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ORIGINAL PAPER

Use of bilateral information to determine the walking direction during orientation to a pheromone source in the silkmoth *Bombyx mori*

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Abstract Odor source localization is an important animal behavior. Male moths locate mates by tracking sex pheromone emitted by conspecific females. During this type of behavior, males exhibit a combination of upwind surge and zigzagging flight. Similarly, the male walking moth *Bombyx mori* responds to transient pheromone exposure with a surge in movement, followed by sustained zigzagging walking. The initial surge direction is known to be influenced by the pheromone input pattern. Here, we identified the sensory input patterns that determine the initial walking direction of males. We first quantified the stimulus by measuring electroantennogram values, which were used as a reference for subsequent tests. We used a brief stimulus pulse to examine the relationship between sensory stimulus patterns and the turning direction of initial surge. We found that the difference in input timing and intensity between left and right antennae affected the walking direction, indicating that *B. mori* integrate bilateral pheromone information during orientation behavior. When we tested pheromone stimulation for longer periods, turning behavior was suppressed, which was induced by stimulus cessation. This study contributes toward understanding efficient strategies for odor-source localization that is utilized by walking insects.

Keywords Bi-antennal · Odor · Surge · Turning behavior · Programmed behavior

Introduction

Male moths exhibit mating behavior in response to sex pheromones emitted by conspecific females (Butenandt et al. 1959; Kennedy 1983; Vickers 2000; Cardé and Willis 2008). Inside an odor plume, pheromones do not form a continuous concentration gradient, but are discontinuously distributed (Murlis and Jones 1981; Murlis et al. 1992). The discontinuous portion of the pheromone is termed the filament, with moths detecting individual filaments as a pheromone input. Several candidate strategies for odor-source localization have been proposed (Kennedy 1940; Farkas and Shorey 1972; Baker 1990; Mafra-Neto and Cardé 1994; Vickers and Baker 1994; Vergassola et al. 2007). According to the optomotor anemotaxis hypothesis, male moths exhibit flight behavior in response to sex pheromones, modifying their flight path by visual feedback (Kennedy 1940; Kennedy and Marsh 1974; Baker et al. 1984). It has also been proposed that male moths intrinsically generate sustained flight during plume tracking (Baker and Kuenen 1982; Willis and Baker 1987; Willis and Arbas 1991; Vickers and Baker 1992). In addition to sustained flight, phasic movement, termed upwind surge, has been observed during mating behavior by several moth species, and is thought to have an important role in odor-source localization.

A precise role of phasic behavioral response for contact with pheromone filaments to set an upwind course has been proposed (Baker 1990). The upwind surge after interception of a pheromone filament in a plume, or a single puff of

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pheromone, has been reported (Vickers and Baker 1992, 1994, 1996; Mafra-Neto and Cardé 1994, 1995). Due to surge flight, moths are able to detect filaments at various frequencies (Vickers and Baker 1992). At frequencies higher than 5 filaments/s, moths show reiterative upwind surge, resulting in straighter upwind tracks (Mafra-Neto and Cardé 1994; Vickers and Baker 1994). Hence, many researchers support that flying moths exhibit a combination of optomotor anemotaxis and intrinsically programmed behavior, i.e., upwind surge followed by sustained zigzagging flight.

The silkmoth *Bombyx mori* exhibits pheromone-triggered walking behavior that comprises wing fluttering and body bending, termed a mating dance (Butenandt et al. 1959; Kaissling 1971; Kramer 1975, 1986; Obara 1979; Ono 1980), with the major sex pheromone bombykol alone being sufficient to trigger full sexual behavior. After brief exposure to bombykol (100 ms), moths show a surge in movement followed by a zigzagging walking pattern (Kanzaki et al. 1992). During zigzagging walking, moths show repeated turning behavior, with the inter-turn interval increasing over time. When the moth receives supplementary pheromone input during zigzagging walking, the walking behavior is re-initiated. When a pheromone plume contains many filaments, the moth repeats the initial phase and, as a result, the walking trajectory becomes almost linear as the relative frequency of surge increases (Kanzaki et al. 1992). This locomotion pattern by *B. mori* seems to be the counterpart of upwind surge and zigzagging flight by flying moths.

Walking direction is important for orientation behavior. We previously reported that the direction of initial surge is affected by pheromone input on the antennae of *B. mori*, whereby the moth exhibits turning behavior in the direction from which the antenna was stimulated (Kanzaki et al. 1992). Because moths do not change body angle during the surge period, initial surge corresponds to the first turning behavior in the mating dance. During zigzagging walking, the turn direction is usually opposite to the previous turn, and hence it is predictable. However, under natural conditions, the odor plume has a complex distribution, with the time sequence of the stimulation on both antennae possibly being different. Hence, the key stimulus features that determine the direction of the first turn have still not been fully investigated.

Here, we focused on the first turning behavior. The objective of the present study was to identify the stimulus features that determine the direction of the first turn in more detail. We evaluated the following three conditions that might affect the behavioral pattern of the initial phase: (1) difference in the timing of pheromone detection between antennae, (2) difference in pheromone concentration between antennae, and (3) background wind direction. The relationship between sensory input and the direction of the first turn was analyzed. Pheromone input was quantified by using electroantennogram (EAG). To discuss the basic strategy of odor-source localization in *B. mori*, we then examined the behavioral response of male moths to sustained pheromone input, and analyzed their walking trajectories.

Materials and methods

Animals

We used the adult male silkmoth *Bombyx mori* L. (Lepidoptera: Bombycidae). Pupae were obtained from Katakura Kougyo Industries Co., Ltd. (Tokyo, Japan) and reared at 26°C and 50–60% relative humidity under a 16:8 h light:dark photoperiod. Animals were used within 2–4 days after eclosion.

Odorants

Synthetic (E,Z)-10,12-hexadecadien-1-ol (bombykol), the principal pheromone component of *B. mori*, with a purity of >99% (confirmed by gas chromatography), was dissolved in HPLC-grade *n*-hexane. The odorant (via a 5 µL solution) was applied to a piece of filter paper (1 × 2 cm) and inserted into a glass stimulant cartridge (1 mm tip diameter). Airflow was generated by an air compressor. Compressed pure air was filtered through cotton, charcoal and water, and then passed into an air delivery tube. In this study, pheromone concentration is expressed as the amount of stimulant applied to filter paper in the stimulus cartridge. The distance between the filter paper and cartridge exit was approximately 7 cm. All stimulant cartridges were sealed with a Teflon sheet, stored at −20°C and brought to room temperature prior to a recording session. We used solenoid valves (YDV-3-1/8; Takasago Electric, Nagoya, Japan) and electric stimulators (SEN-3201; Nihon Kohden, Tokyo, Japan) to control stimulus duration and interval. The order of pheromone stimulation on both antennae was randomized. We showed the stimulus duration by using an electrical trigger pulse, which controls the opening of the solenoid valve.

