© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 12-16 doi:10.1242/jeb.123356

SHORT COMMUNICATION

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

Toshiya Yamashita[1], S. Shuichi Haupt[2,3], Hidetoshi Ikeno[4] and Hiroyuki Ai[1,*]

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 alternate 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 odor, 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.

KEY WORDS: Proboscis extension response, Differential conditioning, Olfactory learning, Trackball locomotion recording

INTRODUCTION

Learned odors influence the behavior of bees. For example, bees that were trained to associate an odor at a particular concentration with a 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 complex odors and are learned by proboscis extension response (PER) conditioning (Laska et al., 1999). We compared properties of odor-induced locomotion, in particular differences in locomotion during and after exposure to the odor stimuli, to clarify 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 olfactory conditioning began. Bees were placed upright by inserting the tether into a Styrofoam board and were offered a second wooden 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^{-1}, with the cartridge tips placed 1 cm from the antennae of the bees. Bees showing a 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 five trials with a 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 non-reward-associated conditioned stimulus (CS−) or clove as CS+ and orange as CS− (Fig. S1A). We evaluated whether each bee showed PER to CS+ and CS− for the first 3 s of odor application (Fig. S1A). Bees that did not respond to the CS+ during the five training trials (10 min ITI) were categorized as non-learners. The bees that showed 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. S2A). Each tethered bee was placed on the top of the sphere using a micromanipulator, 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. S2B). The movements of the sphere acquired by the mice were transferred to a computer (Lenovo ThinkPad SL500, China) running Linux OS Mandriva v2007.0 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

[1]Department of Earth System Science, Fukuoka University, Fukuoka 814-0180, Japan. [2]Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan. [3]Department of Biological Cybernetics, Bielefeld University, 33615 Bielefeld, Germany. [4]School of Human Science and Environment, University of Hyogo, Himeji, Hyogo 670-0092, Japan.

*Author for correspondence (ai@fukuoka-u.ac.jp)

Received 7 April 2015; Accepted 21 October 2015

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 12-16 doi:10.1242/jeb.123356

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. S3).

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^{-1} through the cartridge. Stimuli were given in a series of five pulses of 0.9 s duration at intervals of 1.2 s starting 5 s after the start of a locomotion-recording trial (Fig. S1B). 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 deg body angle (Fig. 1B). In 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 deg. Turns with amplitudes of absolute value greater than 5 deg 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), a non-learner group (conditioned but not learned the odors) and in differential olfactory learner groups (reward

