is mediated by Gr63a and Gr21a receptors5,21; high concentrations of CO₂ are also detected by an acid-sensitive ionotropic receptor, IR64a22. In our assay, mutant
flies that lack the IR64a receptor showed no significant change in their behaviour compared to wild type (Fig. 4a, b, d). Consistent with previous work, mutants that lack the Gr63a receptor exhibited no aversion to CO₂; however, they were still attracted to CO₂ when active. Mutant flies that are homozygous for both Gr63a and IR64a behaved similarly to the Gr63a mutants. It is noteworthy that the characteristic decaying time course of attraction was unaffected in Gr63a mutants, even though these flies showed no aversion. Thus, the decay in attraction to CO₂ is not caused by an increase in aversion over time.

Given that CO₂ attraction is not mediated by IR64a or Gr21a or Gr63a, we wanted to confirm that the attraction is indeed a chemosensory response. To determine whether CO₂ attraction is mediated by either an olfactory or ionotropic receptor, we tested a mutant that lacks the olfactory and ionotropic co-receptors (Orco, IR25a and IR8a) as well as Gr63a (Fig. 4c). These near-anosmic mutants exhibited no detectable behavioural response to CO₂. Flies in which we surgically removed the third antennal segment also showed no response to CO₂, despite normal levels of activity. Together with our arista ablations (Fig. 3e), these experiments show that CO₂ attraction is mediated by receptors on the third antennal segment. To further confirm this, we tested each co-receptor mutant individually and found that mutants that lack IR25a did not exhibit wild-type CO₂ attraction, whereas Orco and IR8a mutants did (Fig. 4c). Mutant flies that lack Orco, IR8a and Gr63a also exhibit wild-type attraction to CO₂, confirming that the only required co-receptor is IR25a. IR25a has previously been implicated in a wide range of behaviours, including temperature and humidity sensation. We measured the temperature in our arena near the CO₂ port, and found no change in temperature as a result of the stimulus (Extended Data Fig. 5). To eliminate the possibility of a humidity artefact, we tested an IR40a mutant, which still exhibited attraction to CO₂ (Fig. 4c). In summary, our experiments show that CO₂ attraction is mediated by a separate chemosensory pathway from that which governs aversion, and that CO₂ attraction requires the IR25a co-receptor (Fig. 4d). IR25a is the most highly conserved olfactory receptor among insects. It is possible that other insect species that lack Gr63a but that still respond to CO₂ use the same IR25a-dependent pathway. Unfortunately, the GAL4 driver for the IR25a promoter is expressed only in about half of the endogenous IR25a-expressing neurons, which makes imaging experiments that aim to identify which glomerulus is involved difficult.
Our finding that active flies are attracted to CO₂ makes ethological sense, given that CO₂ is generated by yeast—the preferred food of these flies. We considered why it might be that Drosophila avoid CO₂ when in a low-activity state. Flies do not exhibit this state-dependent reaction to ethanol and vinegar (Extended Data Fig. 8); perhaps the aversion to CO₂ at low activity is an adaptation that minimizes encounters with parasites that seek CO₂. Alternatively, the behaviour may help flies to avoid respiratory acidosis when near high concentrations of CO₂ within the environment³⁴ (Extended Data Fig. 9). Previous studies have suggested that CO₂ serves as an aversive pheromone by which stressed flies signal others to flee a local environment³⁵. However, an alternative explanation is that agitated flies release CO₂ not as a social signal but simply because it is present in their tracheal system owing to their process of discontinuous respiration²⁸,²⁹ (Extended Data Fig. 10). Further work on this state-dependent reaction to CO₂ will require experiments that carefully consider the natural ethology of the flies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0732-8.

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METHODS

Statistics and reproducibility. Here we provide the exact number of trials, trajectories, individuals and cohorts for each experiment. For our wind tunnel experiments, each trajectory was treated as an independent sample because it is impossible to keep track of the identity of individual flies in these experiments. In the walking assays, each trial was considered independent, as the inter-trial variability within a cohort of flies over the course of the 20-h experiments was similar to the inter-cohort variability. This is in part due to the changes in activity over the course of the experiments. In all of our figures, we show the trial by trial variance with shaded 95% confidence intervals around the mean or median. These confidence intervals were determined by 1,000 iterations of bootstrapped sampling with replacement. In each experiment, we attempted to collect the largest sample sizes we could, given the time constraints required for behavioural data in which an experiment with one cohort lasts for 24 h. In situations in which we were comparing behaviour under different conditions, we attempted to randomize the temporal sequence with which we collected data to minimize any artefacts due to long-term influences such as season changes in humidity, temperature and so on.

