Walking patterns induced by learned odors in the honeybee, *Apis mellifera* L.

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Abstract

The odor localization strategy induced by odors learned via differential conditioning of the proboscis extension response was investigated in honeybees. In response to reward-associated but not non-reward-associated odors, learners walked longer paths than non-learners and control bees. When orange odor reward association was learned, the path length and the body turn angles were small during odor stimulation and greatly increased after stimulation ceased. In response to orange odor, bees walked locally with alternated left and right turns during odor stimulation to search for the reward-associated odor source.After odor stimulation, bees walked long paths with large turn angles to explore the odor plume. For clove, learning-related modulations of locomotion were less pronounced, presumably due to a spontaneous preference for orange in the tested population of bees. This study is the first to describe how an odor-reward association modulates odor-induced walking in bees.

Introduction

Learned odors influence the behavior of bees. For example, bees that were trained to associate an odor at a particular concentration with sugar reward oriented toward this conditioned odor at the given concentration on a locomotion compensator (Kramer, 1976). In a four-armed olfactometer, bees spent more time in sections with an odor previously associated with a sugar reward (Sandoz et al., 2000). Similarly, bees prefer the conditioned stimulus in a Y-maze (Martin, 1964; Carcaud et al, 2009). These odor-induced orientation behaviors may be indicative of odor source localization strategies however they were not analyzed quantitatively. Honeybees almost exclusively move by flight outside the hive but inside the hive, they must walk towards food resources. Such strategies are presumably important to bees when they are inside the hive walking towards odor sources, rather than outside where they mostly orient in flight. This is the natural context in which orientation toward associatively learned odors by walking could occur.

Here, we asked what kinds of walking patterns odorant stimulation induces after bees were either conditioned or not conditioned to the odor. We used orange and clove as odors because both are the complex odors and are learned by proboscis extention response (PER) conditioning (Laska et al., 1999). We compared properties of odor-induced locomotion, in particular differences in locomotion during and after the odor stimuli for clarifying whether there are any differences in locomotion patterns corresponding to entering and subsequently leaving an odor plume.

Materials and Methods

Honeybees (*Apis mellifera* L.*)* were captured at the entrance of hives in Fukuoka University. After anesthesia by cooling, their compound eyes were covered with aqueous black and subsequently white paint to ensure coverage (POSCA, Mitsubishi Pencil Co., Ltd, Tokyo, Japan) and they were attached with their tergum to the tip of wooden sticks (2 mm diameter, 40 mm length, as tethers) using beeswax. The bees' honey stomach was emptied by gently squeezing their abdomen followed by 1.5 h rest in an incubator (20°C) before starting olfactory conditioning. Bees were placed upright by inserting the tether into a Styrofoam board and offered a second stick placed on their ventral side to be held with their legs. These assemblies with the bees were placed in a dark box and transferred to the olfactory conditioning setup. Two essential oils, orange oil and clove oil (Nacalai Tesque, Inc., Kyoto, Japan) were diluted 1:10 with liquid paraffin (Wako Co. Ltd., Osaka, Japan) and 10 μl aliquots were absorbed onto individual filter papers (5 × 5 mm) that were placed into odorant cartridges (50-ml syringes). Odor stimuli were applied in an air stream at 6 ml/s placing the cartridge tips at 1cm from the antennae of the bees. Bees showing PER to these odors before conditioning (27% of all bees for orange but 0% for clove) were excluded from the study. Control bees received the unconditioned stimulus (US) alone during 5 trials with 10 min intertrial interval (ITI). Bees were trained differentially to associate the PER with either Orange as a reward associated conditioned stimulus (CS+) and Clove as a reward non associated conditioned stimulus (CS-) or Clove as CS+ and Orange as CS- (Fig. S2A). It was evaluated whether the bee shows PER to CS+ and CS- for the first three seconds of odor application (Fig. S2A). Bees that did not respond to the CS+ during the 5 training trials (10 min ITI) were categorized as non-learners. The bees

which show PER to CS+ but not to CS- in at least three consecutive trials including the last trial were classed as learners. These bees were incubated in a dark room (20°C) for 3 h, and then selected control bees and non-learners showing PER to neither CS+ nor CS- and learners showing PER to CS+ but not to CS- were used for locomotion recording experiments in paired trials (i.e. orange and clove).