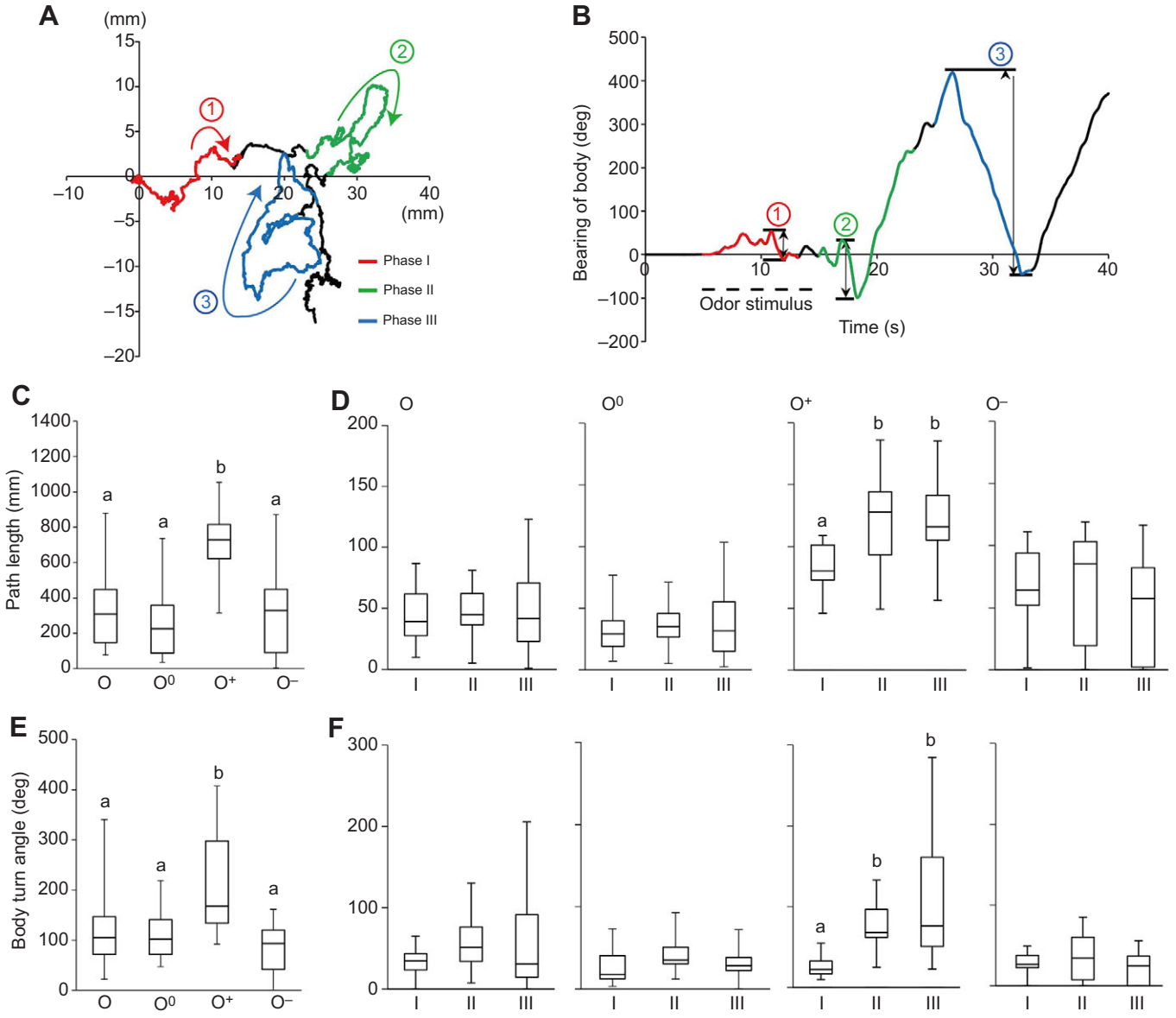

Fig. 1. Locomotion induced by orange odor. (A) Fine structure of a locomotor path induced by the reward-associated orange odor (O^+) in a learner. Three turns to the right are indicated by three arrows. (B) Cumulative body orientation angle of the path shown in A. The turn angle range was relatively small during O^+ stimulation (double-headed arrow 1) and increased in the periods following O^+ stimulation (double-headed arrows 2 and 3). Red line, phase I; green line, phase II; blue line, phase III. (C,E) Comparison of path length (C) and body turn angle (E) caused by an orange odor stimulus (O, control; O^0, non-learner; O^+, reward-associated learner; and O^-, non-reward-associated learner), measured from the start of odor stimulation to the end of the recording, 55 s later. Different letters indicate significant differences (P<0.05). (D,F) Comparison of path length (D) and turn angle (F) among recording phases I, II and III for O, O^0, O^+ and O^- locomotion. Data are shown as boxplots with the median indicated by the middle line in the box; the edges of the box indicate the 25th and 75th percentiles.

Journal of Experimental Biology (2016) 219, 12-16 doi:10.1242/jeb.123356

associated and non-reward associated). The abbreviations used were based on the pre-conditioning of these treatment groups (O, control; O^0, non-learner; O^+, reward-associated learner; O^-, non-reward-associated learner, where O is orange; Table S1). The same system was used 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, 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 versus C: P=0.696, N=24). Locomotion induced by orange odor was compared among the control (O), non-learner (O^0), and the two learner 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: path length O^+ versus O, P<0.001; O^+ versus O^0, P<0.001; O^+ versus O^-, P=0.001; and body turn angle O^+ versus O, P<0.05; O^+ versus O^0, P<0.001; O^+ versus O^-, P=0.001; Fig. 1C,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 that with C, C^0 and C^- (Kruskal–Wallis test with the Steel–Dwass test: C^+ versus C, P<0.001; C^+ versus C^0, P<0.001; C^+ versus C^-, P<0.05; Fig. 2A); however, this was not the case for body turn 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 on the path length.

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^+ stimulus. 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; Movie 1). Median path lengths and body turn angles of locomotion induced by orange and clove were compared among the three recording periods, phases I–III.

The path lengths were not significantly different among phases I–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 phase II and phase III were significantly longer than that in phase I (Friedman's test with Steel–Dwass test: phase I versus phase II, P=0.009; phase I versus phase III, P=0.004, N=16; Fig. 1D). The body turn angles were not significantly different among phases I–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 versus phase II, P<0.001; phase I versus phase 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–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).