We did not use blinding in our data collection design. For experiments described in Figs. 1, 4, and the associated Extended Data Figs., control and test experiments were interleaved with each other.

Additional statistics for Fig. 3. To statistically compare the attraction of flies to CO2 under the different conditions presented in Fig. 3, we used resampling (Fisher’s exact test) to test the significance of the difference in the preference indices exhibited by flies in key experiments. The preference index represents the strength of the flies’ attraction to the odour (for example, CO2), relative to the clean air control. The raw preference index, PI, was first calculated for each point in time: P(1) = (n_odour(t) - n_control(t))/n_healthy. In which n_healthy and n_control are the number of flies within the circular regions of interest around the odour and control ports, respectively, and n_healthy is the total number of flies. To remove baseline biases, we then subtracted the mean preference index for the 5-min period before the odour stimulus to yield the relative preference index, PI:

To make statistical comparisons, we then calculated the average preference index for the first half of the odour presentation period (that is, the first 5 min). We chose this range because it captures the majority of CO2 attraction, and thus focuses the statistical test on the most relevant time period.

These calculations provide a single preference index value for each trial of each cohort. For our resampling algorithm, we used 1,000 iterations to determine the P value, and repeated this calculation 1,000 times to calculate a 95% confidence interval around these P values. The confidence intervals are shown for key comparisons as follows. Figure 3b compared to Fig. 3c: 0.0249 < P value < 0.0276. Figure 3d (top row) dusk compared to Fig. 3d (top row) afternoon: P value = 0.002. Figure 3d (top row) dusk compared to Fig. 3d (second row) dusk: 0 < P value < 0.001. To compare the response of flies to CO2 and ethanol, we used the full 10-min odour-presentation time frame because the differences in behaviour primarily appear in the second half of the odour presentation. Figure 3d (third row) dusk compared to Fig. 3d (fourth row) dusk: 0 < P value < 0.001 (24-h-starved flies). Extended Data Fig. 2e compared to Extended Data Fig. 2g (red traces): 0 < P value < 0.001 (12-h-starved flies).

To eliminate the possibility of pseudo-replication, we repeated our statistics after calculating the average PI(t) for each cohort before calculating PI(t). Thus, for the following statistics, the input to our resampling test was a single preference index value for each cohort of flies. This is a very conservative measure, because there is similar intra-cohort variability compared to inter-cohort variability, in part owing to changes in the flies’ circadian activity. Figure 3b compared to Fig. 3c: 0.0249 < P value < 0.0276 (these experiments were 1 trial per cohort). Figure 3d (top row) dusk compared to Fig. 3d (top row) afternoon: 0.0124 < P value < 0.0141. Figure 3d (top row) dusk compared to Fig. 3d (second row) dusk: 0.0129 < P value < 0.0141. Figure 3d (third row) dusk compared to Fig. 3d (fourth row) dusk: 0.0100 < P value < 0.0120 (24-h-starved flies). Extended Data Fig. 2e compared to Extended Data Fig. 2g (red traces): 0.0035 < P value < 0.0045 (12-h-starved flies).

This definition of preference index was also used for the data presented in Fig. 4.

Flies. Wild-type flies were descendants of a Heisenberg Canton-S stock (HCS). For the aristra-clipped and antennae flies, we cold-anasthetized flies and carefully removed the aristra or third antennal segment with sharpened forceps.

Each mutant used in our study is described in detail below. All experiments were done with mutants in which balancers and markers had been crossed out.