Electroantennogram

The antenna was excised with a razor blade, and the scape and pedicel were removed. The most distal five segments of the flagellum were removed. Nichrome wires with a diameter of 20–40 µm and 150 µm were inserted to the distal and proximal ends of the antenna, respectively. Pheromone stimulations were applied to the anterior side of the antenna facing upwind. The incoming signals were amplified (MEZ-8300; Nihon Kohden) and monitored using an oscilloscope (VC-10; Nihon Kohden). The acquired signals were stored in a computer by using an A/D converter (TL-1-125 interface; Molecular Devices, Sunnyvale, CA, USA) and associated

software (Axotape 1.2.01; Molecular Devices). When testing the dose response, we tested the level of stimulation with ascending pheromone concentrations. When the first sequence was complete, the next sequence was examined, also in ascending order (Fig. 1a, b).

Behavioral experiments with a tethered system

We performed behavioral experiments with a tethered system (Figs. 2, 3, 4).

Tethered system

To apply odorant to different positions on the antennae, we used a tethered system, following the method of Kanzaki (1998). For the experiments shown in Figs. 2, 3, 4, moths were placed on a small Styrofoam ball and tethered to a steel bar on the dorsal thorax with adhesive material (G-17; Konishi, Osaka, Japan). The diameter of the ball was 3 cm and its mass was 0.46 g. Tethering did not disturb wing beating. We recorded moth behavior at 125 frames/s with two cameras (Ektapro EM; Kodak, Tokyo, Japan). Camera recording was triggered by a signal from an electric stimulator (SEN-3201, Nihon Kohden). The same signal also controlled valve opening, such that recording and odorant presentation were synchronized. Cameras were placed 40-cm anterior and 40-cm dorsal to the moth. Images were copied to video tape with a video cartridge recorder (BR-S605B; Victor, Kanagawa, Japan). We tested three types of stimulation:

Stimulation with a time difference

Two cartridges (5.5-mm tip diameter) and two valves were prepared to stimulate left and right antennae independently (Fig. 2a). The cartridge exit was placed approximately 0.5 cm from the antenna. To examine the time difference between the left and right antennae, 200 ng of bombykol was used. Each stimulus was applied at a velocity of 400 mL/min (approximately 28 cm/s) for 200 ms. Two valves were controlled independently, whereby one side (Ta) opens earlier than the other (Tb). We changed the time difference between valve openings by 0, 25, 50 and 100 ms.

Stimulation with different concentrations

Similar to condition I, we used two cartridges and two valves (Fig. 2a). One cartridge (Tq) contained 100 ng of bombykol, the other contained 0, 100, 200 or 400 ng of bombykol (Tp).

Stimulation with different airflow orientations

We used a constant airflow system to examine the effect of airflow on pheromone processing (Fig. 3a; Kanzaki

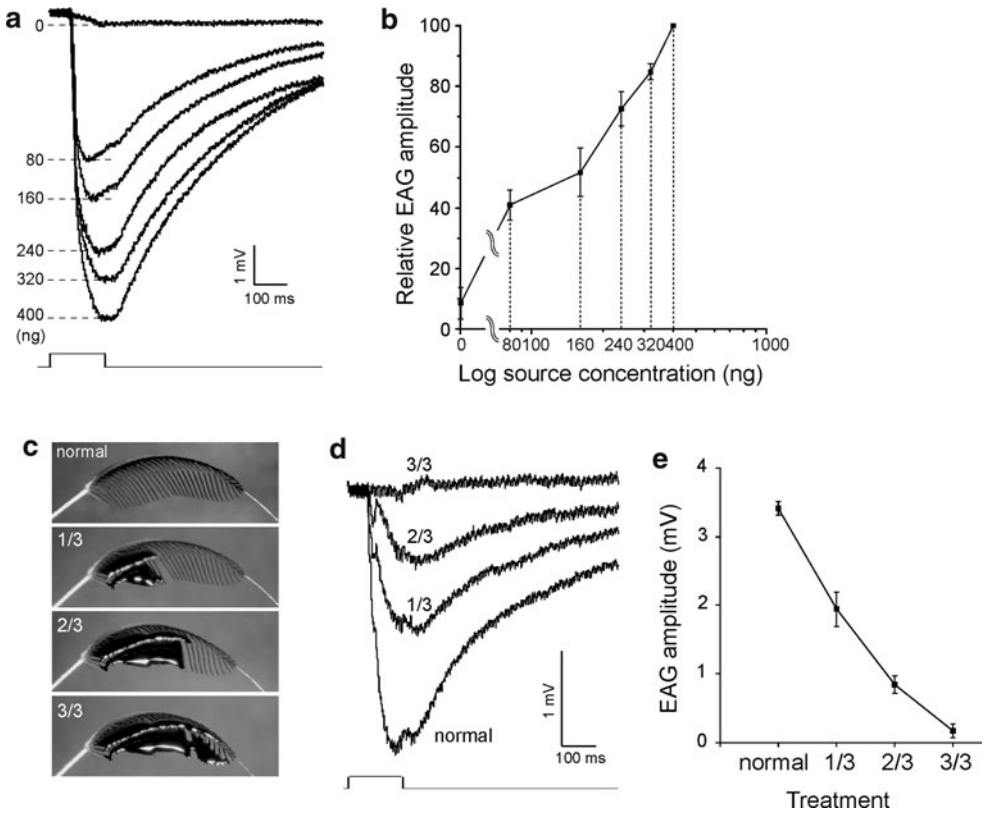

Fig. 1 Electroantennogram and the effect of partial antenna covering. **a** Example of electroantennograms with different doses of bombykol. Six different concentrations were examined. Bottom trace shows the trigger pulse of solenoid valves. Bombykol was applied for 200 ms. **b** Relative amplitude of electroantennogram as a function of the amount of bombykol ($n = 8$). The value was normalized by the absolute amplitude of the response to 400 ng of bombykol. The amplitude increased with increasing amounts of bombykol. **c** Partial and full covering of antenna with cashew. **d** The effect of antenna covering. Stimulation with 200 ng of bombykol was applied for 200 ms. **e** Electroantennogram amplitude as a function of antenna covering ($n = 5$)