Odor-induced locomotion in tethered honeybees

We used a locomotion compensator-based walking simulator similar to other designs (Brandstaetter et al., 2014). It consisted of a Styrofoam sphere (5 cm in diameter, 2.2 g) positioned in a modified plastic funnel and suspended on an air-cushion formed by air flowing into the funnel from its neck (Fig. S3A). Each tethered bee was placed on the top of the sphere using a micromaniplator, allowing virtual locomotion in any direction by turning the sphere below it. Locomotion recording was started after spontaneous walking ceased. To monitor the movements of the sphere caused by locomotion the motion sensors of two laser mice (Logicool G5, Logitech) were placed around the equator of the sphere, <1 mm from the surface and arranged at right angles with one at the rear and one laterally, with respect to the experimental animal (Fig. S3B). The movements of the sphere acquired by the mice were transferred to a computer (Lenovo ThinkPad SL500, China) running Linux OS Mandriva ver. 2007.0 with a patched kernel and were recorded by a real-time locomotion-recording program (Recloc, written in C) developed by one of us (Haupt and Kanzaki 2009). The complete system, including the two mice, was confirmed to operate at a minimum capture rate of 500 Hz and had a spatial resolution of 800 dpi (<5% error at <1 mm sensor-substrate distance, Fig. S4).

The odor-loaded filter papers were individually placed into odorant cartridges (Pasteur pipettes) with the wider opening 1 cm from the tips of the antennae of the animals. Air from an air pump was passed through activated charcoal before entering the stimulus delivery system. Odorant stimulus timing was controlled through the locomotion recorder program via pulses generated at the computer's parallel port, with a timing precision of <0.02 ms with respect to locomotion-recording data capture. Stimulus delivery was operated via solenoid valves delivering puffs at a flow rate of 360 ml/min through the cartridge. Stimuli were given in series of five pulses of 0.9 s duration at intervals of 1.2 s starting 5 s after beginning a locomotion-recording trial (Fig. S2B). The different odorant stimulus conditions were applied in random order across individuals.

Evaluation of body turn angle

In each locomotion recording, the direction of the body axis at the beginning of each recording was defined to be 0° body angle (Fig. 1B). On this study body turn angle was calculated from the difference of the bearing of the bee between an inflection point and the next inflection point. The inflection points were judged by the threshold of the changing value, 5 degrees. Turns with amplitudes of absolute value greater than 5 degrees were extracted to calculate the average body turn angle during each observation phase.

Results and Discussion

   Odor-induced Locomotion

Odor-induced locomotion was recorded in a control group (no conditioning), non-learner group (conditioned but not learned the odors) and in differential olfactory learner groups (reward-associated and reward-non associated). The abbreviations of odors were named based on the pre-conditioning of these treatment groups (O: orange for control, O^0: orange for non-learner, O^+: orange for learner as reward associated, O^-: orange for learner as reward-non associated (Table S1). These are the same for clove (C). To evaluate the effects of olfactory learning on odor-induced locomotion, path lengths and turn angles were compared among these four groups during the whole recording period (55 s, Fig. 1C, E, and Fig. 2A, C). Odorant but not clean air stimulation triggered walking in all tested honeybees (n=87). There were no significant differences in path length between walks induced by the two odors used in control bees (Wilcoxon rank sum test, O vs C: p=0.696, n=24). Locomotion induced by orange odor was compared among the control (O), non-learner (O^0), and the two orange-learned groups (O+ and O-). Path length and body turn angle in locomotion induced by O^+ were significantly longer than in the other groups (Kruskal–Wallis and Steel–Dwass tests: O^+ vs O, $p<0.001$; O^+ vs O^0, $p<0.001$; O^+ vs O^-, p=0.001 (in path length) and O^+ vs O, $p<0.05$; O^+ vs O^0, $p<0.001$; O^+ vs O^-, p=0.001 (in body turn angle), Fig. 1C and E).