Gr63a, IR64a: +/+;Gr63a /- IR64a /- double mutant; this line was generated using recombination by crossing Tl[w+ = Tl]Gr63a3 (Bloomington 9941) to Mi[ET1] Ir64a [Bloomington 26528] (Bloomington 26410). The double mutants were verified using PCR. IR8a, IR25a, Orco, Gr63a (near-amosinic): IR8a /- IR25a /- Orco /- Gr63a /- quadruple mutant; this was a gift from R. Benton and A. Silbering. IR8a, Orco, Gr63a: IR8a /- Orco /- Gr63a /- triple mutant; this line was generated by crossing IR8a;IR25a;Orco,Gr63a to wild-type HCS. Orco: +/+; Orco /-; this line was created by backcrossing an Orco2 (Bloomington 23130) line to the wild-type HCS for five generations, and verified through PCR. IR64a: +/+;IB64a /-; this line was created by backcrossing the Mi[ET1] Ir64a [Bloomington 26528] (Bloomington 26410) line to the wild-type HCS for seven generations, and verified through PCR. IR8a: IR8a /-; IR8a /-; this mutant was a gift from G. Suh27,31. IR25a: +/+;IR25a /-; we used two variants of this mutant ((1) and (2)), along with the bacterial artificial chromosome rescue, all of which were gifts from R. Stanewsky. Figure 4 uses the (2) variant. Gr63a: +/+;Gr63a /-; this mutant is Bloomington 9941. IR40a: IR40a /-; IR40a /-; this mutant was a gift from M. Stensmyr and M. Galiò.

All of the flies were raised on a 16:8 light:dark light cycle at 25°C in standard 300-ml bottles on fly food consisting of water (17.8 l), agar (136 g), cornmeal (1,335.4 g), yeast (540 g), sucrose (320 g), molasses (1.64 l), CaCl2 (12.5 g), sodium tartrate (150 g), tewsophate (18.45 g), 95% ethanol (153.3 ml) and propionic acid (11.5 ml). For all of our experiments, we used 2- to 3-day-old female flies. To sort and starve flies, they were briefly anesthetized on a cold plate, and placed in a test-tube with a wet Kimwipe.

Fermentation and trap assays. We prepared the yeast from 130 ml of apple juice (Treetop brand) and 20 g of cane sugar, warmed to 35°C. Next, we added 130 ml of Cellar Science EC-1118 wine yeast, which produces a neutral flavour and aroma. The fermentation was carried out at room temperature (23°C), under an airlock. All glassware was first sanitized with StarSan. We measured the specific gravity daily with a standard hydrometer, and calculated the alcohol content according to the following equation32:

\[
\text{ABV} = \frac{76.08}{\frac{\text{OG} - \text{FG}}{0.794}} \times \frac{\text{FG}}{1.775 - \text{OG}}
\]

in which ABV is alcohol by volume, OG is the starting specific gravity and FG is the final specific gravity. After 14 days, the fermentation had finished and the yeast flocculated. At this point, we sealed the containers and stored them in the fridge for 6–14 days while waiting for the next active batch of fermenters to reach the desired age.

For the trap assays we let fermentations run for 2, 7, or 12 days. One day before these fermenters were ready, we pulled a flocculated ferment from the fridge, and wet-starved groups of flies (50–150 flies each). For all of our trap assays, each trial we poured the active ferment into one jar, and the flocculated ferment into another jar, and inserted the traps into the jars. The two traps were placed side-by-side in our wind tunnel (~6 cm apart), and a group of flies was released. Two hours later we removed the traps, CO2-anaesthetized the flies, and counted the number of individuals in each trap. A preference index was calculated as: (n_f – n_b)/(n_f + n_b), in which n_f is the number of flies in the active ferment, and n_b is the number of flies in the flocculated ferment. For each condition we used four separate fermenters, each used for three separate trials, for a total of 12 trials per condition.

CO2 measurements of fly bottles. We first modified 500-ml Nalgene bottles by drilling two holes and fitting them with Luer Lock valves (with lock plugs attached). These Nalgene bottles are slightly larger than standard (300-ml) food bottles used by many Drosophila laboratories, and can be fitted with the same standard-sized cotton plugs. For each Nalgene bottle, we melted the food from 1 fly food bottle (50 ml) in the microwave, and poured it inside. Once cooled, we added a measured amount of baker’s yeast, depending on the experiment, and fitted the bottle with a cotton plug and placed it in a 25°C incubator for 2 days. For experiments with flies, we added 10 females and 15 males to each bottle and allowed them to lay eggs in the bottles for two days. Fourteen days later (when the majority of the flies had eclosed, and were ~2 days old), we made our measurements.