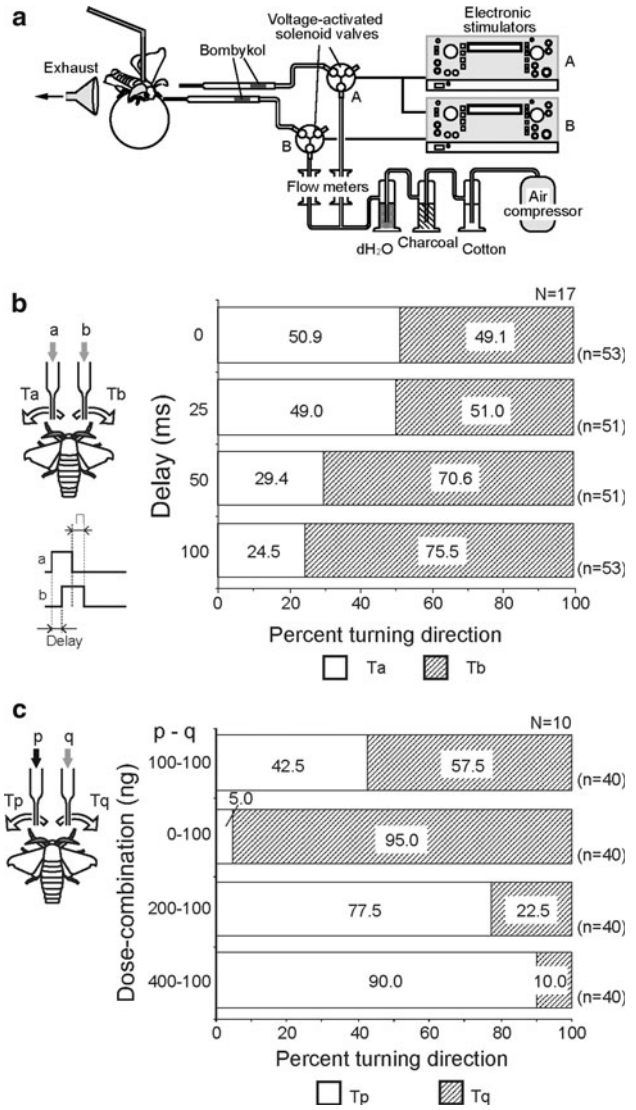

Fig. 2 Relationship between pheromone input pattern and the direction of turning behavior. **a** Odorant delivery system with air puff stimulation to provide different stimulation to the left and right antennae. **b** Direction of the first turn in response to temporally staggered bilateral pheromone stimulation. One antenna (Ta) receives pheromone stimulation prior to the stimulation of the other antenna (Tb) with a delay of 0, 25, 50 or 100 ms (*asterisk in the left graph*). There was no difference between Ta and Tb when the time difference was 0 or 25 ms ($p > 0.1$, χ^2 test). For time differences of 50 and 100 ms, the probability of turn to the Tb side was significantly higher ($p < 0.005$, χ^2 test). **c** Direction of the turning behavior in response to bilateral pheromone stimulation of different concentrations. There was no difference in direction preference when we applied the same concentration of pheromone to both antennae ($p > 0.1$, χ^2 test). When there was a difference in pheromone concentration, the number of turns toward the higher concentration was significantly higher than the number of turns toward the lower concentration ($p < 0.001$, χ^2 test)

et al. 1989). Clean air was divided into main and sub-air streams for odorant delivery. Main stream flow was 5 L/min through a 2.2-cm inner diameter air delivery tube. Substream flow was 1 L/min, and connected with a Pasteur pipette (1-mm tip diameter) through valves. These were inserted into the cartridge for the main air stream. One side contained filter paper containing the pheromone that was controlled by an electric stimulator. Flow rate at the exit was approximately 30 cm/s. An exhaust tube was placed on the opposite side of the stimulant cartridge to remove odorants. The orientation of odorant delivery and exhaust systems was tested at −90, 0 and 90° angle to the moth body axis.

Analysis of the direction of turning behavior

We observed the recorded video in slow motion and noted the direction of initial walking. When moths turned left, they first moved the left foreleg while keeping the right foreleg on the Styrofoam ball. We used this movement as the criterion to determine the initial walking direction.

Behavioral experiments in a wind tunnel

We performed behavioral experiments in a wind tunnel (Figs. 4, 5).

Wind tunnel

The wind tunnel was 88-cm wide, 88-cm high and 150-cm deep (Fig. 4a). In the tunnel, a 52 cm × 92 cm recording arena was prepared. Airflow was induced with negative pressure created by a fan (SP-300; Sanritsu, Tokyo, Japan), and flow speed was adjusted to 0.6 m/s by changing the supply voltage with a transducer (RSA-10; Tokyo Rikosha, Tokyo, Japan). The input area of the wind tunnel was covered by 1 mm × 1 mm mesh filters and a 1.5 cm × 1.5 cm grid to reduce distributed flow or vortex turbulence. This operation stabilizes the shape and position of the pheromone plume. The shape and position of the pheromone plume was simulated by applying $TiCl_4$, which makes a smoke plume. The filter paper containing the pheromone was placed at the same location in the tunnel as the pheromone during an experiment.

Analysis of walking to continuous pheromone exposure

To reveal the effect of sustained pheromone stimulation on male behavior, we performed behavioral experiments in the wind tunnel (Fig. 4b). The cartridge was bent and inserted into the tunnel. The exit of the cartridge was placed 15 cm from the head of the moth and 1.5 cm from the rear end. The cartridge contained 1 μg bombykol, with a stimulation of 5 s. Moths were placed at an angle of −90° (air from the left side), –45, 0° (facing upwind), 45 and 90° (air from the right side) to the wind direction (Fig. 3b). Behavior was