Locomotion induced by clove odor was also compared among control, non-learner and the two groups of odor-learner bees. The path length of locomotion induced by C^+ was significantly greater than in C, C^0 and C^- (Kruskal–Wallis test with the Steel–Dwass test: C^+ vs C, $p<0.001$; C^+ vs C^0, $p<0.001$; C^+ vs C^-, $p<0.05$, Fig. 2A), however not in the case of turn body angle (Kruskal–Wallis test: p=0.205, Fig. 2C). These results

suggest that reward-associated odors activate odor-induced locomotion similarly irrespective of odorant identity.

Temporal patterns of locomotion induced by reward-associated odor

After learning the orange odor associatively with the reward, honeybees walked in a complex trajectory when they received the O^+. During the O^+ stimulus (phase I, Fig. 1A), they moved toward the O^+ source (the air current containing the odor comes from the front, positive X axis in the coordinate). After the stimulus (phase II) and in the period starting 10 s after the stimulus ended (phase III), they walked along an arc. The honeybees turned alternately to the left and right (Fig. 1B). Median path lengths and body turn angles of locomotion induced by orange and clove were compared among the three recording periods, phases I, II and III.

The path lengths were not significantly different among phases I, II and III for locomotion induced by O (Friedman test: $p=0.215$, n=28), O^0 (Friedman test: $p=0.283$, n=19) and O^- (Friedman test: $p=0.076$, n=19). In contrast, the path lengths of the locomotion induced by O^+ in both phases II and III were significantly longer than that in phase I (Friedman's test with Steel–Dwass test: phase I vs phase II, $p=0.009$; phase I vs phase III, $p=0.004$, n=16) (Fig. 1D). The body turn angles were not significantly different among phases I, II and III for locomotion induced by O (Friedman test: $p=0.075$, n=28), O^0 (Friedman test: $p=0.086$, n=19) and O^- (Friedman test: $p=0.085$, n=19). In contrast, body turn angles of the locomotion induced by O^+ in both phases II and III were significantly greater than that in phase I (Friedman's test with Steel–Dwass test: phase I vs II, $p<0.001$; phase I vs III, $p<0.001$, n=16) (Fig. 1F). The path lengths were not significantly different among the three phases for locomotion induced by C

(Friedman test: p=0.351, n=24), C^0 (Friedman test: p=0.298, n=19), C^+ (Friedman test: p=0.135, n=19) and C^- (Friedman test: p=0.269, n=16) (Fig. 2B). The body turn angles were not significantly different among phases I, II and III for the locomotion induced by C (Friedman test: p=0.053, n=24), C^0 (Friedman test: p=0.095, n=19), C^+ (Friedman test: p=0.066, n=19) and C^- (Friedman test: p=0.159, n=16) (Fig. 2D).

In the virtual path observed (Fig. 1A), the bee moved forward with repeated alternated left and right turns with respect to its orientation during O^+ stimulation (phase I). This corresponds to a local orientation toward the reward-associated odor source. We also observed after the odor stimulation (phases II and III) in the virtual path that the bee traced a roughly circular path after O^+ stimulation and finally returned close to the position where the stimulus was received (Fig. 1A). This corresponds to exploratory locomotion toward the odor plume. Such odor-seraching behavior pattern was investigated previously in free-flying honeybees (Chaffoil et al., 2005; Ikeno et al., 2014). In the wind tunnel, honeybees that have learned an odor by PER-conditioning orientate upwind with alternate turning and circling flight (Chaffoil et al., 2005; Ikeno et al., 2014), while outside of the plume, they turn in a large circular arc (Ikeno et al., 2014). Therefore, both the forward locomotion with alternating turns upwind to the reward-associated odor and circular locomotion outside of the plume seem to be common features of searching strategies in walking and flying toward the reward-associated odor source in honeybees.