To measure the CO2 content, we first pressed the cotton plug into the bottle far enough to twist on the original Nalgene cap, sealing the contents of the bottle inside. Meanwhile, we prepared our CO2 analyzer—the LiCorCo2–6262—by running CO2 free air through the system at 201 min^-1. We attached the CO2 analyzer to the Luer valve, slowly replacing the air inside the bottle with CO2-free air. Before connecting the air stream, we started our data acquisition. Data were collected from the LiCor-Co2–6262 using the analogue-to-digital converters on a Phidgets InterfaceKit, connected to an Ubuntu laptop running custom Python code for data acquisition. Preliminary measurements showed that the CO2 content of the bottles was beyond the dynamic range of the LiCorCo2–6262. To resolve this, we added a 500-
For these and all other experiments, we used Teflon tubing. Cohorts of 12 female Nalgene bottles filled with fly food based on their measured peak CO$_2$ concentration, and the actual concentration of the bottles. Using this calibration curve, we were able to calculate the actual CO$_2$ concentration of the Nalgene bottles filled with fly food based on their measured peak CO$_2$ concentrations.

**Free-flight wind tunnel assays.** To record the free-flight behaviour of flying flies, we used the same wind tunnel and 3D tracking system described at length in previous papers.[10,11,13] To observe the flies’ behaviour in response to odours, we added an acrylic platform with two sites for odour release. Air flow was controlled using computer-controlled Alicat mass-flow controllers (0–200 ml min$^{-1}$ range). For these and all other experiments, we used Teflon tubing. Cohorts of 12 female flies were starved for 6 h before starting the experiments at 17:00, 6 h before the flies’ sunset. Starting at 20:00 (3 h before the flies’ sunset), either CO$_2$ or ethanol was released from the landing platform for 30 min, followed by an hour of clean air. This stimulus pattern was repeated seven times. **Regions of interest.** We chose regions of interest to quantify the behaviour of the trajectories shown in the heat maps of Fig. 1c, d. The boundaries of the regions for approaching the dark spot, approaching the platform, and landing on the platform were chosen based on the behaviour of the flies in the presence of the odours. The objective was to compare the behaviour with the different odours and controls, rather than determine absolute numbers. Thus, the exact size and position of the regions is not critical.

The white region of interest was chosen to be roughly in the region in which the odour plume passes, above and behind the dark spot. By comparing how many flies approach the pad or spot to how many flies pass through this white region, we control for the overall change in behaviour of the flies in the presence of the odour. For example, it is possible that the odour causes the flies to spend less time near the top of the tunnel, bringing them closer to the spot or platform—and thus more likely to approach these objects. By always selecting trajectories that passed through the same volume, we control for this overall change in behaviour.

**Free-walking wind tunnel assays.** The 3D tracking system used for the free-flight experiments did not have sufficient spatial and temporal resolution to accurately record the walking behaviour of flies once they had landed on the pad. To examine this behaviour more closely, we developed a 2D real-time tracking system designed for general-purpose applications. Our Python-based software and documentation is freely available on GitHub: http://florisvb.github.io/multi_tracker/. The software runs on Ubuntu, and is built on the ROS (Robot Operating System) framework, and takes advantage of open-source packages including OpenCV, scipy, numpy, pandas, h5py and pyQTGraph. A brief overview of the software flow is as follows: (1) image background subtraction; (2) thresholding and contour identification; (3) contours larger than a specified size are broken up into smaller contours (this corrects for cases when two flies come close to one another); (4) data association using a posteriori estimates from a Kalman filter estimator; (5) Kalman filtering of trajectories to (a) smooth position information, (b) estimate velocity and (c) calculate a posteriori estimates for the next data-association step; (6) trajectory data are recorded as an HDF5 file, and the changes from the background in the raw image are recorded as a ROS bag file; and (7) data can then be efficiently analysed using the pandas data structure, and trajectories can be viewed and corrected using a custom pyQTGraph GUI.

**CO$_2$ plume measurements in the wind tunnel.** We measured the CO$_2$ concentration downwind from the landing platform shown in Fig. 2a using a LiCorr-6262. To make accurate point measurements within the plume, we used a 15-cm-long tube with a 1-mm inner radius to minimize disturbances to the airflow. With a bulk air speed of 40 cm s$^{-1}$, the volume flow rate across the cross section of the tube was approximately 75 cm$^3$ min$^{-1}$ (ml min$^{-1}$). We used a mass-flow controller to regulate the suction being passed through the LiCorr-6262 to match this volume flow. After positioning the tube, we let the system equilibrate for several minutes before making a 2-min-long recording of the CO$_2$ concentration.

Because the LiCorr-6262 has a measurement limit of approximately 3,000 p.p.m. (0.3%), we made our measurements at low CO$_2$ flow rates (1–5 ml min$^{-1}$), and used a linear model to calculate the CO$_2$ concentration at larger flow rates (Extended Data Fig. 2a).