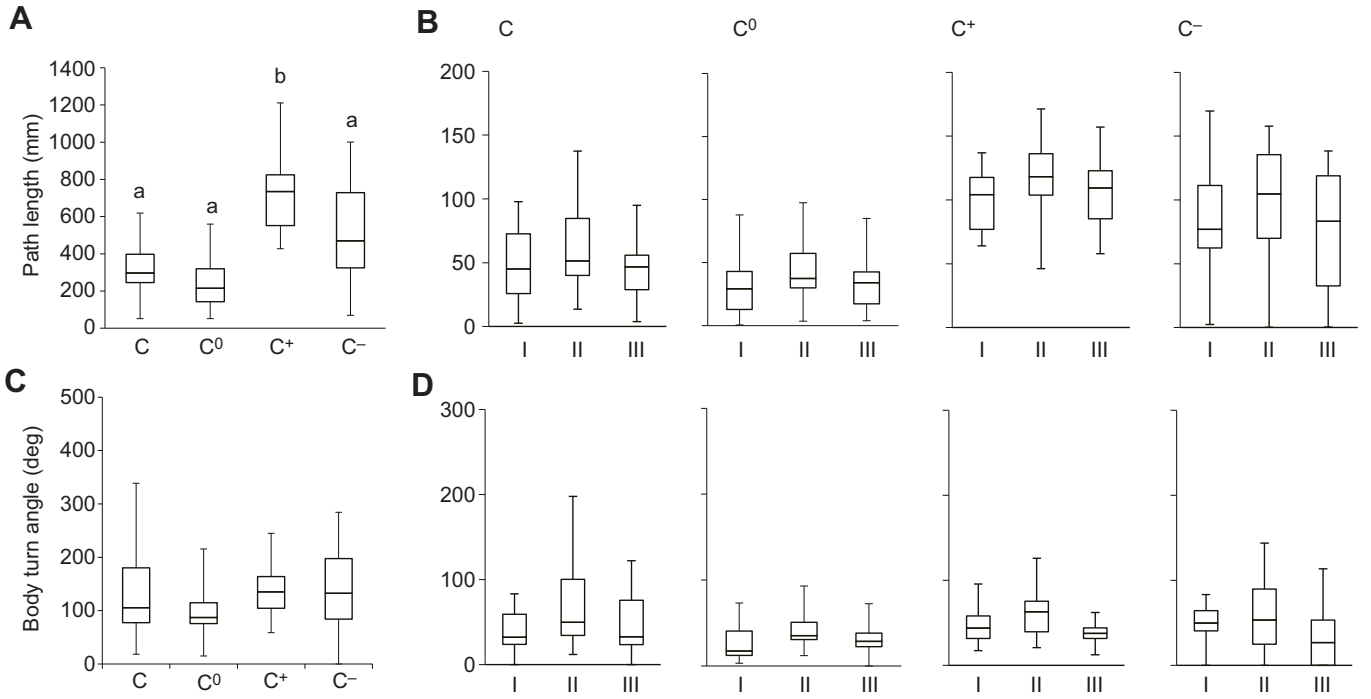

Fig. 2. Locomotion induced by clove odor. (A,C) Comparison of path length (A) and body turn angle (C) following exposure to the clove odor stimulus (C, C^0, C^+ and C^-), measured throughout the recording time (55 s). Different letters indicate significant differences (P<0.05). (B,D) Comparison of path length (B) and turn angle (D) among recording phases I, II and II for C, C^0, C^+ and C^- locomotion.

Journal of Experimental Biology

In the virtual path observed (Fig. 1A), the bee moved forward with repeated alternate 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 to a position close to where the stimulus was received (Fig. 1A). This corresponds to exploratory locomotion toward the odor plume. Such an odor-searching behavior pattern was investigated previously in free-flying honeybees (Chaffiol 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 (Chaffiol 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 would 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 odor–reward association, O^+ might trigger strategic orientation to the O^+ source, which consists of upwind orientation with alternate turns during exposure to 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. S4), despite the 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 to 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). The learning effect of C^+ may have declined at the locomotion recording (after more than 3 h from the PER conditioning). It is therefore possible that differences in extinction play a 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 of an upwind surge toward the odor source. Studies with other odorants and different concentrations are required to clarify this important point.

Acknowledgements
We thank Prof. Tsunao Itoh for helpful comments on the article and Mr Yuta Kimura and Mr Shota Ishibashi for technical advice.

Competing interests
The authors declare no competing or financial interests.