Odor-orientation behavior has been studied in various moths, cockroaches and flies (Kramer, 1975; Baker and Kuenen, 1982; Kaissling and Kramer, 1990; Kanzaki et al., 1992; Budick and Dickinson, 2006; Willis et al. 2008; Schleyer et al 2015). When a moth receives a pulse of pheromone, it performs a stereotypic behavior

consisting of a surge, a zigzag and a loop. This series of movements is reset to the first component, the surge, by the next pulse of pheromone. Because of continuous pheromonal pulses inside the plume, the male moth orientates toward the pheromonal source by surges and zigzagging. If the moth goes outside the plume, the series of movements is not reset and the moth repeatedly loops (Baker, 1990; Kanzaki et al., 1992). However, the honeybee does not show such clear stereotyped movements upon single pulses of reward-associated odor (data not shown). Nevertheless, the orientation behavior of repetitive alternating counter-turns with small angles observed in our study might be partly analogous to the series of movements evoked by sex pheromone in male moths.

We also recorded locomotion induced by CS^- in this study. There were no significant differences in odor-induced locomotion with respect to path length or body turn angle among the locomotion induced by O, O^0 and O^-(Fig. 1) and among those by C, C^0 and C^- (Fig. 2). These observations suggest that unrewarded conditioning has no effect on odor-induced locomotion. Sandoz et al. (2000) suggested that simply blowing odors into the hive has no effect in attracting other hive-mates, however reward-associated odors do induce attraction. In natural hives, foragers return carrying odors from many different flowers. It would be advantageous for hive-mates to detect profitable food with more certainty by orientating only toward the previously reward-associated learned odor. This in turn would lead to recruitment of the conditioned forager to the site advertised by the dancing bee or stimulate the conditioned forager to revisit the foraging location where it was conditioned to the odor (von Frisch, 1967; Reinhard et al., 2004).

Once the honeybee experiences the orange-reward association, O^+ might trigger strategic orientation to O^+ source which consists of upwind orientation with alternative

turns during O^+ stimuli and circular turns after O^+ stimuli. While clear differences in locomotion were observed for both reward-associated odors tested, the clear distinction between stimulus phase and after-stimulus phase was not evident with clove. The asymptotes for PER learning success for clove and orange were identical, suggesting the conditioned PER responsiveness was similar for both (Fig. S1), despite that fact that there is a proportion of bees in our hives that shows spontaneous PER to orange and none to clove. While bees were tested for retention prior locomotion recording and the majority also responded with PER to O^+ on the trackball (75%, n=16), most bees failed to do so for C^+ (5%, n=19). It is therefore possible that differences in extinction play are role in the differences observed for O^+ and C^+ bees. This implies an intrinsic preference and possibly increased sensitivity to O that has an impact on behavior during exposure to stimulation reminiscent to an upwind surge toward the odor source. Studies with other odorants and different concentrations will have to clarify this important point.

Competing interest

The authors declare no conflict of interest.

Author contribution

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: HA. Acquisition of data: TY, HA. Analysis and interpretation of data: TY, HA. Drafting of the manuscript: HA, TY. Critical revision of the manuscript for important intellectual content: HI, SSH. Obtained funding: HA. Setup design and programming: SSH. Study supervision: HA.