To further confirm our extrapolated measurements, we estimated the CO$_2$ concentration on the platform from first principles, as follows. First, we assume that all of the CO$_2$ that enters the wind tunnel is whisked away inside of the boundary layer (Extended Data Fig. 2b). The thickness of the boundary layer can therefore be used to estimate the average CO$_2$ concentration within that layer. The thickness of the boundary layer can be approximated for laminar and turbulent flows as:

$$h \approx \frac{5k}{Re^{1/2}} \approx \frac{0.37k}{Re^{5/7}},$$

where $h$ is the thickness of the boundary layer, $x$ is the distance downwind from the start of the platform and $Re$ is the Reynolds number. With a characteristic length of 9 cm, a kinematic viscosity of $15 \times 10^{-6}$ m$^2$ s$^{-1}$ for air at 20 °C, and a free-stream velocity of 0.4 m s$^{-1}$, the Reynolds number is 2,400. For a value of $x = 6$ cm, the boundary layers for laminar and turbulent flows are 6.1 mm and 4.6 mm, respectively. For simplicity, we will continue our calculations with a boundary layer of 5 mm.

The total volume flow rate over the platform can now be calculated as follows. The mean velocity in the boundary layer is 0.2 m s$^{-1}$ (half the free-stream velocity), the CO$_2$ is released from a 3 cm $\times$ 3 cm patch, and the boundary layer is 5 mm thick; thus, the total volume flow rate of clean air over the platform that is mixed with the introduced CO$_2$ is approximately $0.2 \times 0.03 \times 0.005 = 0.00003$ m$^3$ s$^{-1}$, or 1,800 ml min$^{-1}$. With 60 ml min$^{-1}$ of CO$_2$ added, the concentration comes to 3.2%, which agrees relatively closely with our measurement model.

**Walking assays.** We designed custom walking arenas from sheets of laser-cut acrylic (Extended Data Fig. 3). Before experiments, the cut acrylic was washed with soap (Liquinox) and warm water, and wiped down with ethanol. Between each experiment, the floor and ceiling of the arenas were wiped down with ethanol. All walking experiments were done in darkness. Experiments for Fig. 3b, c were done and flies were sacrificed either before or after their subjective day. We used three different stimulus architectures (Extended Data Fig. 3), all of which provided consistent results. The odours were controlled using a combination of computer-controlled Alicat mass-flow controllers and solenoid valves. Our ROS-based Python control software is available on GitHub at https://github.com/flosrvb/multi_alicat_control. We used three different odour delivery architectures for our experiments as detailed below.

**High flow.** For our high flow (100 ml min$^{-1}$ bulk-flow rate) experiments, we bubbled the 100 ml min$^{-1}$ flow through MilliQ water, and added 5 ml min$^{-1}$ clean dry air, CO$_2$ or clean dry air passed over a liquid ethanol reservoir, to the bulk flow. As a result of this architecture, during the odour presentation the flow rate of one side was slightly increased. However, experiments with clean dry air indicated that the flies did not respond to this change in flow rate. This arrangement was used for Fig. 4e.

**Low flow, constant flow rate and humidity.** At low flow rates (20 ml min$^{-1}$ bulk-flow rate), the architecture used for the high-flow experiments did not work properly, as flies were attracted to the change in the overall flow rate. To overcome this, we re-designed the flow architecture. In this new system, we used additional mass-flow controllers that added 1 ml min$^{-1}$ of clean dry air to the bulk-flow rate. During odour presentations, we used a solvent to switch from 1 ml min$^{-1}$ of clean dry air to CO$_2$, or clean dry air passed over liquid ethanol. This architecture ensured that the flow rate and the humidity on the two sides remained equal and constant. Control experiments in which we added clean dry air instead of CO$_2$ or ethanol confirmed that wild-type flies had minimal responses to the changes in flow. This arrangement was used for Fig. 3d.