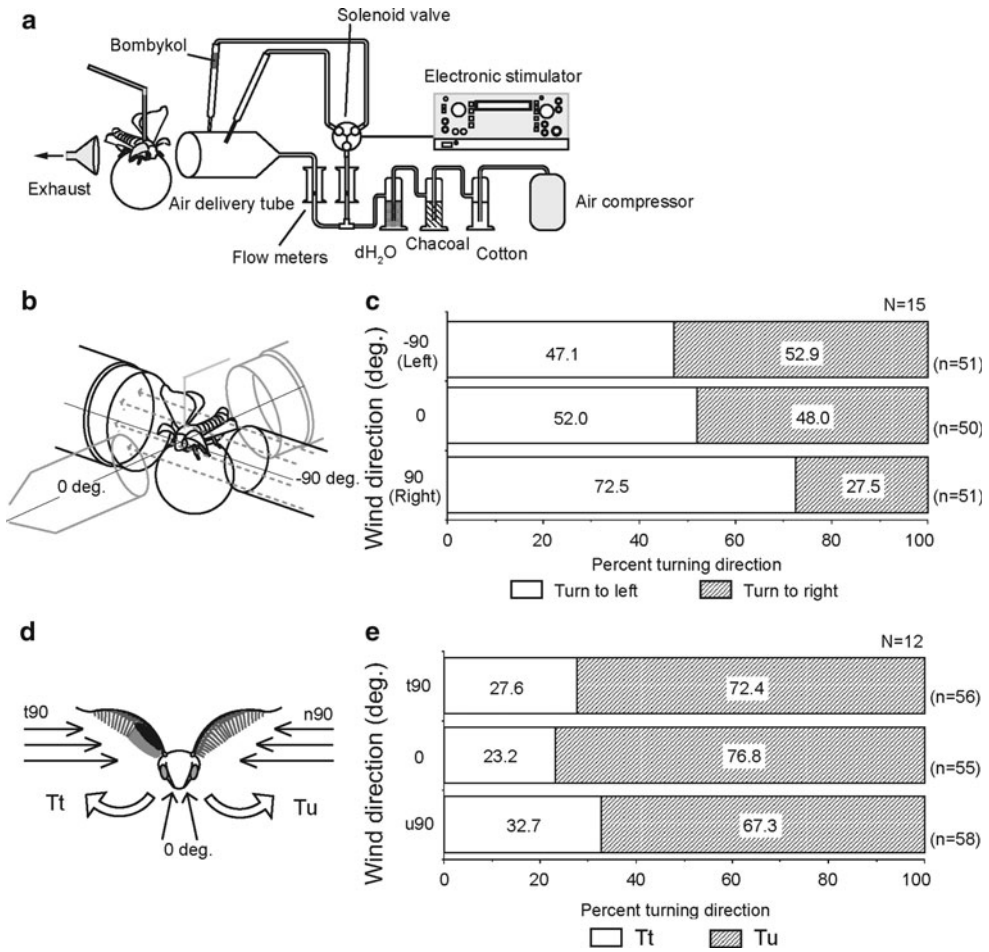

Fig. 3 The relationship between the direction of turning behavior and the direction of the wind. **a** Odorant delivery system with constant airflow to stimulate both antennae simultaneously. **b** A way to change the main stream direction. Total air stream was 6 L/min (approximately 28 cm). We changed the wind direction by rotating the odorant delivery system and the exhaust tube. **c** The direction of turning behavior under airflow from different directions. When we rotated the system 90°, the probability of turning to the opposite side (*downwind*) was significantly higher ($p < 0.005$, χ^2 test). In contrast, there were no significant differences when we applied the wind from the anterior (0°) and left sides (–90°) ($p > 0.1$, χ^2 test). **d** Covering of the antenna on one side. The proximal half of the antenna was covered with cashew. We tested three wind directions: Airflow from the side of the treated antenna (t90), from the anterior (0 deg.) and the side of untreated antenna (u90). **e** The relationship between the initial walking direction and wind direction. In all three cases, the probability of walking toward the untreated antenna (Tu) was significantly higher than that toward the treated antenna side (Tt) ($p < 0.01$, χ^2 test)

recorded with a camera (Ektapro EM, Kodak) at 50 frames/s. To obtain angular velocity, the rotation of a Styrofoam ball was analyzed (Kanzaki 1998). We arbitrarily selected a dot on the ball for each frame (Fig. 4c). Angular velocity was measured by calculating the difference between the angles of the body axis in consecutive frames.

Analysis of the trajectory of an unrestrained moth

To investigate the function of bilateral integration of pheromone information, we analyzed the trajectory of a moth walking in the wind tunnel. The cartridge was bent and inserted into the tunnel. The exit of the cartridge was placed 15 cm from the head of the moth, and 1.5 cm from the rear end. The cartridge contained 1 μg bombykol, and the pheromone was applied constantly. To expand the pheromone plume, a plate (2 cm × 3 cm) was placed 1 cm from the cartridge exit. We examined two conditions: 1. the moth was placed on the windward side, 30 cm from the cartridge (Fig. 5a, b) and 2. under the wind, 15 cm from the cartridge (Fig. 5c, d). We started the stimulus while the moths were stationary. Behavior was monitored by a video recorder at 30 frames/s (DCR-VX1000; Sony, Tokyo, Japan). Images were copied to a video, and analyzed with software (Frame-DIAS; DKH, Tokyo, Japan). We measured the position of the head and the center of the posterior edge of the thorax for each of the five frames (1/6 s). We reconstructed the walking trajectories based on this time-series data.