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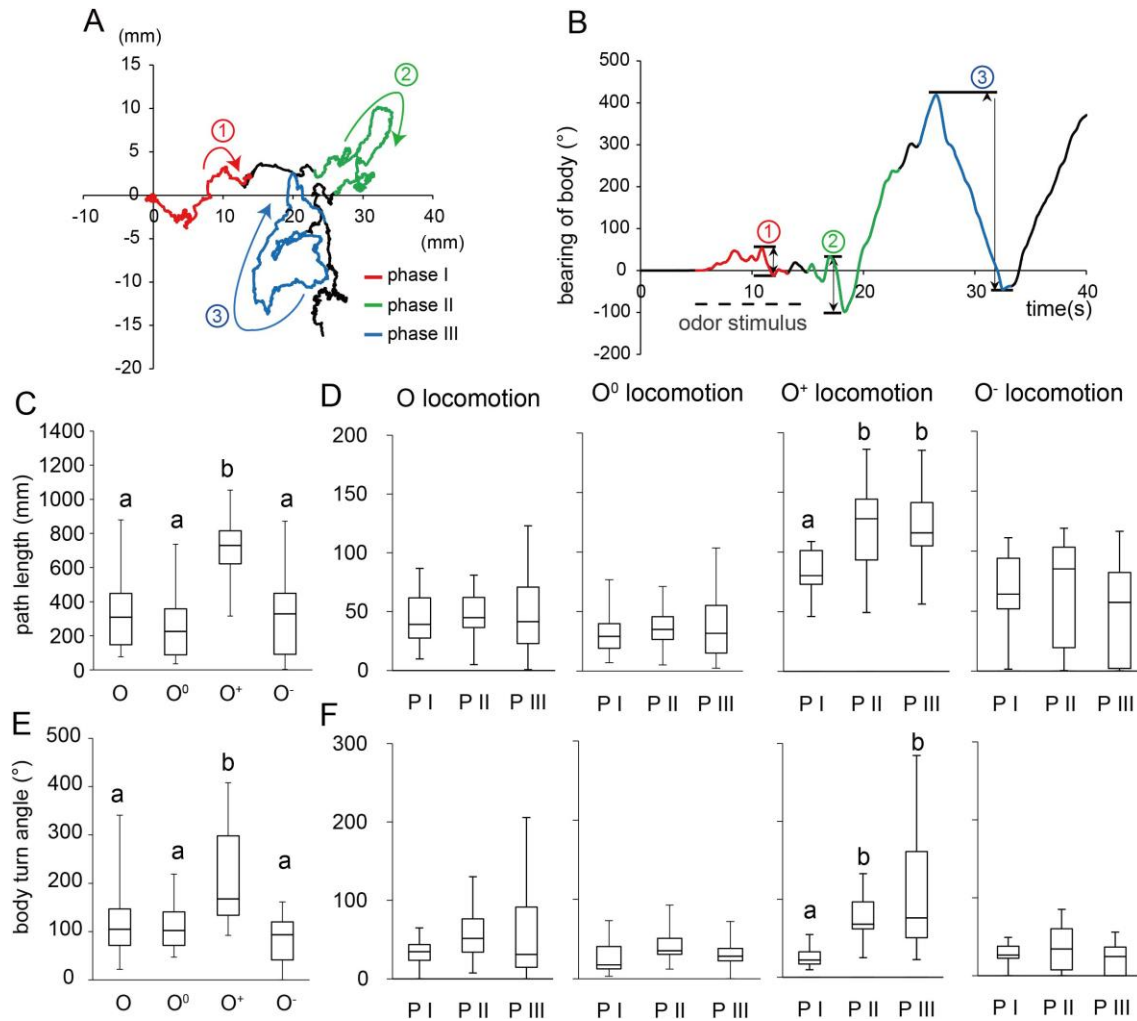
Figures

Fig. 1. **Locomotion induced by orange odor.** A: Fine structure of a locomotor path induced by the reward-associated orange odor (O⁺) in a learner. Three turns to the right are indicated by three arrows. B: Cumulative body orientation angle of the path shown in A. The turn angle range was relatively small during O⁺ stimulation (double-headed arrow of □) and increased in the periods following O⁺ stimulation (double-headed arrow of □ and □). Red line: phase I; Green line: phase II; Blue