**Low flow, symmetric stimulus.** The architecture used above for (‘Low flow, constant flow rate and humidity’) elicited small responses in certain olfactory mutants. To achieve a complete null response in these flies, we re-designed the experimental architecture once more. In this third architecture, we removed the solenoids from the system because the flow transients they created appeared to be responsible for the responses of mutant flies. Instead, we connected two flow controllers to 20 ml min$^{-1}$ bulk-flow lines. One of these flow controllers provided clean dry air, and the other CO$_2$. Both flow controllers were set to zero as a baseline. For each odour presentation, we added 3 ml min$^{-1}$ of flow to both sides of the arena. One side received 3 ml min$^{-1}$ of clean dry air, whereas the other received 2 ml min$^{-1}$ of clean dry air and 1 ml min$^{-1}$ of CO$_2$. In this arrangement, the flies experienced a change in the flow rate during odour presentations; however, the changes were symmetric. Furthermore, this arrangement made it possible to test different CO$_2$ concentrations ranging from 0% to ~15% on the same cohort of flies, providing continuous internal controls for our experiments. For these experiments, we reduced gain of the PID control settings on the mass flow controllers to provide a smooth, low change in flow rates. This is probably the cause of the slightly delayed behavioural responses that we observed. This arrangement was used for Figs. 3b–c, 4a, 4d, and Extended Data Figs. 4–8.

The qualitative—and even, to a large extent, quantitative—results across all three paradigms were consistent: at low activity the flies found CO$_2$ aversive, whereas at
high levels of activity the flies found CO₂ attractive. Finding the same results while working with three different olfactory-presentation architectures provides support for the robustness of our results.

Our experience with flies' sensitivity to changes in flow conditions—in particular at low bulk-flow rates—underscores how sensitive these flies are to odours and flow. Even our low flow rates of 20 ml min⁻¹ are reasonably high relative to the natural flow rates that a fly might experience on the surface or in the cracks of rotten fruit in the wild. The substantial changes in behaviour that we observed by reducing the flow rates to those better approximating field conditions highlights how important it is to consider the natural environments when studying sensory processing.

Temperature measurements. To eliminate any potential temperature-related confounds in our walking experiments, we measured the temperature in the arena near the odour ports using a thermistor rated to ±0.1 °C (Omega brand model number 44031), connected to a Phidgets RTD sensor. Although we detected very small fluctuations in temperature throughout the day, we did not measure any changes in temperature that correlated with the presentation of our CO₂ stimulus (Extended Data Fig. 5).

Flies' fatal attraction to CO₂. During experiments with a 200 ml min⁻¹ CO₂ stimulus in the wind tunnel, some flies that approached the CO₂ were knocked out, as they would be on a typical CO₂ pad that is commonly used for sorting flies (Extended Data Fig. 9). While the average concentration of CO₂ just downwind from the odour stimulus would not have been lethal (10%, following the calculations associated with Extended Data Fig. 2a), the concentration right at the holes in the platform was 66% (200 ml min⁻¹ CO₂ added to 100 ml min⁻¹ of clean air).

CO₂ measurements of shaken insects. To measure the CO₂ produced by flies and mosquitoes when shaken in a vial, we placed 10–20 flies in a vial and pumped 100 ml min⁻¹ of CO₂-free air through the container. After 1 min, we forcefully tapped the vial against the table for 30 s, and measured the concentration of CO₂ in the air leaving the container using a LiCorr-6262. See Extended Data Fig. 10.

Code availability. Custom code is available online at https://github.com/florisvb/drosophila_co2_attraction.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
Processed data are available in a Dryad repository at https://doi.org/10.5061/dryad.2s8422f. Raw data are available from the corresponding author upon request.

