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Insect-Controlled Robot: A Mobile Robot Platform to Evaluate Odor-Tracking Capability of an Insect

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Abstract:	Robotic odor source localization has been a challenging area to which biological knowledge has been expected to contribute, because finding odor sources is an essential task for organisms to survive. Insects are well-studied organisms with regard to odor tracking, and their behavioral strategies have been applied to mobile robots for evaluation. This "bottom-up" approach is a fundamental way to develop biomimetic robots; however, the biological analyses and the modeling of behavioral mechanisms are still ongoing. Therefore, it is still unknown how such a biological system actually works as the controller of a robotic platform. To answer this question, we have developed an insect-controlled robot in which a male adult silkmoth (Bombyx mori) drives a robot car in response to odor stimuli, which can be regarded as a prototype of a future insect-mimetic robot. In the cockpit of the robot, a tethered silkmoth walked on an air-supported ball and an optical sensor measured the ball rotations. These rotations were translated into the movement of the two-wheeled robot. The advantage of this "hybrid" approach is that experimenters can manipulate any parameter of the robot, which enables us to evaluate the odor-tracking capability of insects, and will provide us useful suggestions for robotic odor-tracking. Furthermore, these manipulations are non-invasive ways to alter the sensory-motor relationship of a pilot insect, and will be a useful technique for understanding adaptive behaviors.
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Dear Dr. Sephorah Zaman,

re: "Insect-Controlled Robot: A Mobile Robot Platform to Evaluate Odor-Tracking Capability of an Insect" by Noriyasu Ando, Shuhei Emoto and Ryohei Kanzaki; JoVE54802.

The capability to localize an odor source is necessary for insects to survive, and is expected to be applicable to artificial odor tracking. However, it is still unknown how such an insect system actually works as a controller of a robotic platform. Our insect-controlled robot is driven by an actual silkmoth and enables us to evaluate the odor-tracking capability of insects for the development of biomimetic odor searching as well as for the study on the insect sensory-motor system.

We are most grateful to the editor and all four reviewers for the helpful comments on the original version of our manuscript. We have taken all these comments into account and submit a revised version of our paper. Please note that we also have revised the title as described above.

We hope that the revised version of our manuscript is now suitable for publication in JoVE and look forward to hearing from you at your earliest convenience.

Sincerely yours,

Noriyasu Ando, Ph. D

TITLE:
Insect-Controlled Robot: A Mobile Robot Platform to Evaluate the Odor-Tracking Capability of an Insect

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KEYWORDS:
silkmoth, insect, odor-tracking, pheromone, vision, multisensory integration, insect-machine hybrid robot, biomimetics

SHORT ABSTRACT:
The capability to localize an odor source is necessary for insects survivival and is expected to be applicable to artificial odor-tracking. The insect-controlled robot is driven by an actual silkmoth and enables us to evaluate the odor-tracking capability of insects through a robotic platform.

LONG ABSTRACT:
Robotic odor source localization has been a challenging area and one to which biological knowledge has been expected to contribute, as finding odor sources is an essential task for organism survival. Insects are well-studied organisms with regard to odor tracking, and their behavioral strategies have been applied to mobile robots for evaluation. This "bottom-up" approach is a fundamental way to develop biomimetic robots; however, the biological analyses and the modeling of behavioral mechanisms are still ongoing. Therefore, it is still unknown how such a biological system actually works as the controller of a robotic platform. To answer this question, we have developed an insect-controlled robot in which a male adult silkmoth (*Bombyx mori*) drives a robot car in response to odor stimuli; this can be regarded as a prototype of a future insect-mimetic robot. In the cockpit of the robot, a

tethered silkmoth walked on an air-supported ball and an optical sensor measured the ball rotations. These rotations were translated into the movement of the two-wheeled robot. The advantage of this "hybrid" approach is that experimenters can manipulate any parameter of the robot, which enables the evaluation of the odor-tracking capability of insects and provides useful suggestions for robotic odor-tracking. Furthermore, these manipulations are non-invasive ways to alter the sensory-motor relationship of a pilot insect and will be a useful technique for understanding adaptive behaviors.

INTRODUCTION:

Autonomous robots capable of finding an odor source can be important for the safety and security of society. They can be used for the detection of disaster victims, of drugs or explosive materials at an airport, and of hazardous material spills or leaks in the environment. At present, we rely entirely on well-trained animals (*e.g.,* dogs) for these tasks, and robotic odor source localization has been strongly expected to relieve the workload of these animals. Finding an odor source is a challenging task for robots because odorants are distributed intermittently in an atmosphere[1]; therefore, continuous sampling of the odor concentration gradient is not always possible. Thus, a search strategy using intermittent odor cues is necessary for the achievement of robotic odor source localization[2-4].

Odor source localization is essential for organism survival and includes tasks such as finding food, mating partners, and sites for oviposition. To overcome the difficulty in tracking patchy distributed odorants, organisms have evolved various behavioral strategies consisting of two fundamental behaviors: moving upstream during odor reception and cross-stream during cessation of odor reception[5,6]. These reactive strategies have been well-documented in insects and further combined with other modalities, such as wind direction and vision[5-8]. The insect behavioral models have also been useful examples for robotics[3,9-11], in which behavioral algorithms or neural circuit models are implemented into mobile robots for the evaluation of odor source localization abilities[10,12-15]. From biomimetic perspectives, this "bottom-up" approach is certainly a fundamental way to develop biomimetic robots. However, the bottom-up approach is not a shortcut to obtaining a useful search strategy, because biological analyses are still ongoing, and the modeling of the sensory-motor systems behind insect behaviors has not been completed. Therefore, it is still unknown how such a biological system actually works as a controller of a robotic platform.

In this article, we demonstrate the protocol of a straightforward "top-down" approach to develop an odor-tracking mobile robot controlled by a biological system[16,17]. The robot is controlled by a real insect and can be regarded as a prototype of future insect-mimetic robots. In the robot's cockpit, a tethered adult male silkmoth (*Bombyx mori*) walked on an air-supported ball in response to the female sex pheromone, which was delivered to each antenna through air suction tubes. The ball rotations caused by the walking of the onboard moth were measured by an optical sensor and were translated into the movement of the two-wheeled robot. The advantage of this "hybrid" approach is that experimenters can investigate how the insect sensory-motor system works on the robotic platform where a pilot insect is in a closed loop between the robot and a real odor circumstance. The manipulation of the robotic hardware alters the closed loop; therefore, the insect-controlled robot is a useful platform for both engineers and biologists. For engineering, the robot represents the first steps of applying a biological model to meet the requirements for robotic tasks. For biology, the robot is an experimental platform for studying sensory-motor control under a closed loop.

PROTOCOL:

1. Experimental animal

1.1) Prepare a plastic box to keep the pupae of male silkmoths (*B. mori*) until their eclosion. Put paper towels at the bottom and pieces of cardboard around the inner wall of the box (Figure 1A).

Note: The pieces of cardboard are necessary for the adult moths to hold while extending their wings during eclosion (Figure 1A).

1.2) Put male silkmoth (*Bombyx mor*i) pupae in the box and keep them in an incubator until eclosion under a 16-h:8-h light:dark cycle at 25 °C.

Note: The male and female pupae can be discriminated by the sex markings on the abdomen (Figure 1B).

1.3) Collect adult male moths after eclosion and move them into a new box.

1.4) Keep the adult moths in an incubator under a 16-h:8-h light:dark cycle and decrease the temperature to 15 °C to reduce their activity before the experiment.

2. Tethering a silkmoth

2.1) Fabrication of an attachment for tethering (Figure 2A)

Note: The attachment consists of a copper wire with a strip of a thin plastic sheet at its tip. This ensures the dorsal-ventral movement of the thorax during walking (Figure 2B).

2.1.1) Prepare a strip of a thin plastic sheet, 2 × 40 mm (thickness: 0.1 mm), and fold it in the middle.

2.1.2) Attach the folded strip to the tip of a copper wire with an adhesive.

2.1.3) Bend the tip of the folded strip where the thorax of a silkmoth is attached.

2.2) Use adult moths (2-8 days old) during the light period for the experiment.
Note: The sensitivity to the pheromone strongly depends on the circadian clock[18]. Because *B. mori* is a diurnal moth, the experiment must be performed during the light period.

2.3) Gently remove all scales on the dorsal thorax (mesonotum) using a piece of wet tissue (or cotton swab) and expose the cuticle of the mesonotum (Figure 2C).

2.4) Paste an adhesive on the strip of plastic on the attachment and on the surface of the exposed mesonotum with a small flat-blade screwdriver and wait 5-10 min until the adhesive is no longer sticky.

Note: The adhesive should not touch the wing hinge or the forewing tegulae (Figure 2C).

2.5) Bond the mesonotum to the attachment.

2.6) Keep the moth tethered before placing it inside the cockpit of the robot. Hold the attachment on a stand and put a piece of paper under the legs to rest the moth.

3. Insect-controlled robot

3.1) Design the hardware of the insect-controlled robot based on previous works[16,17,19].

Note: The insect-controlled robot consists of an air-supported treadmill with an optical mouse sensor to capture the insect locomotion, custom-built AVR-based microcontroller boards for processing and motor control, and two DC brushless motors (Figures 3 and 4). The robot can run on the basis of the ball rotation with 96% precision or higher, within a time delay of 200 ms. It also ensures the mobility of maximum forward speed (24.8 mm/s) and angular velocity (96.3°/s) of the silkmoth during pheromone tracking behavior[16]. The airflow of the treadmill (Figure 5A) and odor delivery system (Figure 5B) are designed for the onboard moth to walk smoothly on the ball and to acquire an odor by two antennae. The air intake and flow channel of the treadmill is separated from those of the odor delivery system to avoid contamination of the pheromone.

3.2) Design the software for the onboard microcontrollers based on previous works[16].

Note: The onboard microcontroller calculates the robot movements from the insect locomotion measured with an optical sensor (rotational, Δx; translational, Δy; Figure 6). The travel distance (ΔL) and turn angle ($\Delta\theta$) per unit time of the robot are calculated on the basis of travel distance of each wheel (left, ΔL_L; right, ΔL_R) such as $\Delta L = (\Delta L_L + \Delta L_R) / 2$ and $\Delta\theta = (\Delta L_L - \Delta L_R) / D_{wheel}$, where D_{wheel} is the distance between the two wheels (120 mm). ΔL_L and ΔL_R are further described as $\Delta L_L = \Delta L_{x,L} + \Delta L_{y,L}$ and $\Delta L_R = \Delta L_{x,R} + \Delta L_{y,R}$, where $\Delta L_{x,L}$ and $\Delta L_{x,R}$ are the travel distances of the wheels on the left and right sides controlled by Δx, and $\Delta L_{y,L}$ and $\Delta L_{y,R}$ are those controlled by Δy. Ideally, $\Delta L_{x,L}$ and $\Delta L_{x,R}$ are described as $\Delta L_{x,L} = -\Delta L_{x,R} = G\ \Delta x\ (D_{wheel} / D_{ball})$, and $\Delta L_{y,L}$ and $\Delta L_{y,R}$ are described as $\Delta L_{y,L} = \Delta L_{y,R} = G\ \Delta y$, where G is the motor gain and D_{ball} is the diameter of the ball (50 mm). In practice, the motor gain is independently set by each side (left or right wheel) and by each direction (forward or backward rotation) so as to calibrate the robot movement. The independent gains further allow for the setting of asymmetrical motor rotation to generate a turning bias of the robot (see step 6.1).