line: phase III. C and E: Comparisons of path length (C) and body turn angle (E) caused by odor stimulus of orange (O, O^0, O^+ and O^-), measured from the start of odor stimulation to the end of the recording55 s later. Different letter labels indicate significant differences ($p<0.05$). D and F: Comparisons of path lengths (D) and turn angles (F) among recording phases I, II and II for O, O^0, O^+ and O^- locomotion. Data are shown as boxplots with the median indicated in middle line in box, the edges of the box indicates the 25th and 75th percentiles. P I, phase I; P II, phase II; P III, phase III.
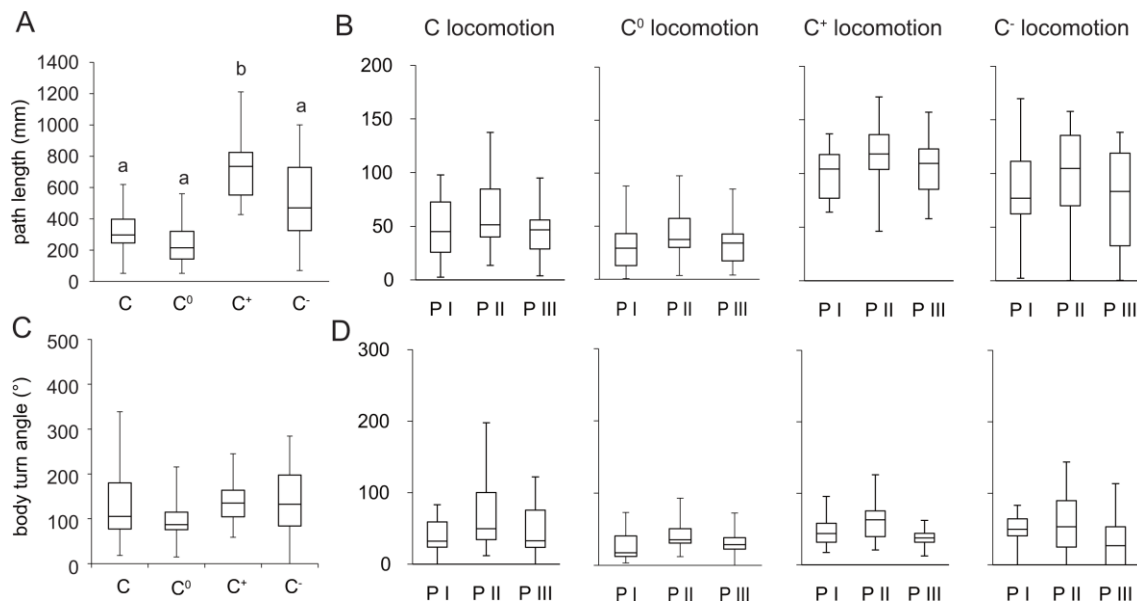
Fig. 2. Locomotion induced by clove odor. A, C: Comparisons of path length (A) and body turn angle (C) caused by odor stimulus of clove (C, C^0, C^+ and C^-), measured during whole recording time (from odor stimulus starting to 55 s). Different letter labels indicate significant differences ($p<0.05$). B and D: Comparisons of path lengths (B) and turn angles (D) among recording phases I, II and II for C, C^0, C^+ and C^- locomotion.
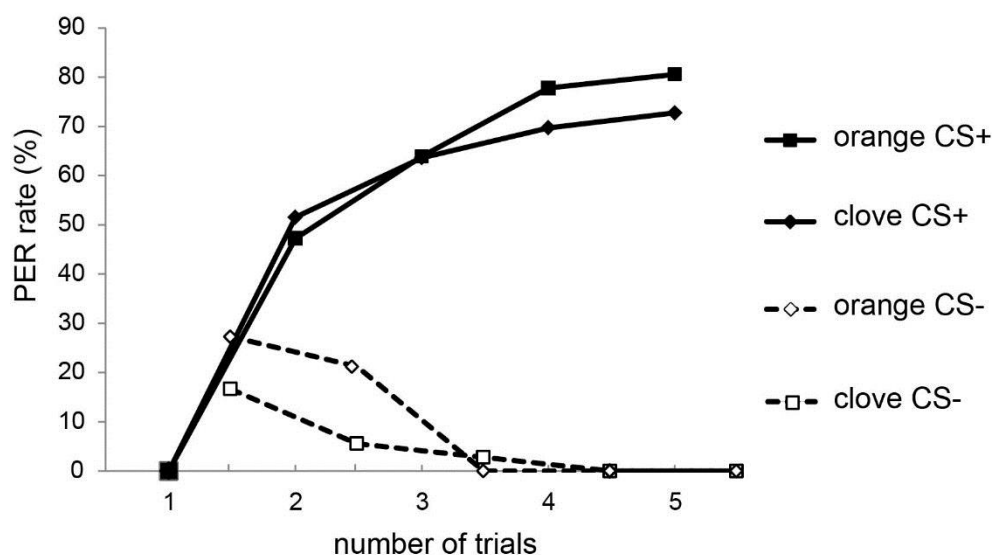
Fig. S1. Proboscis extension response (PER) rates during olfactory conditioning trials (O+ and C-, N=36; C+ and O-, N=54). CS+: reward associated conditioned stimulus, CS- reward non associated conditioned stimulus.
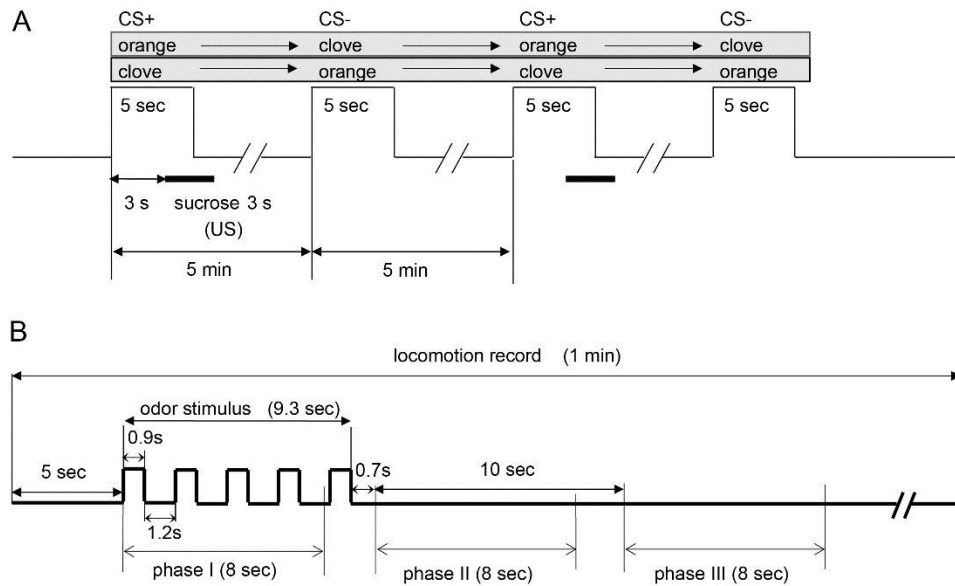
Fig. S2. A: Odor conditioning of PER. For olfactory conditioning, either orange or clove odors (CS$^+$) was applied to the antennae of honeybees for 5 s. Three seconds after the onset of CS$^+$, 1.5 M sucrose solution was applied for 3 s (the unconditioned stimulus, US). After 5 min, the