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Extended Data Fig. 1 | Drosophila prefer early fermentations, at peak CO$_2$ production. a, Alcohol by volume for apple juice and sugar fermented with champagne yeast over the course of two weeks, measured with a hydrometer. CO$_2$ production was calculated from the stoichiometry of fermentation (1 sugar molecule yields 2 ethanol and 2 CO$_2$ molecules), corresponding to the derivative of alcohol by volume. $n = 4$ independent ferments; the results were very consistent. b, Trap assay. c, Preference index exhibited by flies in three two-choice assays, using traps shown in b. Flies were presented with two traps: one was a completed 14-day-old ferment that had been stored in the refrigerator, the second was a fresh ferment aged 2, 7 or 12 days old. The positive preference index indicates a preference for the fresh ferment. The red line shows the linear regression ($P < 0.001, r^2 = 0.28$). $n = 12$ trials per condition. The mean and standard deviation of the total captured flies for each trial was 105 ± 59. d, CO$_2$ concentration in 500-ml fly-rearing bottles under common laboratory conditions. $n = 6$ trials per condition. e, Measurement setup for the data shown in d. f, Time course of CO$_2$ concentration measurement for three bottles filled with different concentrations of CO$_2$. $n = 3$ per calibration gas. g, Peak measured CO$_2$ concentration versus actual CO$_2$ concentration for the calibration gases (black). Coloured lines show the measured peak concentrations for the actual fly-food bottles, and the resulting CO$_2$ concentrations shown in d. In all panels, shading indicates the bootstrapped 95% confidence intervals around the mean.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Responses of flies to odours at different concentrations. **a**, CO₂ concentration on the landing platform (green), and at two distances downwind from the downwind edge of the platform (red and purple). Measurements (shown with points) were made for low flow rates (shown in the inset), and values at larger flowrates were extrapolated based on a linear model for measurements made at the 2-cm distance. This was necessary because the CO₂ sensor could not accurately report concentrations higher than 0.5% CO₂. **b**, Diagram that illustrates the theoretical boundary layer used to confirm our measurements (see Methods). **c**, The responses of flies to odours is consistent across a wide range of concentrations. Data plotted as in Fig. 2e, for additional flow rates. Points indicate individual data points (each trajectory contributes a single point). For each odour, we recorded the following \( n \) = number of trajectories for each of the concentrations (listed left to right): H₂O, 128, 183 and 79; CO₂, 195, 106, 125 and 48; ethanol, 173, 171 and 47; and vinegar, 219, 193 and 248. In all panels, shading indicates the bootstrapped 95% confidence intervals around the median. **d–g**, Comparison of the results from experiments with the landing platform from c and the constrained walking arena used in Fig. 3. Scattergram is repeated from c, 60 ml min⁻¹ CO₂. To compare the data from the wind tunnel experiment to the walking arena from Fig. 3, we calculated a bootstrapped time trace. The time trace is the bootstrapped mean and 95% confidence intervals for the normalized number of flies that would have been on the platform, had all the flies landed simultaneously. The green shading is only provided for reference; the odour was never turned off in these wind tunnel experiments. **e**, Time trace from d overlaid on the normalized number of non-starved flies near the 5% CO₂ source during the dusk time period in the walking arena, copied from Fig. 3d. **f**, Same as d, but for ethanol, 60 ml min⁻¹. **g**, Time trace from f overlaid on the normalized number of non-starved flies near the 5% ethanol source during the dusk time period in the walking arena. Data are not shown, but are very similar to Fig. 3d ethanol case with starved flies. We chose non-starved flies for the comparisons because wind tunnel experiments were done with non-starved flies. We chose the 60 ml min⁻¹ case because the CO₂ concentration in the wind tunnel matches the 5% CO₂ stimulus in the walking experiments.
Extended Data Fig. 3 | Walking arena geometry and odour stimulus. a, Photograph of walking arena, with the lid removed. b, Annotated photograph of the walking arena as seen from above, taken with the machine vision camera that is used for tracking. c, Odour control for the three delivery architectures, with odour off. d, Odour control for the three delivery architectures, with odour on. In our experiments, the port through which odour is delivered was alternated.