3.3) Wash the surface of a white expanded polystyrene ball (mass: approximately 2 g; diameter: 50 mm) with water to remove any possible olfactory or visual cues.

Note: The surface of a new ball should be roughed with fine-grit sandpaper, such as P400, which ensures the grip of the legs on the ball.

3.4) Turn on the blower fan that supplies air at 9 V to the treadmill and floats the ball (Figure 5A). Observe the ball float approximately 2 mm from the bottom of the cup.

3.5) Using a screw, attach the copper wire of the attachment with the moth (see step 2) to a fixture in the cockpit of the robot (see Figure 3 inset). Make sure that the position of the middle legs are at the center of the ball (Figure 7A).

3.6) Adjust the vertical position of the attachment to enable the moth to walk normally on the ball. Keep the ball at the same height before and after attaching the moth (Figure 7B).
Note: A too-low position of the attachment adds pressure on the moth and elicits backward walking to resist the pressure (Figure 7C), whereas a too-high position causes unstable walking and failures of the sensor due to changes in the vertical position of the ball (Figure 7D). To check normal walking behavior, a single-puffed pheromone stimulus is used to trigger walking in the moth (for the pheromone stimulus, see step 4). Note that the test stimulus must be minimal because previous exposure to bombykol habituates silkmoths and decreases their sensitivity (Matsuyama and Kanzaki, unpublished data).

4. Odor source preparation
Note: Male *B. mori* are sensitive to the major component of the conspecific female sex pheromone (bombykol: (E,Z)-10,12-hexadecadien-1-ol)[20]. Any contamination of experimental equipment with bombykol elicits the odor-tracking behavior and affects the responsiveness of the moth.

4.1) Drop 10 µL of the bombykol solution dissolved in n-hexane (200 ng/µL) on a piece of filter paper (approximately 10 mm × 10 mm). The amount of bombykol per piece of filter paper is 2,000 ng.
Note: To check the normal walking behavior of the moth, prepare a pheromone stimulus cartridge in this step. The cartridge is a glass Pasteur pipette with one piece of filter paper containing 2,000 ng of bombykol. Pushing a bulb puffs the air containing bombykol.

5. Odor source localization experiment
5.1) Turn on the fan of a pulling-air-type wind tunnel (1800 × 900 × 300 mm, L × W × H; Figure 8) and set the wind speed to 0.7 m/s. Ensure that the temperature is more than 20 °C.

5.2) Set the odor source (the piece of filter paper containing bombykol) upstream of the wind tunnel.
Note: The plume width should be confirmed prior to the experiment by using $TiCl_4$[17,19].

5.3) Turn on the microcontroller board of the robot and establish a serial connection to a PC via Bluetooth.

5.4) Launch a custom-made Java program called "BioSignal," which provides an interface between the PC and the robot.
Note: The main window includes buttons for sending commands to the robot, text windows for displaying the input and output of the serial communication, and small boxes to configure parameters. The subsequent commands are sent by clicking corresponding buttons in this program, except for video capturing.

5.5) Click on the "about device" button to confirm the connection by sending a command to the robot via the specified COM port and check that a message is returned by the robot.

5.6) Click on the “memory erase” button to erase previous locomotion data left on the onboard flash memory.

5.7) Click on the “drivemode1” button to send the default motor gains to the robot.
Note: The manipulations of the motor gains and the time delay between insect locomotion and robot movement are applied after this step (see steps 6.1 and 6.3, Figure 9).

5.8) Click on the “don’t drive” button to send a command to immobilize the robot until the experiment starts.

5.8) Put the robot at a start position (600 mm downstream from the odor source) and turn on the switch of the motor driver board.

5.9) Push the recording button of the camcorder to start video capture.

5.10) Click on the “rec start” button to send a start command to initiate the robot with a simultaneous recording of the ball rotation on the onboard flash memory. Observe that the robot starts to move and tracks the odor plume.

5.11) Click on the “rec stop” button to send commands to stop both the robot movement and the recording if the robot localizes the odor source.

5.12) Push the recording button of the camcorder to stop video capture.

5.13) Download recorded locomotion data from the onboard flash memory to the computer via a serial connection. Close the program.

6) Manipulation of the insect-controlled robot
Note: The timing of each manipulation is indicated in Figure 9.

6.1) Manipulation of motor gains
Note: This manipulation alters the translational and rotational velocity of the robot. Asymmetrical motor gains generate a turning bias, which can be used to investigate how insects compensate for the bias[17].

6.1.2) Define the rotational gains for forward and backward rotation of the motor on each side[17] (Figure 6B) by editing the configuration file named “param2.txt” using a text editor.

6.1.3) Click on the “set param2” to read the edited configuration file in the software program. Then, click on the “drivemode2” to send the manipulated gains to the robot.

6.2) Inversion of the motor output
Note: This manipulation provides a condition similar to the inversion of bilateral olfactory input (see step 6.4) and can be used to investigate the significance of bilateral olfaction. However, the inversion of motor output also inverts self-induced visual motion of an

onboard moth. The impact of the inverted self-induced visual input can be evaluated by a comparison with the inverted olfactory input[19].

6.2.1) Invert the bilateral motor control by crossing the control cables for each motor.

6.3) Manipulation of the time delay between insect locomotion and robot movement.
Note: This manipulation allows for the investigation of the acceptable period of time spent on sensory-motor processing for the robotic odor-tracking. The microcontroller stores the locomotion data on a buffer memory and then processes it after the specified time delay. Note that the robot has a maximal internal time delay of 200 ms; therefore, the actual time delay is expected to be the specified time delay plus 200 ms[16,17].

6.3.1) Input a number (from 0-10) in a small box of the main window to specify a time delay from 0-1,000 ms at 100-ms steps.

6.3.2) Click on the "set delay" button to apply the time delay.

6.4) Manipulation of the olfactory input.
Note: This manipulation can be used to investigate the significance of bilateral olfactory input. The surge direction of silkmoths is biased on the higher-concentration side[22].

6.4.1) Change the gap between the suction tube tips or invert their positions to alter the difference in odor concentration acquired by each antenna.

6.5) Manipulation of visual input
Note: This manipulation is to investigate the role of visual input for odor-tracking.

6.5.1) Cover the canopy with a white paper that occludes 105° and 90° of the horizontal and vertical visual field of the onboard moth, respectively.

REPRESENTATIVE RESULTS:
We present here the basic characteristics of the insect-controlled robot required for the successful localization of an odor source. The comparison between the robot and silkmoths, the effectiveness of the odor delivery system, and the significance of accurate bilateral olfactory and visual inputs are examined.

The comparison of odor-tracking behaviors between freely-walking moths and the insect-controlled robot is shown in Figure 10A and B. Under the same odor circumstances, both the walking moths and the robots scored success rates of 100% (walking moth, 10 trials by $N = 10$ moths; robot, 7 trials by $N = 7$ moths). Though the robot exhibited broader trajectories compared to those of the walking moths, there was no significant difference in the time to localization between the walking moths and the robot ($P > 0.05$, Wilcoxon rank sum test; moth, median = 46.5 s, IQR = 36.7, 69.6; robot, median = 48.1 s, IQR = 44.9, 61.9).

The odor delivery system (Figure 5B) is necessary for supplying the odorant flow near the floor to the antennae of the onboard moth placed 90 mm above the floor. Without this system (suction tubes, fans, and the canopy), the robot could not orient toward the odor source and continued circling until it stopped (all 10 trials by *N* = 5 moths failed, Figure 10C). According to programmed silkmoth behavior, continuous circling is a typical behavior when a silkmoth fails to contact the pheromone during orientation[21,22].

Figure 11 shows the representative results demonstrating the manipulations of the robot. The effectiveness of a bilateral olfactory input for odor-tracking was evaluated by changing the position of the tube tips (step 6.4) or by inverting the motor output (step 6.2). The robot achieved success rates of 100% with two different gaps between the left and right tubes (wide gap [control], 90 mm, 10 trials by *N* = 10 moths; narrow gap, 20 mm, 10 trials by *N* = 10 moths; Figure 11A, B), and there was no significant difference in the time to localization between these two tube positions ($P > 0.05$, Steel's test; Figure 11E). On the other hand, the inversion of tube tips (each antenna received the odorant from the contralateral side, tube gap = 90 mm) broadened trajectories along the crosswind direction and slightly increased the median of time to localization, although there was no significant difference ($P > 0.05$, Steel's test; Figure 11C, E). The inversion of motor output provides a similar situation as the inverted olfactory input; furthermore, it also inverts the self-induced visual motion received by the onboard moth. Because of the inverted negative visual feedback (*i.e.,* positive feedback), the robot continued circling, even in the odor plume (Figure 11D), which significantly lengthened the time to localization ($P < 0.01$, Steel's test; Figure 11E). The success rates of the inverted olfactory input (C) and the inverted motor output (D) were 80% (10 trials by N = 10 moths) and 90.9% (11 trials by 11 moths), respectively. A detailed discussion of sensory-motor control in silkmoths is described in the previous work[19].

FIGURE LEGENDS:

Figure 1. Storing of silkmoth pupae. (A) Male pupae are stored in a plastic box (left). The adult moths hold the cardboard around the inner wall of the box during eclosion (right). (B) Sex markings of pupae. Each arrow indicates a small spot on the ventral side of ninth abdominal segment of the male and an "X" mark with a fine, longitudinal line on the ventral side of the eighth abdominal segment of the female.

Figure 2. Tethering a silkmoth. (A) Fabrication of an attachment for tethering a silkmoth. The three steps are described in 2.1.1 to 2.1.3 (see text). A two-fold strip of thin plastic sheet was attached at the tip of the copper wire, which absorbs the dorsal-ventral movement (see Figure 2B) of the mesonotum during walking. The other, curved tip of the wire is for handling. (B) Higher and lower attitudes of a silkmoth during pheromone tracking (see the angle between the femur and the tibia of the forelegs [arrows]). (C) Removal of the scales on the mesonotum (indicated by arrowheads). The left and right pictures show before and after the removal of scales, respectively. The forewing tegulae were intact (surrounded by dashed lines).

Figure 3. Insect-controlled robot. The inset shows a magnified view of the cockpit. (1) A tethered silkmoth on a treadmill (an air-supported ball, see inset), (2) two fans for supplying an odor to the moth (air speed, 0.5 m/s), (3) suction tubes for taking the odor, (4) DC motors and wheels, (5) microcontroller boards, (6) an air intake for supplying air to the ball, (7) tracking markers for offline video analyses, (8) two LEDs to keep constant illumination in the

cockpit (280 lx), (9) an attachment for tethering the silkmoth, and (10) a fixture of the attachment.

Figure 4. Hardware diagram. The rotation of the air-supported ball in the treadmill was measured by an optical mouse sensor with a resolution of 0.254 mm at a sampling rate of 1.5 kHz. The microcontrollers calculated the trajectory of the silkmoth from the sensor output and controlled two DC motors on the left and right sides. The motors were driven by pulse-width modulation at 1 kHz, with position feedback from built-in Hall sensors. The optical sensor output (*i.e.,* behavior of the onboard moth) was stored on an onboard flash memory (8 Mbit) at a sampling rate of 5 Hz. These data were used for comparing the behavior of the onboard moth with robot movements. The wireless communication between a computer (PC) and the robot was achieved via Bluetooth, which was only used for sending commands to start and stop the robot, or to manipulate the motor properties of the robot.