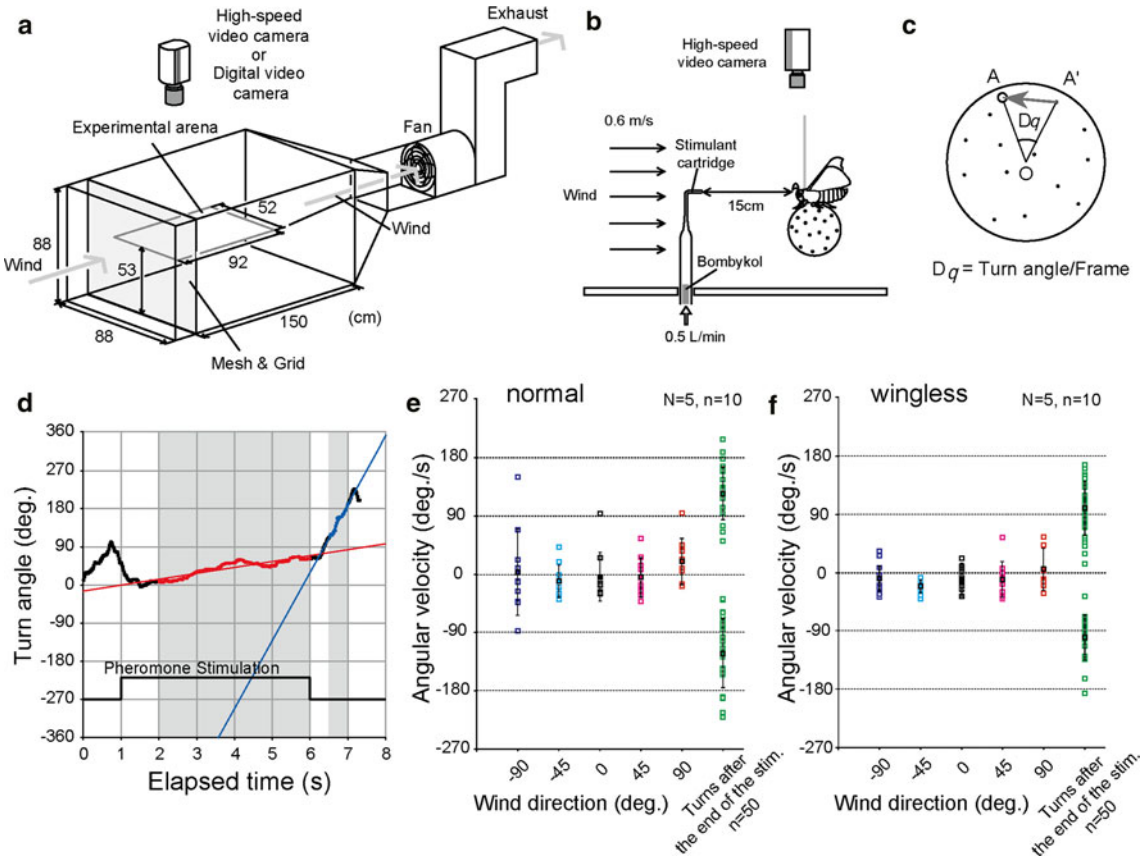

Fig. 4 Analysis of walking behavior in the wind tunnel. **a** A schematic diagram of the wind tunnel. Airflow was induced with negative pressure created by a fan and flow speed was adjusted to 0.6 m/s. **b** Schematic of the behavioral experiment. The tethered system was placed on the arena in the tunnel. **c** Measuring the angular velocity of moth walking. We took the difference of the angles for each pair of consecutive frames. **d** Example of a change of body angle in response to long pheromone stimulation. We applied the pheromone for 5 s to a moth which exhibited the mating dance. *Red* and *blue* lines represent the fitted curve of 1–4 s after stimulus onset and 0.5–1 s after the stimulus ended. The slope of these lines represents the angular velocity. **e** Angular velocity as a function of wind direction. Wind directions of −90, −45, 0, 45 and 90° were tested ($n = 10$ trials in each direction with each of 5 moths) with no significant differences observed ($p > 0.05$, one-way ANOVA). Angular velocity after stimulation cessation was significantly different from that during stimulation ($p < 0.00001$, one-way ANOVA; $p < 0.00001$, Scheffé's F test). **f** Angular velocity as a function of wind direction in wingless moth. Wind directions of −90, −45, 0, 45 and 90° were tested ($n = 10$ trials for each direction with each of 5 moths) and there were no significant differences observed ($p > 0.05$, one-way ANOVA). Angular velocity after stimulation cessation was significantly earlier than that during stimulation ($p < 0.00001$, one-way ANOVA; $p < 0.001$, Scheffé's F test). There were no significant differences between untreated and wingless moths

Data analysis

The probability of turning direction was analyzed by using the χ^2 test (Fig. 4). For multiple comparisons among groups, the amplitude of EAG (Fig. 1), the probability of turning behavior (Figs. 2, 3) and the angular velocity (Fig. 4) were compared by one-way analysis of variance (ANOVA), followed by Scheffé's F test. Data are reported as mean ± standard deviation. Statistical analysis was performed using MATLAB and a Statistics Toolbox (Mathworks, Natick, MA, USA).

Results

We first examined whether the amount of bilateral pheromone input could be controlled by the antenna-covering treatment. We then focused on the relationship between the direction of initial turning behavior and bilateral pheromone input, under several different conditions. To investigate the effect of bilateral pheromone input, we used an air puff system (Fig. 2a). The system has two stimulus cartridges to present pheromones to the left and right antennae independently. The response to sustained pheromone stimulation and locomotion trajectory in restrained conditions was recorded in a wind tunnel.

Quantification of pheromone input by EAG

To present the pheromone input on each antennae with different intensity, we first examined a method to control input intensity to the antenna with covering treatment.

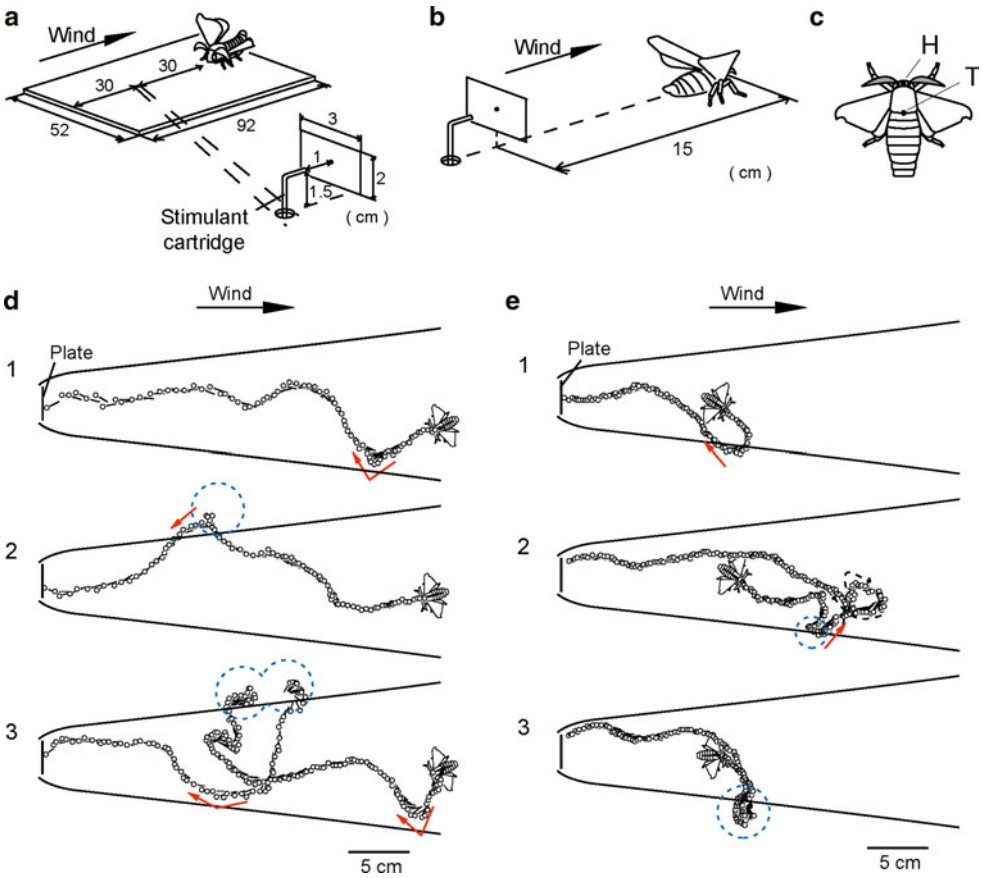

Fig. 5 Free walking in the wind tunnel. **a** A method to generate a pheromone plume. The tip of a stimulant cartridge was bent and placed at a 1.5-cm height downwind. Moths faced the wind, 30 cm from the cartridge. **b** Moths facing away from the wind, 15 cm from the cartridge. **c** The positions of the head (H) and the border between thorax and abdomen (T) were noted in each image. **d** Trajectories of moths placed on the windward side. The position of the head (H) is represented by *circle*. Line segments represent the line connecting points 'H' and 'T'. Broken lines represent zigzagging behavior. The moth figure represents the initial position. Lines indicate the average boundaries of the pheromone plumes simulated by $TiCl_4$ smokes. **e** Trajectories of moths placed downwind

Dose–response

We prepared stimulus cartridges containing 0, 80, 160, 240, 320 and 400 ng of bombykol and recorded the EAG (Fig. 1a). Each dose was tested eight times. The normalized response amplitude is shown in Fig. 1b. Each value was normalized by the amplitude of the response to 400 ng stimulation. The relative response amplitudes were 8.00 ± 5.30, 40.9 ± 5.00, 51.7 ± 8.09, 72.6 ± 5.86, 84.8 ± 2.57 and 100 for 0, 80, 160, 240, 320 and 400 ng stimulations, respectively. The amplitude of the EAG increased with pheromone dose. The same results were obtained for all four specimens.