Extended Data Fig. 4 | Responses to CO₂ are strongest at 5% concentration and are unaffected by social dynamics. a, Control and 5% CO₂ responses for individual flies. For these experiments, we starved a single two-day old wild-type (HCS) female fly for either 24 h or 3 h before starting the experiment. In every other way, the data are plotted as in Fig. 4. The data shown were collected from \( n = 29 \) individual flies, in which each fly was subject to a 20-h long experiment with \( n = 14 \) 5% CO₂ stimuli and \( n = 10 \) control stimuli. b, CO₂ responses exhibited by flies to three concentrations of CO₂. For these experiments, we starved groups of 10 flies for 24 h before starting the experiment. Flies were presented with 0%, 1.7% or 5% CO₂ in one set of experiments, and 0% or 15% in another set. Data are plotted as in Fig. 4. \( n = 20–170 \) trials per condition. To explain the complex dynamics of the approach behaviour under the different CO₂ concentrations, we made a very simple agent-based model with the pseudocode shown in c; see Supplementary Information for additional discussion. d, Dynamics of the CO₂ attraction of flies can be explained by the simple agent-based model described in c. Preference indices are shown for the results of \( n = 100 \) iterations of the model, under three different CO₂ concentrations. The data are plotted in the same manner as b. The key insight offered by this model is that although our agents were programmed to exhibit the same behaviour towards 1.7% and 5% CO₂, the decreased likelihood of them detecting the lower concentration CO₂ in conjunction with the long-term aversion results in an apparent indifference towards low concentrations of CO₂. e, To show that flies are indeed attracted to the low (1.7%) concentration of CO₂, we used a different analysis that calculated the number of times that flies approached the CO₂ source during the course of each 10-min stimulus. Pairwise statistics were determined with the two-sample Kolmogorov–Smirnov test (test statistics were 0.57, 0.83 and 0.41 for comparisons between 0% and 1.7%, 5%, and 15%). f, Time course of the number of times that flies approach the CO₂ source, in 5-min intervals. In each panel, the shading shows the bootstrapped 95% confidence intervals around the mean.
Extended Data Fig. 5 | Temperature measurements in the walking arena show no correlation with CO₂ or clean air stimuli. a, Temperature over the course of 16 h (see Methods). As in our experiments, every 40 min a 10-min CO₂ stimulus identical to that used in Fig. 4 was applied either to the side of the arena with the temperature probe (green shading) or to the opposite side of the arena (blue shading). b, c, Data from a time-aligned and baseline-subtracted for CO₂ and control trials, respectively.
Extended Data Fig. 6 | Flies do not respond to a stimulus of clean air (without CO₂). Data plotted as in Fig. 4, but for a 0% CO₂ stimulus. \( n = 17 - 81 \) trials per condition. Shading indicates the bootstrapped 95% confidence intervals around the mean.
Extended Data Fig. 7 | IR25a is required for CO₂ attraction and IR40a is not. As in Fig. 4, the data from each experimental group are sorted according to the mean speed during the reference period of 5 min before the odour stimulus. In addition, for each mutant we show two sets of panels corresponding to: (1) flies that were starved for 24 h or 3 h before experiments conducted at 23 °C, and (2) flies that were starved for 3 h before experiments done at 32 °C. This arrangement is in contrast to Fig. 4, in which data from the two temperature groups are combined. a, Responses of two IR25a mutants and a bacterial artificial chromosome rescue to a 5% CO₂ stimulus (a) and a 0% CO₂ stimulus (b). c, d, Responses of an IR40a mutant to a 5% CO₂ stimulus (c) and a 0% CO₂ stimulus (d). n = 4–78 trials per condition. Shading indicates the bootstrapped 95% confidence intervals around the mean.
Extended Data Fig. 8 | IR25a is required for ethanol attraction but not vinegar attraction. Data plotted as in Fig. 4. Experiments were done with 24-h-starved flies only. a, b, Responses to 3 ml min⁻¹ air passed through a bottle of pure ethanol added to 20 ml min⁻¹ clean air. c, Control responses with 3 ml min⁻¹ of clean air added to 20 ml min⁻¹ of clean air. d, e, Responses to 3 ml min⁻¹ air passed through a bottle of pure vinegar added to 20 ml min⁻¹ clean air. f, Control responses with 3 ml min⁻¹ of clean air added to 20 ml min⁻¹ of clean air. n = 14–70 trials per condition. Shading indicates the bootstrapped 95% confidence intervals around the mean.
Extended Data Fig. 9  *Drosophila* are attracted to fatal levels of CO$_2$.

Top, Photograph of two flies that were fatally attracted to a 200 ml min$^{-1}$ CO$_2$ stimulus. Bottom, trajectories for these two flies before they became anaesthetized and died. Colour encodes time, starting at purple and ending at green or yellow.
Extended Data Fig. 10 | Flies and mosquitoes both increase CO$_2$ production when shaken. Red shading indicates the time during which the vial was shaken. We tested four groups of 10–20 animals for flies (black) and mosquitoes (blue). CO$_2$ was measured with a LiCorr-6262. See Methods for details.
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- Data exclusions: The only data that were excluded from our primary analysis were the experiments done at 32 deg C with IR25a BAC rescue flies and IR40a mutants. We discuss this exclusion in the text, and show the excluded data in Extended Data Figure 7.

- Replication: We used 4 completely independent assays (trap assay, free flight wind tunnel assay, wind tunnel walking assay, and constrained walking assay) to verify our primary results, and discuss the consistency of these results throughout the manuscript.

- Randomization: In Fig. 3, controls were done with a separate group of animals. In every other figure, we interleaved control and test stimuli randomly on the same set of individuals.

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