Figure 5. Airflow designs for the treadmill and the odor delivery system. (A) Airflow to support the ball of the treadmill. The air was taken from the air intake behind the cockpit by a blower fan; it then flowed through a channel and blew out from small holes (1-mm diameter) on a custom-made FRP cup (inset). The top view of the cup surrounded by a red rectangle is shown in the inset. Red arrows indicate airflow; the white arrow, the optical sensor with an LED transmitter; and the black arrow, the cup with small holes. (B) The airflow of the odor delivery system. The air containing the pheromone was suctioned from the tip of a flexible polyethylene tube on each side, separated with a partition in the canopy, and delivered to the antenna on the ipsilateral side. Airflow on each side is indicated by red or blue arrows. This figure has been modified from Ando and Kanzaki[19].

Figure 6. Calculation of the robot movement from insect locomotion. (A) A schematic drawing of the robot (ΔL) and wheel movements (left, ΔL_L and right, ΔL_R). $\Delta\theta$, turn angle of the robot. (B) Parameters for the calculation. Δx and Δy represent the rotational and translational movements of a ball (a positive value indicates the clockwise or forward direction); D_{ball}, the diameter of the ball; D_{wheels}, the distance between wheels; $G_{FW,L}$ and $G_{BW,L}$, motor gains of forward (FW) or backward (BW) rotation of the left wheel (L); $G_{FW,R}$ and $G_{BW,R}$, motor gains of forward or backward rotation of the right wheel (R).

Figure 7. Adjustment of the position of a tethered moth on the treadmill. (A) The lateral view of a tethered moth on a ball. The middle legs should be placed at the top of the ball (black arrow). (B) The appropriate vertical position of the moth. The optical sensor behind the moth faces the center of the ball. Normal forward walking rotates the ball clockwise (as viewed from the left side). (C) The vertical position is too low (downward arrow). The silkmoth extends the forelegs to resist the pressures and rotates the ball backward (counterclockwise rotation). (D) The vertical position is too high (upward arrow). The moth holds the ball and lifts it up. Although the moth can perform forward walking in this situation[23] (clockwise rotation), it lifts up the ball and shifts its position. The vertical shift of ball position increases the gap between the ball and the optical sensor, which results in a failure of sensor reading.

Figure 8. Wind tunnel. The air was filtered with a mesh panel (red arrow); it then entered the recording area of a camcorder, 1,500 (L) × 900 (W) mm. The odor source was placed upstream of the recording area and the pheromone-contaminated air was exhausted outside by a fan (blue arrow). The wind tunnel was made of extruded polystyrene foam. The ceiling was a transparent acrylic sheet, and the floor was a rubber mat to avoid slipping of the robot wheels. The odor source was placed at the center of the crosswind position and 250 mm downwind from the mesh panel.

Figure 9. Timings of manipulations of the robot in the protocol.

Figure 10. Odor source localization test. Each panel shows the trajectories of the silkmoths (A; 10 trials by N = 10 moths; data from Ando *et al.*[17]), the insect controlled robot (B; 7 trials by N = 7 moths), and the robot without the odor delivery system (C; 10 trials by N = 5 moths). The moths or the robot started 600 mm downwind (arrowhead) from an odor source (cross mark, a piece of filter paper containing 2,000 ng of bombykol). The trials with the shortest or the longest time taken for localization are indicated as red and blue lines, respectively. The other successful trials are colored gray, and failed trials are green. A circle indicates the goal area for judging success in localization. The radius of the goal area was defined on the basis of the size of the robot, equivalent to the closest distance between the onboard moth and the odor source[17]. An arrow indicates wind direction (wind speed: 0.7 m/s), and dashed lines indicate the boundaries of the pheromone plume.

Figure 11. Manipulation of the olfactory input and the motor output. Each panel shows successful trajectories of the robot (the position of the onboard moth) with a wide-tube gap (A; control, 90 mm, successful in all 10 trials by N = 10 moths), a narrow gap (B; 20 mm, successful in all 10 trials by N = 10 moths), an inverted wide-tube gap (C; successful in 8 of 10 trials, N = 10 moths), and a wide-tube gap with inverted motor output (D; successful in 10 of 11 trials, N = 11 moths). The repetitive air puffs through a piece of filter paper containing 2,000 ng of bombykol were released from the cross mark. The gray and white arrows with the robot indicate the orientations of bilateral olfactory input and motor output. The other experimental conditions and figure descriptions are the same as in Figure 10. (E) Time to localization of the robot under the four conditions (A-D). Individual data are summarized in a box plot. The left and right sides of the box indicate the first and third quartiles, and the bar represents the median. The whiskers indicate the 1.5× interquartile range. Asterisks indicate a significant difference from the control data (A), according to Steel's test (**$P < 0.01$).

DISCUSSION:

The most important points for the successful control of the robot by a silkmoth are letting the moth walk smoothly on the air-supported ball and the stably measuring the ball rotation. Therefore, tethering the silkmoth and mounting it on the ball at the appropriate position are the critical steps in this protocol. Inappropriate adhesion of the moth to the attachment or inappropriate positioning of the moth on the ball will cause unnatural pressure on it, which perturbs its normal walking behavior and/or causes a failure of the optical sensor to measure the ball rotation. Roughening the polystyrene ball is also important to prevent the moth from slipping. The locomotion of the tethered moth in response to odor stimuli and the subsequent robot movement should be carefully checked prior to the odor-tracking test (see step 3.6).

The use of a larger ball is better because it decreases the curvature of the treadmill, which provides a nearly flat plane for the insect legs. The 50-mm diameter ball used here is relatively small compared to that used in the conventional treadmill setup for silkmoths (diameter: 75 mm)[24]. However, a larger (and heavier) ball must be used with care, because the inertia of the ball is not negligible during robot movements. If an onboard moth cannot restrain the inertial-force-induced rotation of a ball during robot movements by its legs, the robot oscillates continuously without any walking by the moth. When experimenters consider the use of other insect species, therefore, the ball size should be selected on the basis of the strength of their leg grips as well as their sizes. During odor source localization, experimenters should also check the behavior of the moth—whether an onboard moth walks smoothly on the ball and the robot quickly responds as the moth moves. The silkmoth exhibits backward walking when it receives too much pressure from the attachment (a too-low position, see Figure 7) and repetitive movements of the forelegs if they slip on the ball or touch an object (such as the partition in front of the head, Figure 5B). Poor responsiveness of the robot to insect locomotion is due to inappropriate ball position or the depletion of the batteries (the batteries last for approximately 30 min).

The limitation of the insect-controlled robot is that the onboard moth is definitely situated under unnatural circumstances. The treadmill, the odor delivery system, and the 90-mm height of the cockpit provide different sensory information (mechanosensory, olfactory, and visual) from those acquired by freely-walking moths. These differences became obvious when we compared the behaviors of the insect-controlled robot with those of freely-walking silkmoths. For example, though the same performance for odor source localization was observed between the robot and freely-walking silkmoths, the trajectories of the robot were sparse along the crosswind direction, whereas those of the freely-walking silkmoths converged as they reached the odor source, according to the decrease of plume width (Figure 10A, B). This difference is simply due to the different sizes of the robot and moths. In particular, the distance between the onboard moth and the tube tip determines the range for searching odorants; therefore, the larger distance (robot: 100 mm; moth: approximately 10 mm from the thorax to the antenna tip) enable the robot to activate even outside the plume. Furthermore, the moth in the canopy cannot receive the wind direction from the external environment. Although the significance of the wind direction for odor-tracking has not yet been determined in silkmoths[22], the use of flow direction is a fundamental strategy for odor-tracking in other organisms[5,6]. Because of the imposed airflow generated by the odor delivery system, it is also difficult to account for “active sensing,” such as the effect of wing flapping that generates airflow and facilitates odor reception in silkmoths[25]. Because of these limitations, if experimenters employ this technique to explore the use of multiple modalities, it should be discussed whether the results obtained by these robot experiments can be applied to intact insects in natural conditions[19].

The insect-controlled robot fulfilled three requirements for the evaluation of the odor-tracking capability of insects: 1) direct interfacing of insect motor commands to robot control, 2) testing in a real odor plume, and 3) allowing the manipulation of the insect’s sensory-motor system. First, regarding the interface between an insect and a robot, the use of neural signals for controlling a robot, such as a brain-machine interface[26], is an alternative technique. Several studies on insects use neural signals or electromyograms for control of a robot and closed feedback loops[27-30]. However, this approach requires the decoding of neural signals to extract meaningful motor commands, which is an important and ongoing research subject in neuroscience. Therefore, the use of actual walking behavior of insects for robot control is a direct and simple way to interface the insect's motor commands to a robot. Second, regarding the environment in which the robot behaves, the use of virtual reality would be an alternative[13,31-33]. Virtual reality enables us to conduct behavioral experiments under more controlled situations and is most successful in the study of vision, where the air-supported treadmill has been used for tracking animal locomotion and generation of visual circumstances[24,34-36]. However, closing the feedback loop of olfactory information is technically difficult because it requires precise flow control. Although the application of optogenetics to activate olfactory receptor neurons[37-40] will overcome the limitations of virtual reality in olfaction, the use of a mobile robot in a real odor plume would be a reliable way to establish an olfactory closed loop at present. Finally, regarding the manipulation of an insect's sensory-motor system, alternative approaches would be surgical manipulations of the insects (*i.e.,* cutting or covering sensory organs or appendages[41]). However, our robotic manipulation (step 6 and Figure 11) is a non-invasive and reversible way to alter the sensory-motor system of insects, achieved by the manipulation of the robot platform[19], and the controllability of various parameters of the robot enables us to test its performance under various circumstances.

The insect-controlled robot has two major directions for future applications. The first direction is for engineering. As an autonomous robot controlled by the insect sensory-motor system, the insect-controlled robot will be a reference for mobile robots implemented with biological models, ranging from simplified Braitenberg vehicles[42] to large-scale neural networks. The insect-controlled robot will also be a useful platform for testing possible combinations of other modalities with insect odor-tracking, such as the implementation of a camera and an algorithm for collision avoidance to explore collision-free odor-tracking algorithms. Furthermore, fine-tuning of the robot properties may improve the odor-tracking performance better than intact insects. Such translation of the insect capability might lead to the practical use of this robot itself for finding hazardous materials, if we imitate the transgenic silkmoths[43] that respond to characteristic chemicals in a target material. On the other hand, the insect-controlled robot will also raise an important question: How should we use biomimetic algorithms for robotic applications that extend beyond the difference between insects and robots? For example, insect olfactory receptors have an outstanding ability to acquire high-speed temporal dynamics of odor concentration[44-46], which is responsible for insect olfactory processing and odor source localization, but are far beyond the capabilities of conventional gas sensors[4,29,47]. How to modify the biomimetic algorithm to meet the sensory ability of robots should also be explored as a future direction. The other major direction is definitely for biology. The insect-controlled robot can be regarded as a closed-loop experimental platform. In addition, robotic manipulation, a non-invasive way to alter the insect's sensory-motor relationship, will be further applied to investigate how the small insect brain can respond, learn, and adapt to new circumstances.

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The authors have nothing to disclose.