alternative odor was applied without sucrose solution (CS$^-$). These two series of differential conditioning, shown as the upper and lower bars, were applied at 5 min intervals for up to five repeats of the cycle. It was evaluated whether the PER was induced during 3 s after the onset of odor stimuli. **B: The time schedule of locomotion recording**. Five odor pulses were applied over about 10 s, commencing 5 s after the onset of recording. Locomotion was recorded for 1 min. The time-course of the locomotion were compared during three 8-s phases. phase I, during the odor stimuli; phase II, commencing at the end of the odor stimuli; and phase III, 8 s commencing 10 s after the end of the odor stimuli.
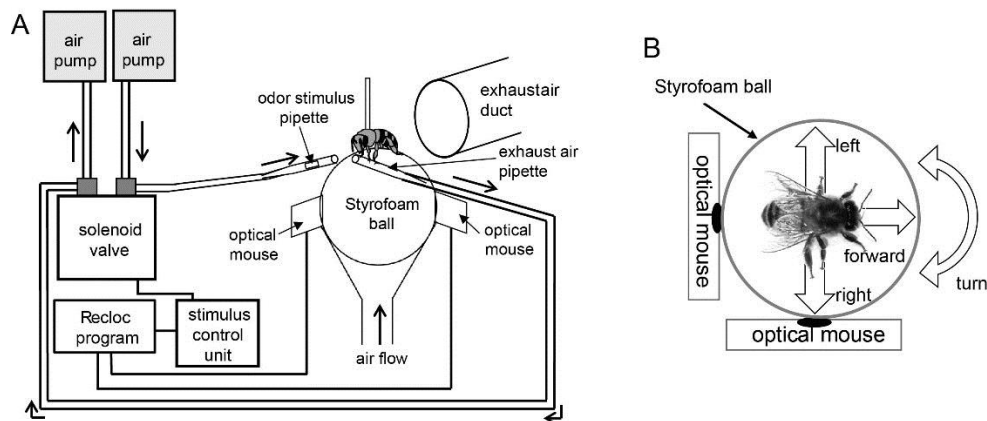
Fig. S3. Locomotion recording setup. A: A honeybee was placed on top of a Styrofoam ball suspended by air flow. The position was adjusted to enable the honeybee to walk naturally. After the honeybee ceased walking on the ball, the odor was applied with intermittent pulses from the pipette in front of honeybee. To prevent interference from odor leaking from the pipette, a second exhaust pipette was situated in front of the honeybee. Both stimulus timing and locomotion recording were controlled by a locomotion recording program (Recloc). B: Two optical mice were placed close to the surface of the ball laterally and posteriorly to the honeybee. The rotation of the ball caused by the honeybee's locomotion was used for quantitative analysis by Recloc.
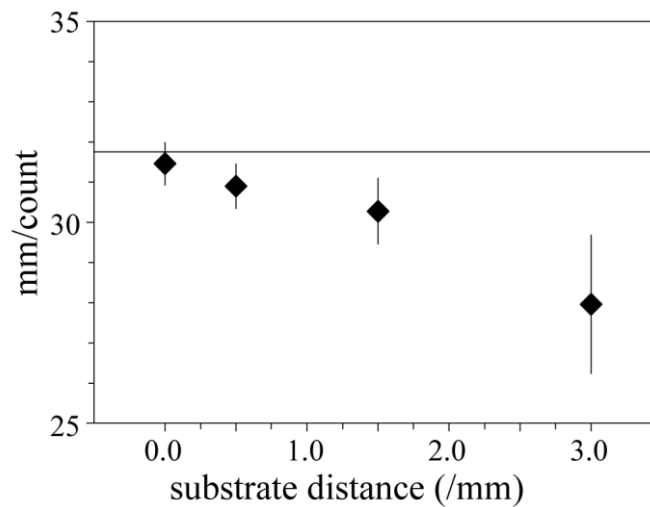