Antenna-covering treatment

To control the amount of pheromone input, we examined the effect of covering the antenna with synthetic resin coating (cashew). We examined four conditions: 0 (no treatment), 1/3, 2/3 and 3/3 covered (Fig. 1c). The EAGs are shown in Fig. 1d, e. The maximum amplitudes were 3.41 ± 0.10, 1.95 ± 0.25, 0.85 ± 0.12 and 0.17 ± 0.10, for the 0, 1/3, 2/3 and 3/3 covered conditions, respectively. The amplitudes significantly differed between conditions ($p < 0.000001$, one-way ANOVA; $p < 0.05$, Scheffé's F test). As the covered area increased, the EAG amplitude decreased. The same results were obtained for all specimens. These data indicate that the input intensity of the pheromone could be controlled by covering the antenna.

Relationship between bilateral olfactory input and the direction of the first turn

Effect of time difference

Using an air puff system (Fig. 2a), we stimulated both antennae with time differences of 0, 25, 50 and 100 ms ($N = 17$). For descriptive purposes, the antenna that was first stimulated was termed Ta, and the antenna that was stimulated later was termed Tb. The probabilities of turning behavior to Ta were 50.9 ± 0.29, 49.0 ± 0.17, 29.4 ± 0.11 and 24.5 ± 0.23% for time differences of 0, 25, 50 and 100 ms, respectively (Fig. 2b). The results for the 50 and 100 ms time differences were significantly different from those for the 0 and 25 ms time differences ($p < 0.005$, one-way ANOVA; $p < 0.05$, Scheffé's F test); however, they were not significantly different from one another. The probability of turning behavior to Tb was significantly higher than to Ta for time differences of 50 and 100 ms ($p < 0.05$, χ^2 test). These results indicate that the moths first turn toward the side of later input.

Effect of intensity difference

To investigate the effect of bilateral pheromone input with different input intensity, we used two cartridges containing different amounts of the pheromone. We stimulated one antenna with a cartridge containing 100 ng of bombykol (Tq) and the other with a cartridge containing 0, 100, 200 or 400 ng of bombykol (Tp). We examined the probabilities of turning behavior to Tp and Tq ($N = 10$; Fig. 2c). When we stimulated both antennae with the same amount of bombykol (100 ng), the probabilities of turning to Tp or Tq were 42.5 and 57.5%, respectively. When different amounts of bombykol were used for Tp (0, 200 and 400 ng), the probability of turning behavior to Tq was 5.0 ± 0.11, 77.5 ± 0.14 and 90.0 ± 0.13%, respectively. Hence, there was a significantly higher probability of walking toward the side stimulated by greater amounts of pheromone compared to when the same amount of pheromone was used on both sides ($p < 0.00001$, one-way ANOVA; $p < 0.001$, Scheffé's F test). These results indicate that the moths walk toward the side receiving the larger amount of pheromone.

Effect of background wind direction

To examine the effect of background wind direction, we used a constant airflow system (Fig. 3a). The system has a single large cartridge (air delivery tube) for the main air stream (2.2-cm tip diameter), and two normal cartridges for the substream (1.0-cm tip diameter). One substream cartridge contained the pheromone, which was supplied for 200 ms by controlling a solenoid valve. The diameter of the main air stream cartridge was large, to allow both antennae to be stimulated simultaneously. We tested three different airflow angles by rotating the odorant delivery and exhaust systems ($N = 15$): specifically, wind from the left side ($-90°$), the anterior (0°) and the right side (90°) (Fig. 3b). At -90 and 0°, there was no significant difference in the probability of walking toward the left or right sides ($p < 0.005$, one-way ANOVA; $p > 0.8$, Scheffé's F test; Fig. 3c). When the wind direction was 90°, the probability of walking toward the right (windward) was significantly higher than that to the left ($p < 0.05$, Scheffé's F test). This result indicates that background airflow partially influences the direction of the first turn. This result was unexpected, but there was no other indication of asymmetry.

We next examined the effect of background airflow with stimuli of different concentrations ($N = 12$). One-third of the antenna area was covered, and the experiment was repeated (Fig. 3d). The probability of walking toward the side of the untreated antenna (Tu) was significantly higher than toward the side of the treated antenna (Tt) for all wind directions ($p < 0.001$, χ^2 test; Fig. 3e). There was no significant difference among the three tested wind directions ($p > 0.2$, one-way ANOVA). These results suggest that variable pheromone input on the two antennae, rather than background wind direction, is important for determining the walking direction of the moth.

Response to continuous pheromone input

When moths lose a plume, it is important to relocate it by some strategy of odor-source localization. We replicated these conditions in restrained *B. mori* that exhibited the mating dance, following stimulation by long exposure to the pheromone (~ 5 s), and examined the direction of the first turn after removal of the stimulation.

The first turn after pheromone stimulation

We used the wind tunnel (Fig. 4a) with a tethered system in the arena (Fig. 4b). The angular velocity was measured by evaluating the difference among the locations of a specific dot for each pair of consecutive images (Fig. 4c). Figure 4 d provides an example of the temporal change in body angle. The increment and decrement of this value indicate walking toward the left and right, respectively. Moths that had shown a pheromone response were used in this experiment. Fitting curves of 2–6 s and 6.5–7 s are shown as red and blue lines. The slope of the graph represents angular velocity. We summarized the population data in Fig. 4e. We plotted the angular velocity from 2 to 6 s at wind directions of -90, -45, 0, 45, and 90° ($N = 5$ animals, $n = 10$ trials for each animal). The angular velocity from 6.5 to 7 s (after removal of the stimulus) is shown on the right ($n = 50$). Individual (open squares) and averaged values (filled squares) are shown for each condition. The angular velocity after the removal of the stimulus was plotted as the average of positive and negative values independently. The angular velocities during pheromone stimulation were 3.0 ± 66.8, -10.4 ± 24.8, -4.3 ± 37.8, -5.0 ± 30.7 and 19.7 ± 35.4 degrees/s for -90, -45, 0, 45 and 90°, respectively, with no significant differences ($p > 0.05$, one-way ANOVA). After stimulus removal, the angular velocities were 124.9 ± 40.8 for the left turn and -122.0 ± 53.1 for the right turn, and were significantly different from the values during stimulation ($p < 0.00001$, one-way ANOVA; $p < 0.00001$, Scheffé's F test). These results indicate that turning behavior is suppressed by long pheromone stimulation and that the removal of the stimulation induces the first turn.