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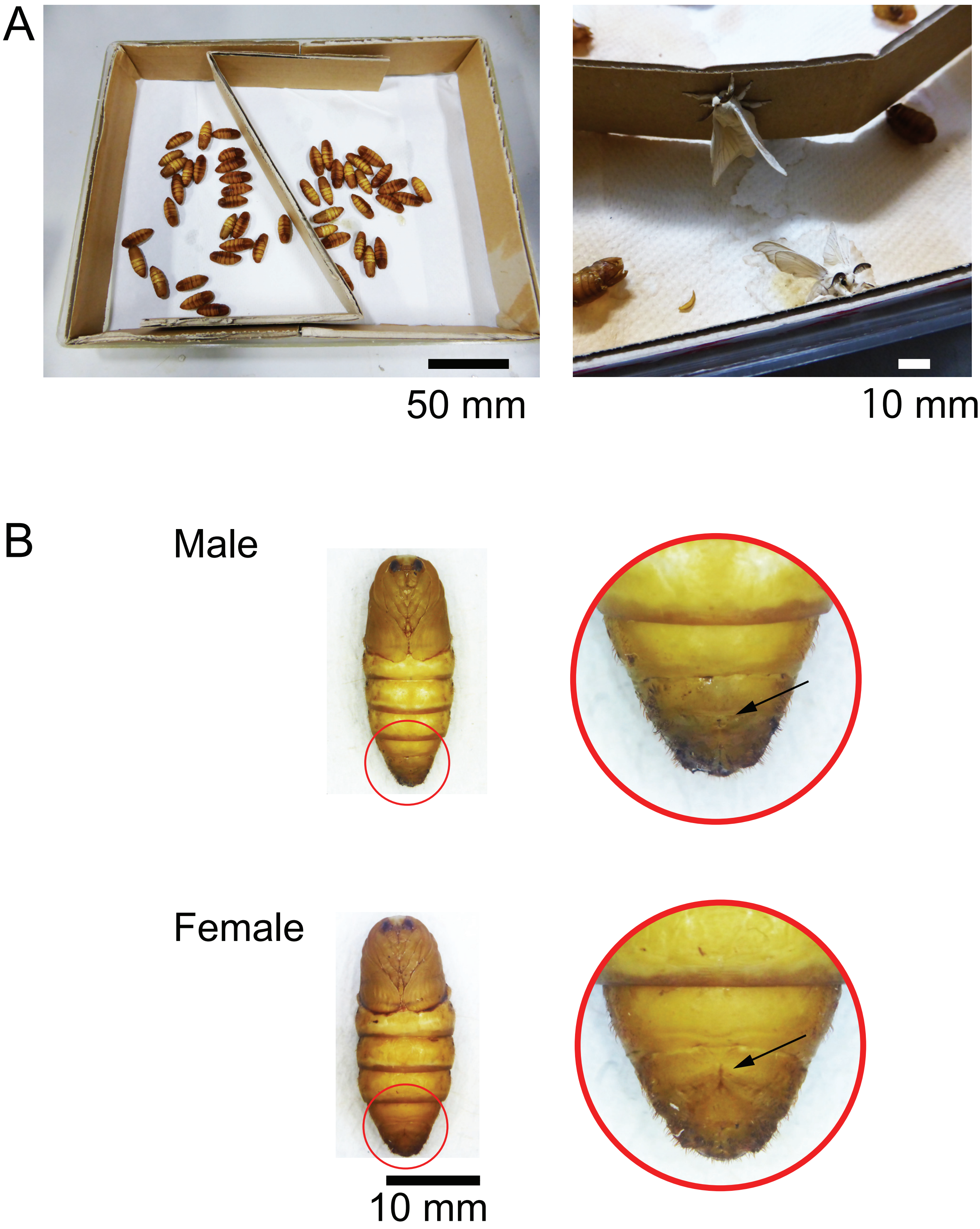

A
50 mm
10 mm
B
Male
Female
10 mm

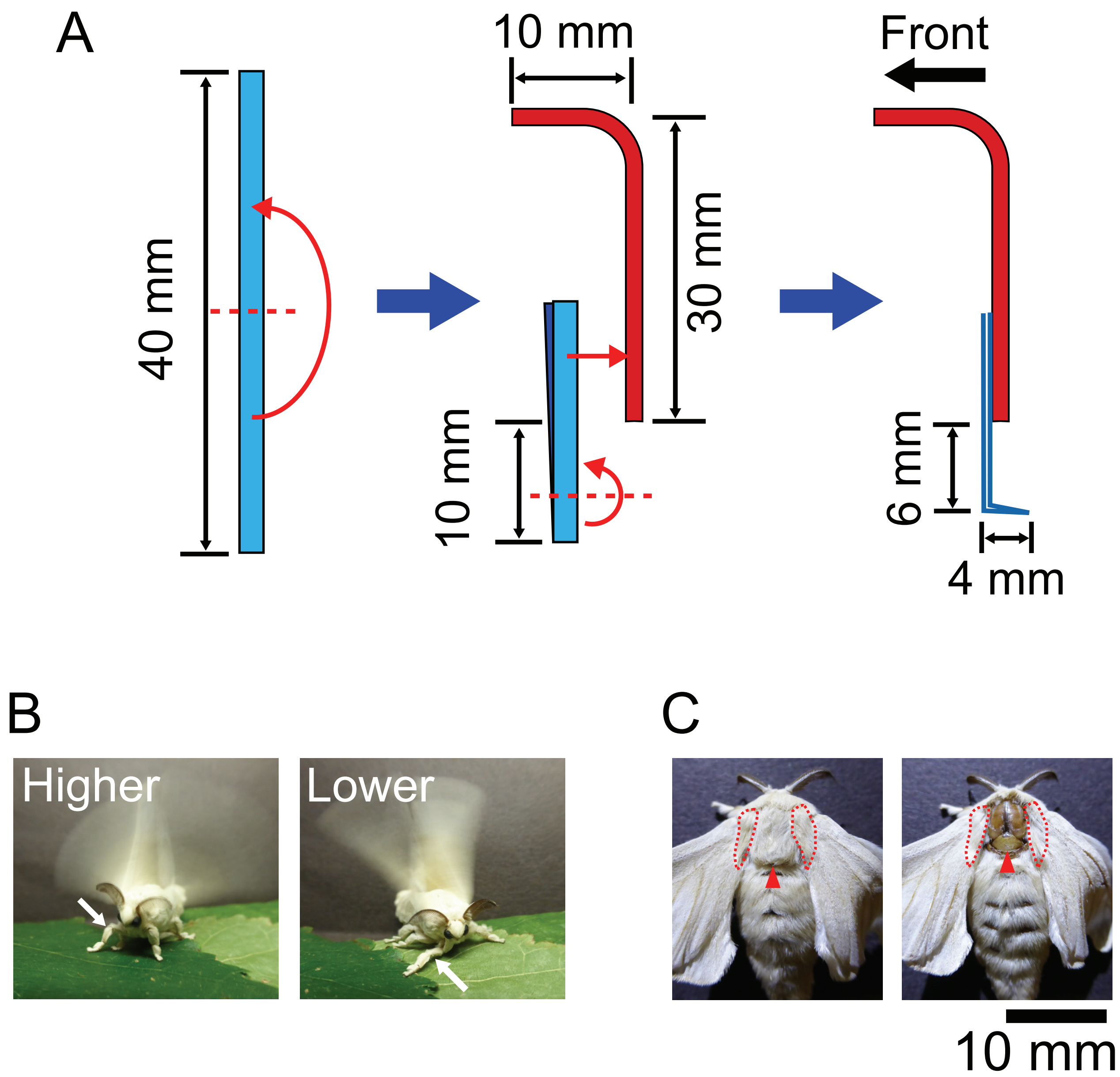
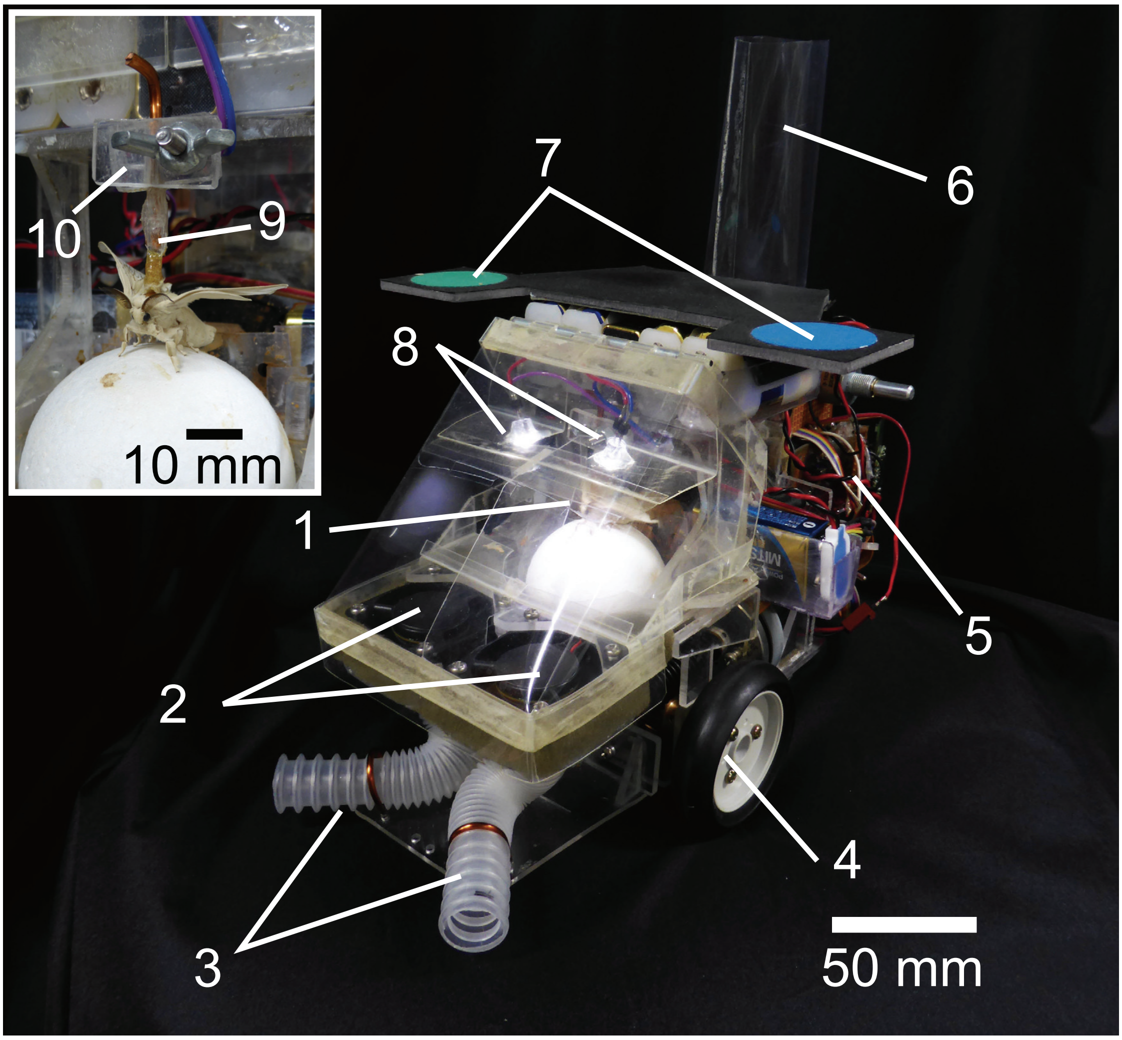
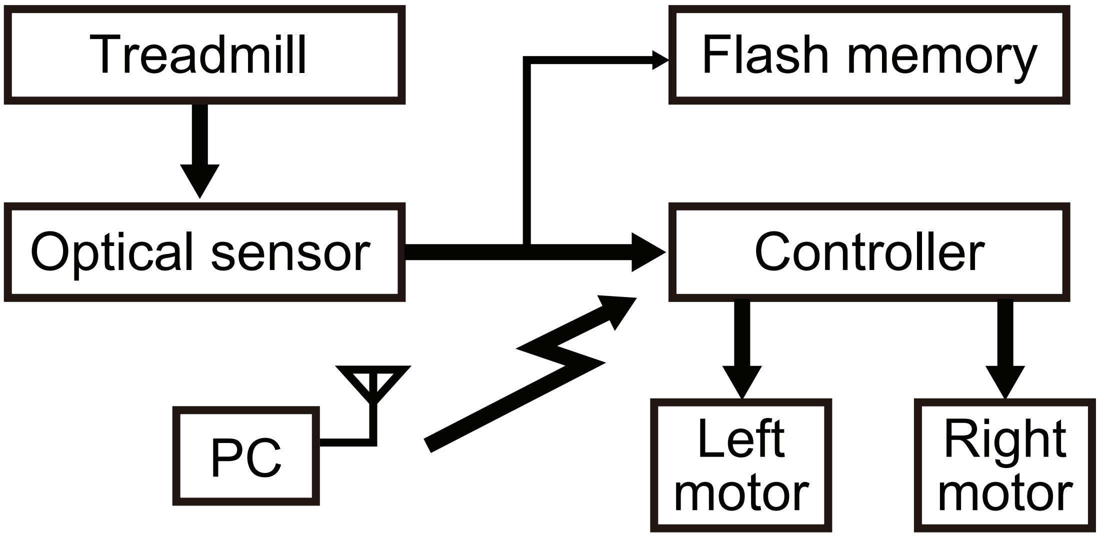
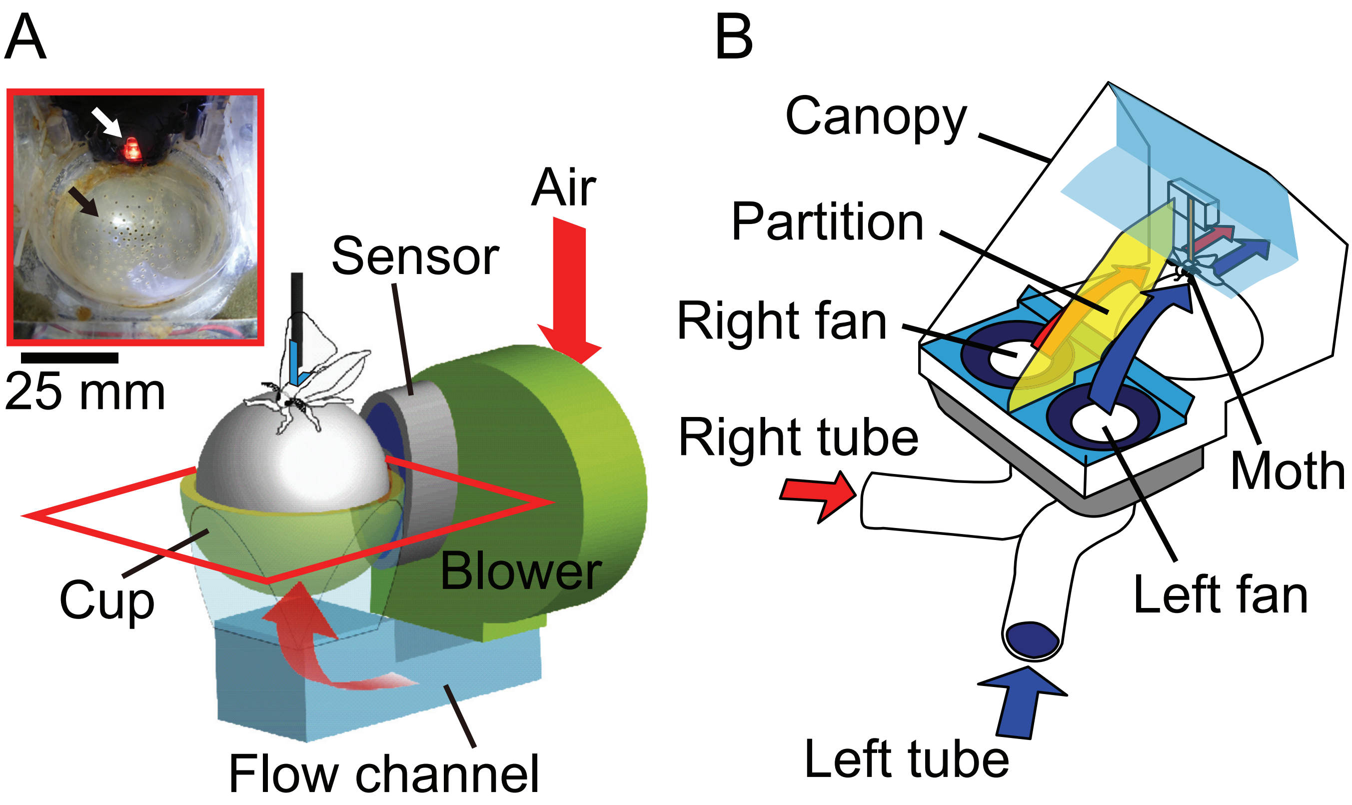
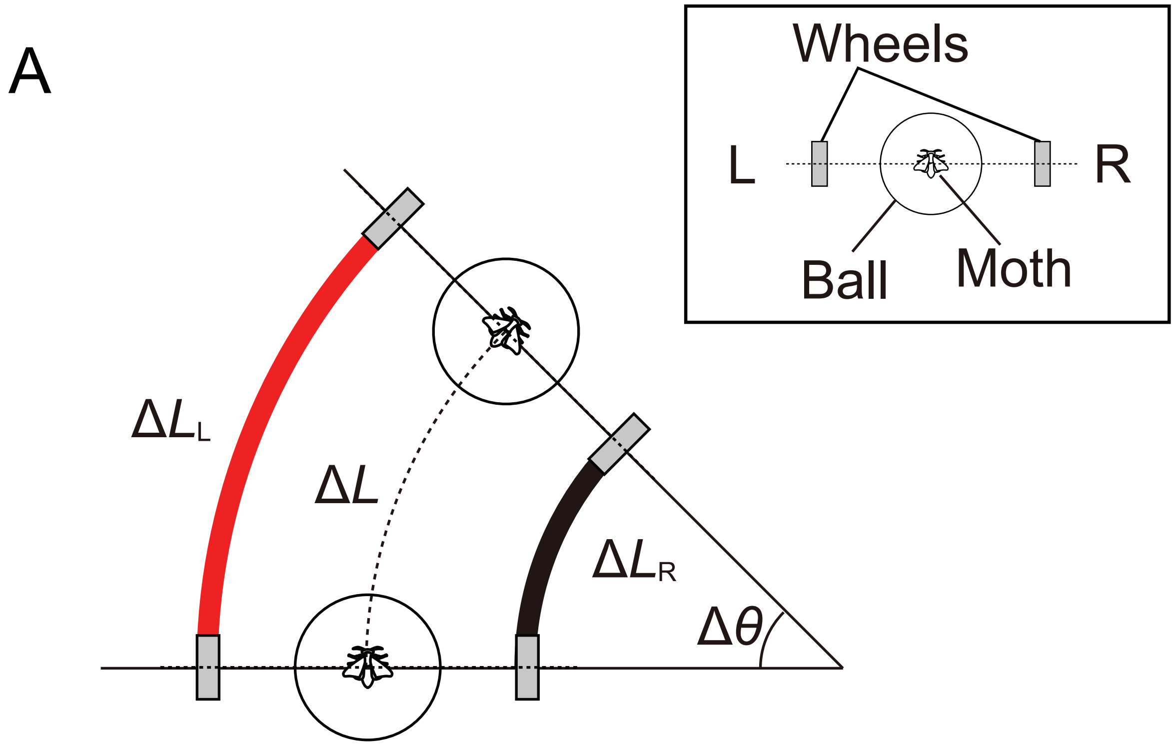