Fig. S4. Mouse sensor movement detection. Displacement information obtained from optical mice (Logitech G5) moved over a Styrofoam substrate at different distances from the substrate. Data represent means and standard deviations obtained from 20 sessions per point. Nominal resolution was 31.75μm/count (800cpi) as indicated by a horizontal line. Displacement was controlled via 0.1mm/turn leadscrews of a micromanipulator (MX-1, Narishige, Tokyo, Japan) with the x/y-axes aligned as well possible to the mouse sensor axes and compensating for backlash before every trial. At zero substrate distance, readings varied from 30.30 to 32.26μm (standard deviation 0.54μm, n=20), resulting in approximately 5% maximum error with respect to the nominal value. While average reported displacement declined with increasing substrate distance, the variance around the mean reported value only started to increase at substrate distances over 1mm. Systematic tests on the dynamic performance of displacement detection by the mouse sensors could not be carried out. Readout was 100% reliable with submicrosecond jitter at 500Hz under the experimental conditions as confirmed by saving time stamps obtained from the PC's real-time clock immediately after obtaining valid mouse data using optional real-time functionality of the Linux kernel (2.6.18 or 2.6.29).

Table S1. Abbreviations of odor used as stimulant.

Odor group	Control	Non-learner	Learner	
			Orange CS＋ Clove CS－	Clove CS＋ Orange CS－
Orange	O (n=28)	O^0 (n=19)	O^+ (n=16)	O^- (n=19)
Clove	C (n=24)	C^0 (n=19)	C^- (n=16)	C^+ (n=19)

Movie 1. Typical example of locomotion induced by O+. When a stimulus pulse was applied, the LED was turned off. Locomotion was always recorded in a dark room. The compound eyes were also masked by paint.