Because wing fluttering might affect the dynamics of a pheromone plume (Obara, 1979), we repeated the experiment using wingless moths. As with the untreated moths, the angular velocities during pheromone stimulation did not significantly differ (-9.17 ± 18.7, -21.0 ± 10.5,

-10.8 ± 25.6, -10.3 ± 27.3 and 5.21 ± 32.8 degrees/s for -90, -45, 0, 45 and 90°, respectively; $p > 0.05$, one-way ANOVA), but the angular velocities after stimulation were significantly different from the velocities during stimulation, which supported our findings with the untreated moths (104.8 ± 37.1 degrees/s for the left and -91.7 ± 24.5 degrees/s for the right; $p < 0.00001$, one-way ANOVA; $p < 0.001$, Scheffé's F test). There were no significant differences between each pair of values for untreated and wingless moths. These results suggest that the direction of walking after the removal of pheromone stimulation does not require wing fluttering.

Walking trajectory in unrestrained moths

To investigate the strategy of odor-source localization further, we recorded the walking trajectory of moth orientation behavior in the wind tunnel. We tested two conditions: moths were placed 30-cm upwind (Fig. 5a) and 15-cm downwind of the source (Fig. 5b). Acquired images were analyzed, and the positions of the head and the border between the thorax and abdomen were manually noted for each frame (Fig. 5c). In each case, the moths exhibited three behavioral features (Fig. 5d, e): (1) The first turn with <45° body angle was observed in the plume, with walking direction being unrelated to wind direction; (2) turning toward the plume at the putative border (arrows); and (3) zigzagging and looping behavior outside the plume (circles with broken line).

Discussion

In the present study, we analyzed the mating dance of *B. mori*, with particular focus on the walking direction during turning behavior, which has not been systematically investigated thus far. Cessation of the pheromone was found to trigger turning behavior (Fig. 4). Moths exhibited turning behavior at the boundary of the pheromone plume (Fig. 5), which is similar to the casting flight behavior of flying moths. These results indicate the similarity in the strategy of odor-source localization between flying and walking moths. Furthermore, we revealed a novel aspect in the initial phase of odor-localization behavior regarding the direction of the first turn, whereby moths integrate information from the left and right sides before turning (Figs. 2, 3).

Possible use of bilateral information during odor-source orientation behavior

To address the functional role in the integration of bilateral pheromone information on male moth turning behavior, we discuss the strategy for odor-source localization by *B. mori* under the following three conditions: (1) moth in a plume, (2) moth at the boundary of a plume and (3) moth outside a plume.

Behavior in a plume

The surge response in the initial phase of mating behavior by *B. mori* shares significant similarity with that of upwind surge by flying moths. In the present study, most of the trajectories in a plume were rectilinear (Fig. 5), as in flying moths. When a moth is in a plume, the difference in input on the left and right antennae might become very small. At this point, moths exhibit straight walking, maintaining a consistent body angle.

Although the function of turning behavior for odor-source localization in a plume is unclear, one possibility is trajectory modification. In a plume, the trajectory was actually not a straight line, but a line with fluctuation. The complex distribution of pheromone filaments probably causes differential input on each antenna, even in a plume. For example, the probability of contact might increase as the distance between moth and odor-source decreases. If intensity information reflects distance information, turning toward the side of higher intensity possibly generates a course correction (Fig. 2c), facilitating efficient localization.

Behavior at the boundary

When moths are at the boundary of a plume, they sometimes exhibit turning behavior (arrows in Fig. 5). Turning behavior at the boundary of the odor plume has also been reported for other walking insects (Kojima et al. 2003; Willis and Avondet 2005). The pheromone input on the left and right antennae are likely to differ at the boundary. The side of pheromone plume might have a larger latency than the opposite side. According to our results (Fig. 2b), in such cases, moths turn toward the side of the antenna that receives pheromone input with larger latency, i.e., inside the plume. We often observed moths turn toward the inside of the plume from the putative boundary (Fig. 5d, 1, 3, arrows). At the boundary, integration of bilateral pheromone input seems to be important to decide the direction of locomotion. Determining the direction of the first turn seems to be especially important for tracking pheromone plumes.

Zigzagging walking outside a plume

Flying moths that have lost the plume of a pheromone exhibit programmed behavior, characterized by zigzagging flight (Kuenen and Baker 1982; Baker et al. 1984; Baker

and Haynes 1987; Willis and Baker 1987; Willis and Arbas 1991). One function of zigzagging flight is thought to be to scan the environment when a moth has lost the plume (Kennedy 1983). For individuals of *B. mori*, zigzagging locomotion is rarely observed when a moth is in the plume, but is frequently observed outside the plume (Fig. 5d, e). In Fig. 5d3, a moth lost a plume and exhibited zigzagging walking (circles with broken line). This behavior might be interpreted as being similar to the casting behavior used by flying moths to relocate a lost plume (David et al. 1983; Kennedy 1983; Baker and Haynes 1987; Kuenen and Cardé 1994; Vickers and Baker 1996). In the present study, *B. mori* showed casting-like behavior at the boundary of a plume, which is consistent with the concept that the zigzagging walking behavior is important for relocating a lost plume.

In instances when moths could not detect the difference between pheromone input on the left and right antennae, they might exhibit straight walking, with a high probability of losing the plume. Moths exhibit zigzagging walking behavior until they detect a difference between pheromone input on both antennae. In this regard, deciding the direction in which to turn when initiating behavior is also important for relocating a lost plume.

Bilateral integration to determine the direction of locomotion

We quantified the relationship between pheromone input and the direction of turning behavior in the present study. Although moths change direction irrespective of stimulus pattern during zigzagging walking (Kanzaki et al. 1992), the direction of the first turn was affected by the stimulus pattern (Figs. 2, 3). For the 0.5 and 1-s time delays, moths preferentially walked toward the side receiving the later stimulus, rather than toward the side receiving the earlier stimulus (Fig. 2). When bilateral pheromone stimulation of different concentrations was applied, moths preferentially walked toward the side receiving the stronger stimulus (Fig. 3). These results indicate that the olfactory system of *B. mori* is able to discriminate temporal and intensity differences. The precise role of bilateral integration in pheromone input on pheromone-source localization is unknown. One explanation for the function of bilateral integration in pheromone input is to improve odor-source localization efficiency in the density gradient of a pheromone filament in a plume. This concept is consistent with the behavioral observation that, even in a plume, moths show modest changes in body axis (Fig. 5).

The bilateral integration of olfactory input from the left and right olfactory organs facilitates odor-source localization in humans (Porter et al. 2007), rodents (Rajan et al. 2006) and fish (Johnsen and Teeter 1980; Gardiner and Atema 2010). Two antennae are required to facilitate odor localization in insects (Bell and Tobin 1981; Borst and Heisenberg 1982; Louis et al. 2008; Duistermars et al. 2009; Steck et al. 2010). Furthermore, bilateral information is also employed in robot odor localization (Webster et al. 2001). In contrast to *B. mori*, sharks use temporal differences only to determine the initial turn direction (Gardiner and Atema 2010). Sharks can discriminate a 100 ms difference in odorant arrival time, whereas *B. mori* may be able to discriminate a difference of as little as 50 ms. Furthermore, sharks turn toward the side receiving the first stimulus, whereas *B. mori* turns toward the later stimulus. The reason why these species use different strategies is unknown, but it might be due to several factors, including body size, locomotion speed, activity time (diurnal vs. nocturnal) and habitat environment (in water vs. air).