A
40 mm
10 mm
30 mm
10 mm
Front
6 mm
4 mm
B
Higher
Lower
C
10 mm

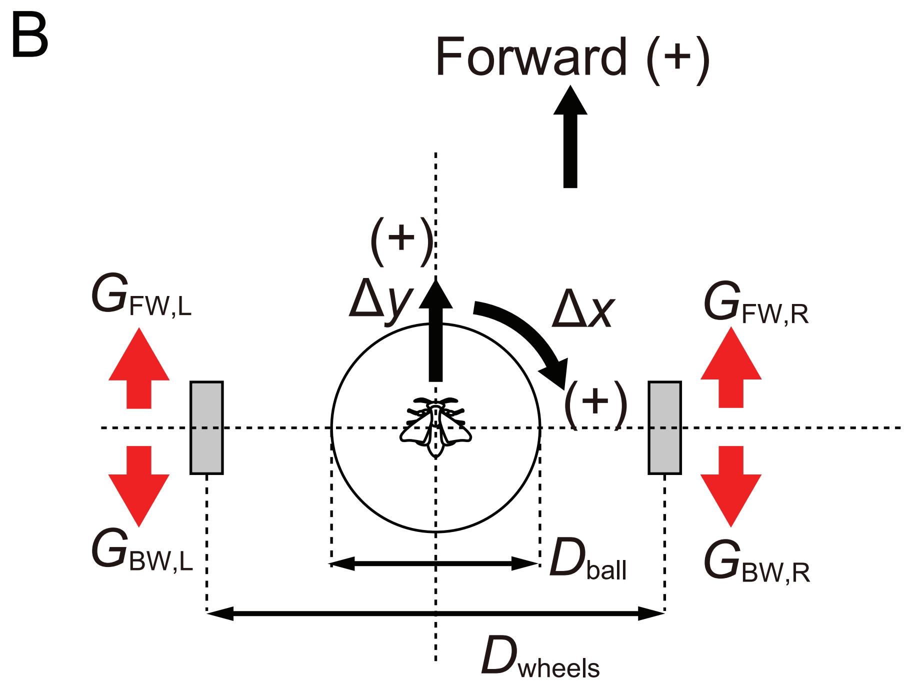
Figure3 Click here to download Figure Fig3J.pdf