Author contributions
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. H.A. conceived and designed the study; T.Y. and H.A. acquired, analyzed and interpreted the data, and drafted the manuscript; H.I. and S.S.H. critically revised the manuscript for important intellectual content; H.A. obtained funding; S.S.H. carried out setup design and programming; H.A. performed study supervision.

Funding
Financial support was provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan, grant number 22570079 and 15K14569, and by the Central Research Institute of Fukuoka University (no. 151031) to H.A. S.S.H. was initially supported by a Japan Society for the Promotion of Science (JSPS) post-doctoral scholarship (P07144).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.123356/-/DC1

References
Baker, T. C. (1990). Upwind flight and casting flight: complementary phasic and tonic system used for location of sex pheromone sources by male moths. In *Proceedings 10th International Symposium Olfaction and Taste*, (ed. K. B. Døving), pp. 18-25. Oslo.

Baker, T. C. and Kuenen, L. P. S. (1982). Pheromone source location by flying moths: a supplementary non-anemotactic mechanism. *Science* **216**, 424-427.

Brandstaetter, S., Bastin, F. and Sandoz, J.-C. (2014). Honeybee drones are attracted by groups of consexuals in a walking simulator. *J. Exp. Biol.* **217**, 1278-1285.

Budick, S. A. and Dickinson, M. H. (2006). Free-flight responses of *Drosophila melanogaster* to attractive odors. *J. Exp. Biol.* **209**, 3001-3017.

Carcaud, J., Roussel, E., Giurfa, M. and Sandoz, J.-C. (2009). Odour aversion after olfactory conditioning of the sting extension reflex in honeybees. *J. Exp. Biol.* **212**, 620-626.

Chaffiol, A., Laloi, D. and Pham-Delègue, M.-H. (2005). Prior classical olfactory conditioning improves odour-cued flight orientation of honey bees in a wind tunnel. *J. Exp. Biol.* **208**, 3731-3737.

Haupt, S. S. and Kanzaki, R. (2009). Behavioural analysis of pheromone orientation in the silkmoth. 80th annual meeting of the Zoological Society of Japan, Shizuoka, September 2009. http://www.zoology.or.jp/news/img/f_users/r_3421967img20090924203937.pdf

Ikeno, H., Akamatsu, T., Hasegawa, Y. and Ai, H. (2014). Effect of olfactory stimulus on the flight course of a honeybee, *Apis mellifera*, in a wind tunnel. *Insects* **5**, 92-104.

Kaissling, K.-E. and Kramer, E. (1990). Sensory basis of pheromone-mediated orientation in moths. *Verh. Dtsch. Zool. Ges.* **83**, 109-131.

Kanzaki, R., Suga, N. and Shibuya, T. (1992). Self-generated zigzag turning of *Bombyx mori* males during pheromone-mediated upwind walking. *Zool. Sci.* **9**, 515-527.

Kramer, E. (1975). Orientation of the male silkmoth to the sex attractant bombykol. In *Mechanisms in Insect Olfaction* (ed. D. A. Denton and J. Coghlen), pp. 329-335. New York: Academic Press.

Kramer, E. (1976). The orientation of walking honeybees in odour fields with small concentration gradients. *Physiol. Entomol.* **1**, 27-37.

Laska, M., Galizia, C. G., Giurfa, M. and Menzel, R. (1999). Olfactory discrimination ability and odor structure-activity relationships in honeybees. *Chem. Senses* **24**, 429-438.

Martin, H. (1964). Zur Nahorientierung der Biene im Duftfeld. Zugleich ein Nachweis für die Osmotropotaxis bei Insekten. *Z. Vergl. Physiol.* **48**, 481-533.

Reinhard, J., Srinivasan, M. V. and Zhang, S. (2004). Olfaction: scent-triggered navigation in honeybees. *Nature* **427**, 411.

Sandoz, J. C., Laloi, D., Odoux, J. F. and Pham-Delègue, M. H. (2000). Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. *Anim. Behav.* **59**, 1025-1034.

Schleyer, M., Reid, S. F., Pamir, E., Saumweber, T., Paisios, E., Davies, A., Gerber, B. and Louis, M. (2015). The impact of odour-reward memory on chemotaxis in larval *Drosophila*. *Learn. Mem.* **22**, 267-277.

von Frisch, K. (1967). *The Dance Language and Orientation of Bees*. Cambridge, MA: The Belknap Press of Harvard University Press.

Willis, M. A., Avondet, J. L. and Finnell, A. S. (2008). Effects of altering flow and odor information on plume tracking behavior in walking cockroaches, *Periplaneta americana* (L.). *J. Exp. Biol.* **211**, 2317-2326.