Combined with the experimental evidence across phyla, we speculate that bilateral pheromone information is also integrated and used to determine the direction of orientation behavior in species of flying moths. However, the way that flying moths use bilateral information might differ compared to walking moths, because they have different strategies of locomotion. The locomotion velocity of flying moths is much faster than in walking moths, with the time resolution for determining the turning direction possibly being smaller compared to *B. mori*.

Neural mechanisms to determine the turn direction

Neural mechanisms for pheromone source orientation behavior have been studied for *B. mori* (Kanzaki 1996, 1998; Kanzaki and Mishima 1996; Mishima and Kanzaki 1998, 1999; Wada and Kanzaki 2005). Males show synchronized head movements when exhibiting zigzagging turning behavior in response to pheromone stimulation. When a male turns to the right, its head shows sidewise movement to the right and then left. The timing of turning behavior is consistently synchronized to that of the sidewise movement of the head (Kanzaki and Mishima 1996). Specific subsets of descending interneurons (DNs), which connect the brain and other ganglions, show state-dependent "flip-flop" neural activity thought to be the command signal for zigzagging walking (Olberg 1983; Kanzaki et al. 1994; Mishima and Kanzaki 1999). The flip-flop activity found in the lateral accessory lobe of *B. mori* appears to be correlated with the activity of neck motor neurons (Mishima and Kanzaki 1998; Wada and Kanzaki 2005). According to the potential synaptic connectivity estimated by use of confocal microscopy, group-IIA and group-IID DNs, both of which have smooth processes in the lateral accessory lobe, are believed to be important in correlating activity between the walking direction and the head angle (Wada and Kanzaki 2005). In addition, group-IIC DNs are

considered as one of command neurons for surge behavior, because they do not exhibit a state-dependent response, but show brief excitatory response to the pheromone and are probably connected to a neck motor neuron (Wada and Kanzaki 2005). In the present study, we found that sustained pheromone input suppressed the expression of the turning behavior (Fig. 4). Furthermore, straight walking behavior terminated after the cessation of sustained input, with the onset of turning behavior being triggered (Fig. 4). If group-IIC is one of the command neurons for surge, it is predicted that the neuronal activity of the command signal for surge, i.e., group-IIC DNs, might be activated during sustained pheromone stimulation and suppressed after the cessation of stimulation. Recently, the presence of OFF olfactory receptor neurons, in which the discharge rate is increased by declining odorant concentrations and vice versa, has been reported for the cockroach (Tichy et al. 2005; Burgstaller and Tichy 2011). Some projection neurons in the antennal lobe exhibit the OFF response after stimulus termination in *B. mori* (Namiki and Kanzaki 2011a). Although such neurons have not been found in the pheromone processing pathway of *B. mori* (Kanzaki et al. 2003), they are potential candidates that might trigger surge.

Neural mechanisms for odor-source localization behavior have been investigated for *Manduca sexta*, which is a closely related species of *B. mori*, (Kanzaki et al. 1991; Vickers et al. 2001; Lei et al. 2009). By monitoring behavior with pharmacological treatment, Lei et al. 2009 reported that the suppression of the counterturning program requires intermittent pheromone stimulation. Furthermore, zigzagging flight pattern was observed during constant odorant presentation. This finding directly contrasted with our results, whereby we did not observe zigzagging behavior during sustained pheromone stimulation. One possible reason for this difference between the two studies is the difference in locomotion, i.e., flight in *M. sexta* and walking in *B. mori*. Another explanation might be differences in the timescale of the response. For example in Lei et al. 2009, the bursting activity of projection neurons, which are second-order olfactory neurons, was blocked by injecting a drug into the olfactory center and neuronal activity was altered for a longer time (over 10 min). In comparison, we used 5-s stimulation in the current behavioral experiment with *B. mori*.

One unanswered question is the location of the circuit for the integration of bilateral pheromone input. We consider neuropils, which are revealed to be involved in pheromone-source orientation behavior for *B. mori*. Pheromonal information is first received by olfactory receptor neurons on the antenna, which is then transmitted into the first-order olfactory center, the antennal lobe. In the fly *Drosophila melanogaster*, sensory neurons from the antennae project to both ipsi- and contralateral antennal lobes, with the integration of bilateral input occurring at the level of second-order olfactory neurons (Agarwal and Isacoff 2011). However, the sensory neurons from the antennae of *B. mori* only project to the ipsilateral antennal lobe (Koontz and Schneider 1987; Ai and Kanzaki 2004). Furthermore, it has been shown that the sensory neurons expressing sex-pheromone receptors project to the macroglomerular complex in the ipsilateral antennal lobe (Sakurai et al. 2011). One glomerulus in the antennal lobe receives bilateral input from a pair of labial pit organs (Kent et al. 1986), but the sensory neurons do not respond to the sex pheromone. Bombykol-sensitive projection neurons with contralateral axonal projection have not been identified thus far (Kanzaki et al. 2003; Namiki et al. 2008; Namiki and Kanzaki 2011b), suggesting that the integration of bilateral input occurs downstream of the antennal lobe. Based on these anatomical data, we are able exclude the possibility that the integration of bilateral pheromone information occurs at the first- or second-order neuron level for *B. mori*.

The delta area of the inferior lateral protocerebrum (ΔILPC), which is the major area where projection neurons respond to the sex pheromone (Seki et al. 2005), might be a possible neuropil for the integration of bilateral input. This is because some output neurons from ΔILPC, which are third-order olfactory neurons, project to the contralateral hemisphere (Namiki and Kanzaki 2005). In addition, it is possible that bilateral integration is performed by other areas of the protocerebrum, such as the mushroom body and central complex (Kanzaki et al. 1991; Lei et al. 2001; Ritzmann et al. 2008; Heinze et al. 2009). Finally, there is a possibility that the lateral accessory lobe might integrate pheromone information. Many interneurons have processes in the lateral accessory lobes of both hemispheres (Iwano et al. 2010). Most neurons in this neuropil exhibit a response to stimulation on both the left and right antennae (Namiki and Kanzaki, unpublished observation). According to available anatomical and physiological data, all or a part of protocerebral neuropils including the mushroom body, central complex, ΔILPC and lateral accessory lobe might be involved in the integration of bilateral pheromone input. The acquisition of physiological recordings from the neurons in these neuropils, following the same stimulation protocol used in the current experiment, might be required to reveal the underlying neural mechanisms.

In the present study, we identified specific stimulus features that determine the walking direction during pheromone-source orientation behavior of *B. mori*. Initial turning direction is affected by bilateral input pattern on both antennae, with differential intensity or time delay. This study contributes toward understanding efficient strategies for odor-source localization that is utilized by walking insects.

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