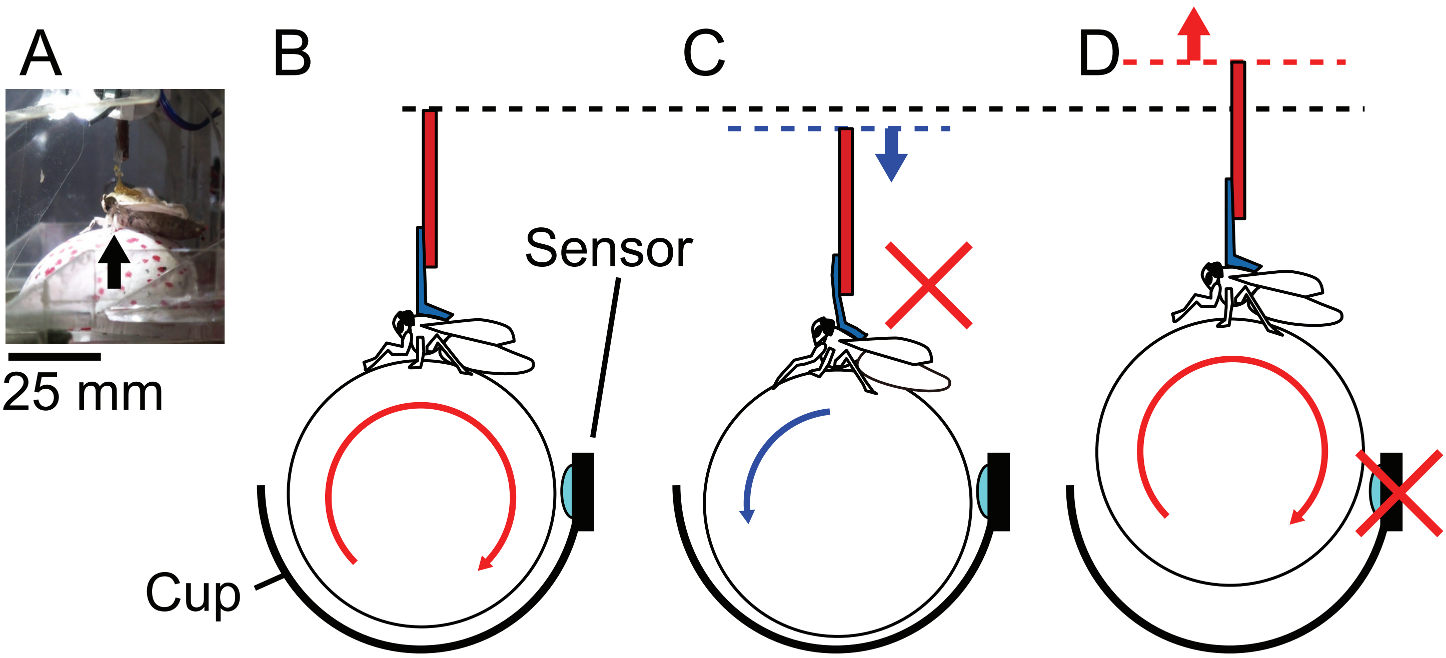


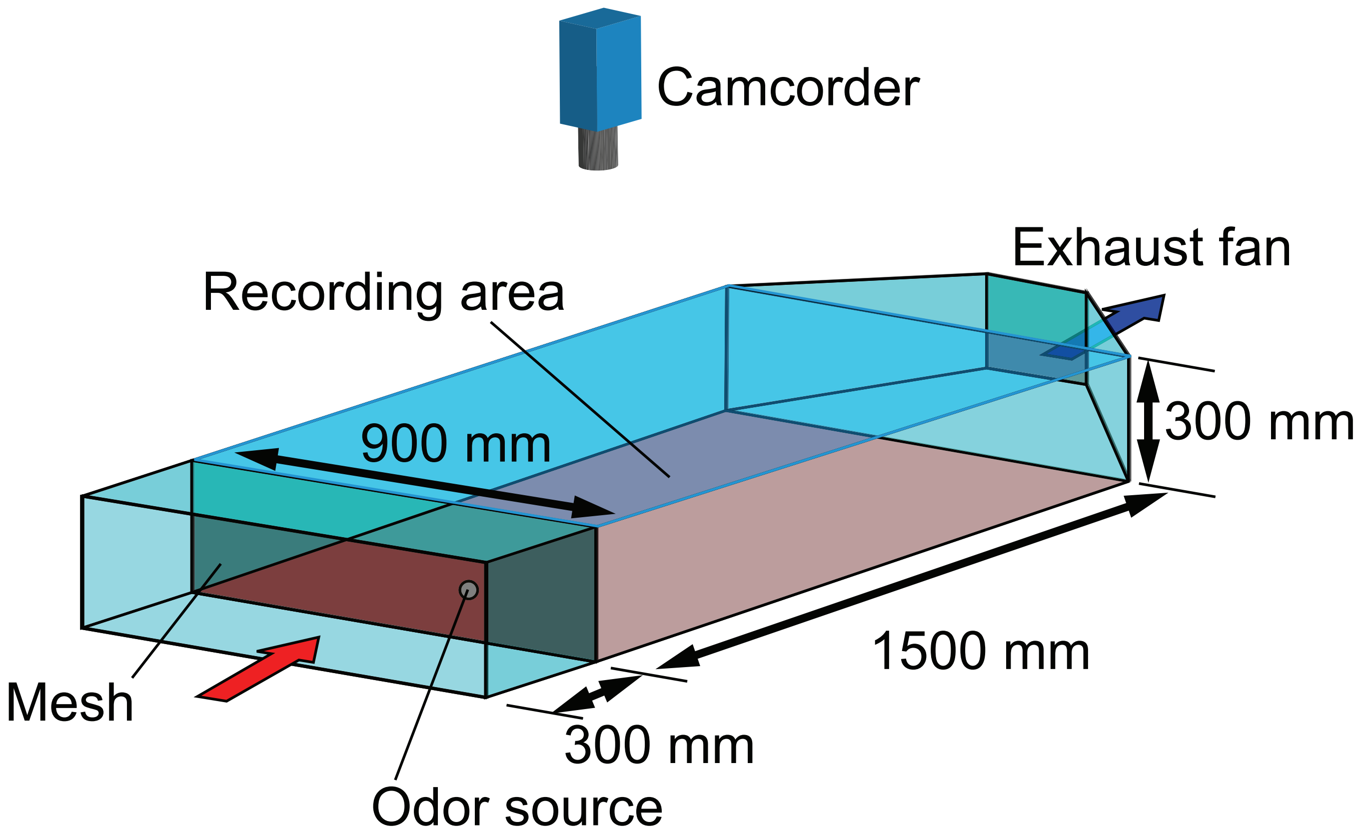

Treadmill
Flash memory
Optical sensor
Controller
PC
Left motor
Right motor

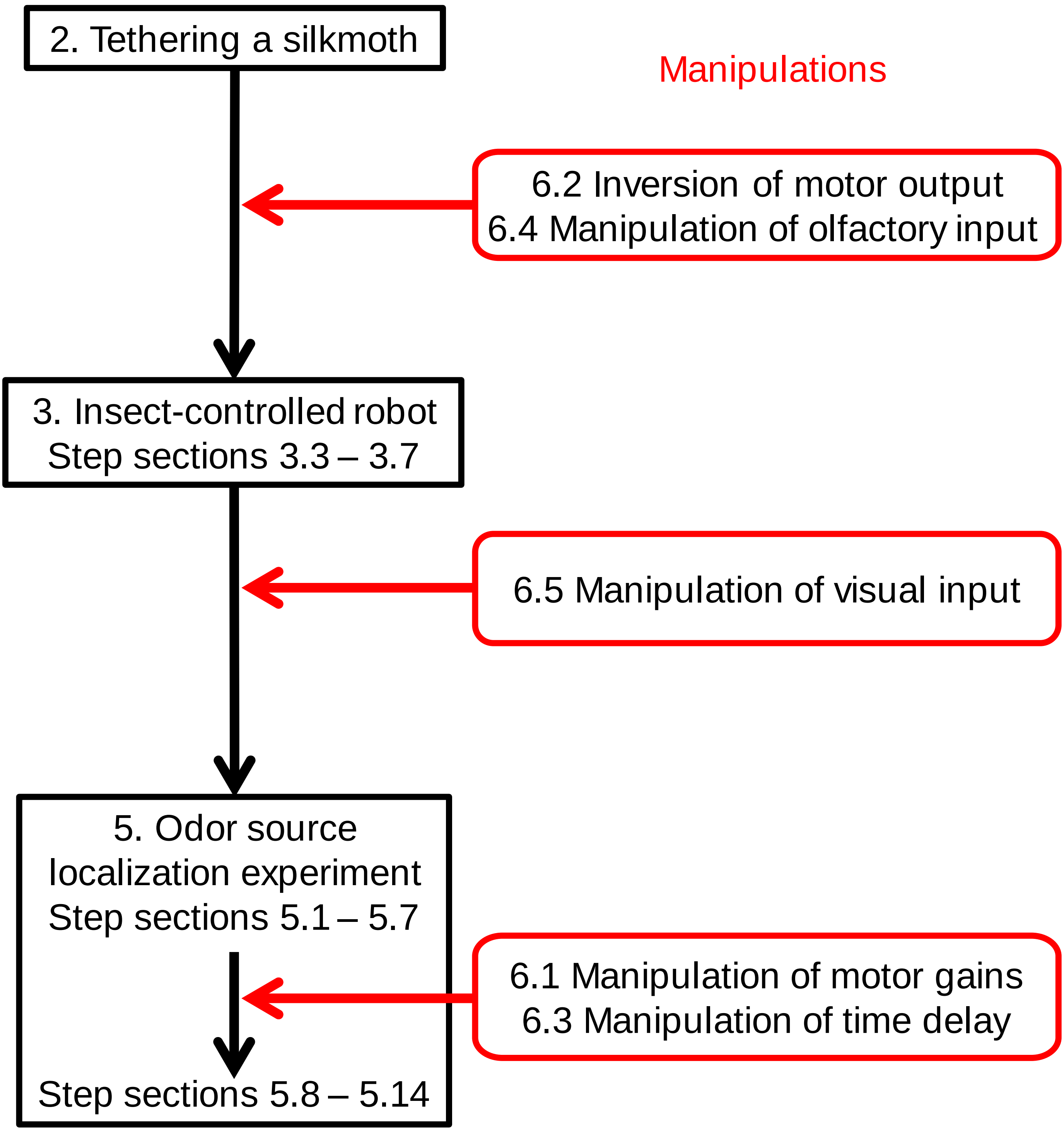

A
25 mm
Air
Sensor
Cup
Blower
Flow channel
B
Canopy
Partition
Right fan
Right tube
Moth
Left fan
Left tube

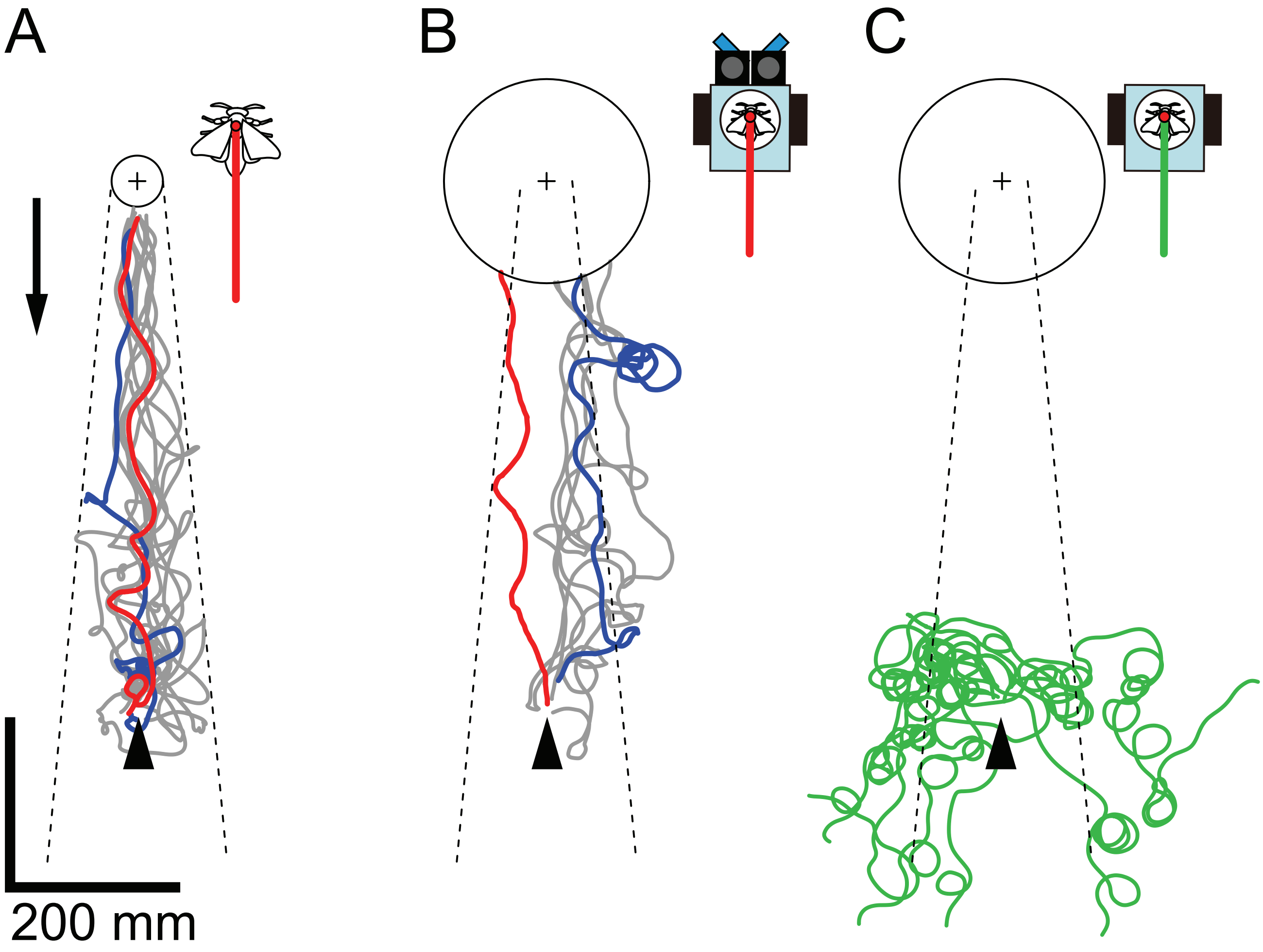

A
Wheels
L
R
Ball
Moth
ΔL_L
ΔL
ΔL_R
Δθ

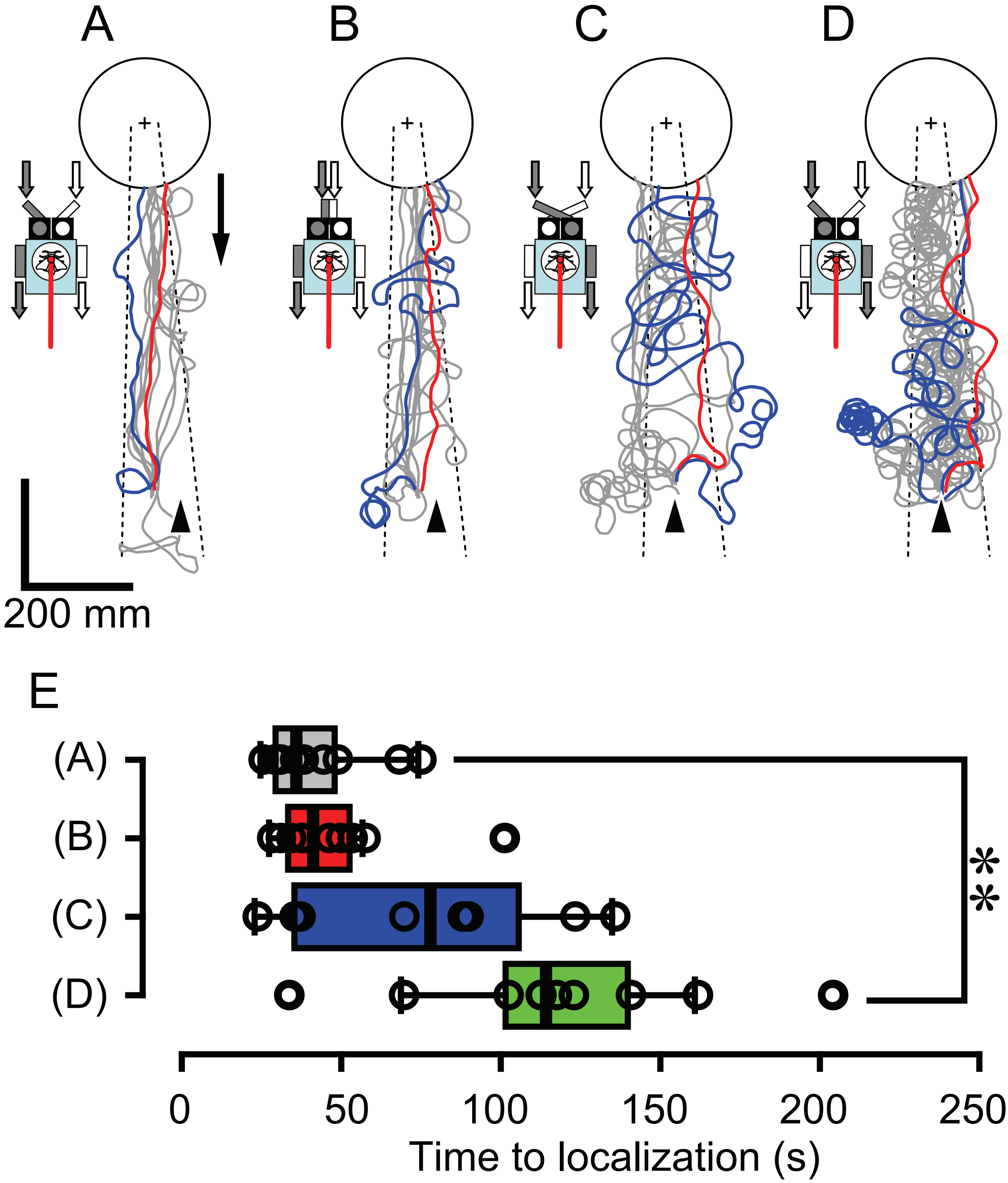

B
Forward (+)
(+)
Δy
Δx
(+)
G_FW,L
G_FW,R
G_BW,L
G_BW,R
D_ball
D_wheels


A
25 mm
B
C
D
Sensor
Cup


Camcorder
Recording area
Exhaust fan
900 mm
300 mm
1500 mm
300 mm
Mesh
Odor source


2. Tethering a silkmoth
Manipulations
6.2 Inversion of motor output
6.4 Manipulation of olfactory input
3. Insect-controlled robot
Step sections 3.3 – 3.7
6.5 Manipulation of visual input
5. Odor source
localization experiment
Step sections 5.1 – 5.7
6.1 Manipulation of motor gains
6.3 Manipulation of time delay
Step sections 5.8 – 5.14


A
B
C
200 mm


A
B
C
D
200 mm
E
(A)
(B)
(C)
(D)
**
0
50
100
150
200
250
Time to localization (s)

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Male adult silkmoth *(Bombyx mori)*			Rear from eggs, or purchase as pupae.
Incubator	Panasonic	MIR-254	Store pupae or adult silkmoths at a constant temperature, 238 L.
Plastic box	Sunplatec	O-3	Store pupae or adult silkmoths, 299 × 224 × 62 mm L × W × H.
Copper wire			2-mm diameter for the attachment. Any rigid bar can be used as an alternative for making the attachment to tether a silkmoth.
Plastic sheet	Kokuyo	VF-1420N	Sold as overhead projector film with thickness of 0.1 mm. Use at the tip of the attachment.
Forceps	As one	5SA	Remove scales on the thorax.
Adhesive	Konishi	G17	Bond a silkmoth to the attachment.
Insect-controlled robot	Custom		Bearing an air-supported treadmill, an optical sensor, custom-built AVR-based microcontroller boards, and two DC brushless motors. It is powered by 8 × AA and 3 × 006P batteries.
Microcontroller	Atmel	ATMEGA8	A component of the insect-controlled robot.
DC blower	Nidec	A34342-55	A component of the insect-controlled robot for floating a ball in an air-supported treadmill.
DC fan	Minebea	1606KL-04W-B50	A component of the insect-controlled robot for suctioning air containing an odor.
Optical mouse sensor	Agilent technologies	HDNS-2000	A component of the insect-controlled robot, obtained from an optical mouse (M-GUWSRSV, Elecom, Japan).
Brushless motor	Maxon	EC-45	A component of the insect-controlled robot for driving a wheel.
White polystyrene ball			A component of the insect-controlled robot. Diameter 50 mm, mass approximately 2 g.
Bombykol: (E,Z)-10,12-hexadecadien-1-ol	Shin-Etsu chemical		Custom synthesis.

n-hexane	Wako	085-00416	Solvent for bombykol.
Wind tunnel	Custom		Pulling-air type, sized 1800 × 900 × 300 mm L × W × H.
BioSignal program	Custom		A program to establish serial communication between the insect-controlled robot and a PC via Bluetooth. Used for sending commands to start/stop the robot or configuring its motor properties.
Camcorder	Sony	HDR-XR520V	Capture robot movements.

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Date:	3/31/2016

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•Formatting:
-Line 191: "Confirm the plume width by using TiCl4 smoke prior to the experiment" – this action occurs out of time sequence, and therefore would be best conveyed as a note. I.e. "Note: Plume width should be confirmed prior to the experiment by using TiCl4."

Response: We have modified as the editor's suggestion and put this action out of time sequence (step section 5.2).

-Please include spaces between all paragraphs.

Response: We have included spaces between all paragraphs.

•Grammar:
-2.3 - "The adhesive does not touch…" should be "The adhesive should not touch…"
-2.6 – ""Hold the tethered moth at a stand and put a piece of paper under the legs to give it a rest before getting inside the cockpit of the robot." Rewrite to "…under the legs to rest the moth, before placing it inside the cockpit of the robot."

Response: We have revised those sentences (step sections 2.4 and 2.6).

-Line 179 – "Any contaminations"

Response: We have revised "Any contamination" (step section 4).

•Additional detail is required:
-1.1-1.3 is there a way to sex silk moth pupae? Can you provide representative images? Or are all pupae just kept together until eclosion, and then separated?

Response: We discriminate males from females at pupal stage, based on the sex marks on the abdominal tip. We have added the explanations and representative images in step section 1.2 and Figure 1.

-3.2 clean the surface with what? Do you use anything in particular to clean the ball?

Response: We wash the ball with water. We have added the explanation (step section 3.3).

-3.3 is there any particular speed, power or setting for the blower fan to maintain hovering at 2mm?

Response: We have added “the fan is supplied by 9 V” in step section 3.4.

-3.5 is there an example height that seems to work well? Assuming an average sized male moth?

Response: It is difficult to show an example of appropriate height, but the important points are (1) keep the same height of the ball before and after attaching the moth, and (2) observe its walking behavior in response to the pheromone. We have described these points in step 3.6, and have added a protocol for the preparation of a pheromone stimulus cartridge in step 4.1, which is used for triggering walking behavior of the moth.

-6.1.1 – How is the file edited?

Response: The file which defines the motor gains is a text file, therefore it can be edited by a text editor. We have added this explanation in 6.1.2.

•Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In 'Insect-controlled robot: a method to evaluate odor-tracking performance of a future insect-mimetic robot' the authors describe a mobile odor-tracking apparatus that is controlled by an on-board silkmoth. The advantages of having an insect control the apparatus are that: 1) The behavior of the 'robot' when under the control of the silkmoth can be compared to behavior of a robot under self-control in order to identify successful odor-tracking strategies. 2) Sensory inputs and motor outputs of the moth can be modified to investigate what input/output mappings are being used by the moth for odor tracking.

Major Concerns:
-The paper is well written and clear, and certainly describes an interesting idea for investigating both biological and artificial odor tracking. However I'm unsure if it is suitable for publication in JOVE. The most fundamental and vital apparatus for all of the experiments described in the paper is, of course, the robot itself and the computer program used to interface with and control it. I understand that the technical details describing how to build the robot would be long and complex, but without these details it is absolutely impossible for anyone to reproduce these experiments. From my understanding, the ability to reproduce the experiments in the primary focus of JOVE.

Response: As the reviewer commented, the ability to reproduce the experiment is the primary focus of JoVE. However, it is difficult to describe throughout experimental system in particular if the system is a custom-built. In this article, therefore, we described a basic design and characteristics of the robot in the text and will focusing on showing how it works in the JoVE video format. The spherical treadmill is a well-known method for analyzing animal locomotion and related papers have also published in JoVE, therefore, the robotic part should be well described in the text. We have added explanations about the transformation from insect locomotion to motor rotations in step section 3. We have also added several important components of the robot in the table of materials and equipments

-Section 5: Odor source localization experiment
No information is provided on construction of the robot. The section contains minor details like names of interface buttons but is missing any information on how to build the robot or write the computer program to control it.

Response: We have added explanations in detail, focusing on the basic design of the robot and how to control its parameters from a computer program in step section 3.

-Step 5.7) The function of the button "param1" is not defined anywhere and is meaningless without the computer program that controls the robot.
-Step 6.1.2) The name of the button "param2" is meaningless without the computer program that controls the robot.

Response: We have added explanations describing what these buttons mean in step sections 5 and 6.

Minor Concerns:
-Step 6.4.2) This step actually inverts the motor output of the robot, not the visual input of the moth. There is a large difference between these two manipulations and they shouldn't be confused.

Response: We have corrected "to invert the motor output" (step section 6.2).

-Figure 6: The placement of the odor source should be illustrated in the figure.

Response: We have illustrated the location of the odor source in Figure 8 (figure 6 in original manuscript).

-L388: If roughening the floating polystyrene ball is important (as stated in Discussion) then it should be mentioned in the methods.

Response: We have added a note in step 3.3.

-L392: If the moth habituates to the odor then this should be mentioned in the methods.

Response: we have moved this sentence to step section 3.6.

-L121: 'which' should be deleted
-L123: '40 m' should be '40 mm'
-L333: 'allows' should be 'arrows'
-L342: do the authors mean 'left side' rather than 'lateral side' (which literally means 'side side')
-L413: 'hight' should be height

Response: We have corrected them.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
This manuscript introduces an interesting insect-controlled robot for odor tracking using silkmoth's inborn ability to move towards pheromone. The content is well organized and the experiment protocol is elaborately illustrated. The result shows that this insect-driven robot car is capable of tracking designated odor and the tracking performance is related to the direction as well as distance of the two tubes. However, there are some shortcomings that need to be further illustrated.

Major Concerns:
1. The manuscript seems to be similar to a previous work of the authors (Ando N, Emoto S, Kanzaki R. Odour-tracking capability of a silkmoth driving a mobile robot with turning bias and time delay[J]. Bioinspiration & biomimetics, 2013, 8(1): 016008.). I think it might be necessary for the authors to show clearly the differences between these two researches and the novelty of the present one.

Response: The JoVE publishes expanded descriptions of techniques that have previously appeared in results-based journal. Therefore the manuscript is based on our previous papers. The main focus of this article is to provide detailed information about our experiment using the insect-controlled robot, which should be the difference between this manuscript and previous ones. We have added detailed description about the robot in step section 3.

2. The application of this method seems quite limited now. The authors are expected to illustrate how does this method work if the odor sources are not interested by the moth but are significant for human beings, such as explosives and drugs?

Response: It is a very important point. Our colleagues succeeded to generate transgenic silkmoth which express different olfactory receptors on the pheromone receptor neurons. If we avail such transgenic silkmoths that respond to some characteristic chemicals of explosive or drugs, and use them as pilots of the insect-controlled robot, our method would be a useful robotic platform to find these chemicals. We have described this point in the last paragraph in discussion.

Minor Concerns:
3. The manuscript and pictures show that there are two separate air circuits in the robot (one to support the ball in cockpit, the other to collect odors from the outside environment). Is it necessary to isolate these two air flow, and if so, how to isolate them in the robot?

Response: The air intake for the air supplying the spherical treadmill must be separated from those for the odor delivery. Without the separation of air intakes, the pheromone contaminates into the air supplying the treadmill, which may apply non-directional odor cues to the moth. This point was omitted in the original manuscript so that we have added the explanations in step section 3.1.

.

4. In Fig. 7A, the authors show the result of tracking the moving trajectories of silkmoth, but no such technique is introduced in the manuscript.

Response: The data is from our previous study, which is cited in the figure legend. The method was a conventional method, simply tracking the center of gravity of a moth in each frame after the experiments. We therefore think that it is not necessary to describe in the manuscript.

5. In Fig. 7 and 8, the goal area is indicated by a circle. How big are the circles to determine that the robot has found the odor sources?

Response: The radius of the goal area was defined as the length between the moth and the tip of odor suction tubes. Because each trajectory indicates positions of an onboard moth, the radius is approximately equivalent to the closest distance between the onboard moth and the odor source. We added this explanation and citation in the legend of Figure 10.

Additional Comments to Authors:

6. In the test session, wind is exerted to the experiment tunnel. What if no wind is applied? Can the robot find the odor source simply by volatilization? The research would be better if some interference odors are involved to testify its odor tracking ability.

Response: We thank the reviewer for the valuable comment. We have been conducted the odor tracking experiments only in a windtunnel where the odor flow is well-controlled. As the next step, we will test in odor circumstances without wind or with turbulence, which would be an important step to investigate the insect odor tracking capability in a real environment.

Reviewer #3:

Manuscript Summary:

The authors present a method for controlling a wheeled robot with a silkmoth as a pilot walking on a rubber ball that controls the movement of the robot. The rotations of the ball are tracked using an optical sensor and this information is translated into wheel movements. They explain the steps through the experiments for building the robot and evaluating its behavior. They then compare odor tracking performance of this robot to a walking silkmoth performing the same task. This allows evaluating the behavior of the robot in response to odor detection by the moth.

Major Concerns:

The experiments are very interesting but the authors are narrowing down the possible impact of the paper by presenting it as an evaluation method for a future bio-mimetic robot the way they emphasized in the Title and the Abstract. On the other hand in the Discussion they mention the tasks fulfilled for evaluating odor tracking capability of insects, but not a bio-mimetic robot. Therefore it is not clear to me whether the main rationale is to evaluate a bio-mimetic robot or an insect. Moreover, the method presented here cannot be a direct and sole evaluation of a future bio-mimetic robot as claimed in the Title and the Abstract, as the authors are only evaluating the the motor behavior of a robot performing an odor tracking task, without considering the problems that may arise related to any possible sensory acquisition performance. This does not hinder the quality of the paper and I understand that this method is a step forward in evaluating a bio-mimetic robot, but it would be more useful either if they could present the paper in this direction mentioning in what sense this method provides an evaluation of a bio-mimetic robot, also briefly discussing what other questions should be addressed for evaluating a bio-mimetic robot (like use of chemosensors, control program etc.) not only in the Introduction but also in the Discussion, or change the Title to a more explicative one rather than discussing the future goal or a possible application field of the paper in the Title.

Response: We thank for the reviewer's suggestion. As the reviewer pointed out, the title and the former part of the original manuscript focused on biomimetics perspective, which was not consistent throughout the manuscript. We have changed the title as "Insect-Controlled Robot: A Mobile Robot Platform to Evaluate Odor-Tracking Capability of an Insect" for covering broader perspectives and have modified long abstract and introduction.

- It is not clear to me what the authors mean by "manipulation of time delay" in 6.2 (line 237). This is the delay between what, and how is it controlled? Is it about sending the signal to the robot after a movement is observed by the optical sensor? Neither the motivation for applying a time delay, nor its implications on behavior are discussed in the paper.

Response: The time delay means the response delay of the robot movement. The aim of this manipulation is to investigate the acceptable period of time spent for sensory-motor processing which would be helpful information for the biomimetic odor tracking. This manipulation was achieved by storing locomotion data into a buffer memory during the specified time delay. We have added the explanations in step section 6.3.

- It would be clearer if the positions in Fig. 5 are mentioned in quotes in the text (such as "'good" position' instead of 'good position'), or to enumerate the three positions as A,B,C and refer to the positions with these labels.

Response: We have enumerated three positions in alphabetic order in Figure 7 (originally Figure 5).

- In Fig. 7, it is not clear to which trajectory the yellow trajectories correspond to. There does not seem to be any yellow lines that correspond to the red trajectory in B for example, unless the wheel rotations (if any) of the robot allow it to move in a direction where it is not facing the odor plume. Are all the 7 trials shown with yellow traces?

Response: All yellow lines including those belonging to the red line are overlaid. Because the silkmoth often perform pinwheeling, it might be difficult to find the corresponding trajectories of the yellow lines (tube tips). We presented the yellow lines to show the difference in searching areas between a small-sized silkmoth and the large robot. However, there are so many lines and it is hard to discriminate each of them. Therefore we have deleted the yellow lines in the revised manuscript.

- How is the gap between the tubes controlled? Is the distance measured between the tips? Does the gap length have an effect on the angle between the tube entrances and the odor plume direction?

Response: The gap indicates the distance between the odor suction tubes on the left and right. Either the wide (90 mm) or narrow (20 mm) gaps can be set. It is sure that the change of the tube gap alters the angle of the tube entrance, but their effects on odor acquisition have not yet been evaluated. We checked the air flow around the tube tip with $TiCl_4$ smoke, which showed that the different tube gaps effectively alter the odor sampling areas (Ando, N. & Kanzaki, R. J. Exp. Biol. 218, 3845-3854, doi:10.1242/jeb.124834 (2015)).

Additional Comments to Authors:
There are some ambiguous sentences, such as "The concept of this robot is simple: the evaluation of the performance of insect-
mimetic odor tracking is possible if an actual insect, instead of bio-mimetic models,
drives a robot." in the Abstract. The authors should be more explicative when making such statements.

Response: This sentence has been deleted in the revision of long abstract according to the major comment.

Reviewer #4:
Manuscript Summary:
Ando et al. presents a very cool apparatus to manipulate environmental cues involved in odor tracking by insects. The authors find that a moth driven robot can track an odor plume and less so when bilateral odor cues are altered or if motor output is inverted. Overall, the study is clear and provides an interesting bio-robotic interface to ask questions about odor localization. Furthermore, the discussion describes pitfalls and limitations and puts the apparatus in the broader context of research on olfactory search behavior. I think the story is solid and should be published with minor revision.

Major Concerns:
N/A

Minor Concerns:
-I think the manuscript could add appeal and breadth by considering and lightly discussing Braitenberg vehicles and olfactory search (See Gomez-Marin et al.). Obviously the apparatus has important parallels and considerations in light of such speculation could be fruitful. Also, there is little discussion of the results in 8D. Are there any studies that report a deficit in insect odor tracking when visual input is manipulated or removed?
-Gomez-Marin A, Duistermars B, Frye M.A, Louis M. Mechanisms of odor-tracking: Multiple sensors for enhanced perception and behavior. Frontiers in Cellular Neuroscience. 2010;4(6)

Response: We thank the reviewer for the valuable comment, and the insect-controlled robot can be used for this aim. We have added the explanation and the citation in the last paragraph of discussion. Regarding the significance of visual input for odor tracking, we reported in the following papers. The former reported the optic flow input is required for compensation of turning bias, and the latter reported that the self-induced optic flow acts as reafference and is compared to putative collorary discharge signals that encodes surge directions. We did not discussed in detail about Figure 11, but have cited the reference at the end of the last paragraph in representative results.

1) Ando, N., Emoto, S. & Kanzaki, R. Odour-tracking capability of a silkmoth driving a mobile robot with turning bias and time delay. Bioinspir Biomim 8, 016008, doi:10.1088/1748-3182/8/1/016008 (2013).

2) Ando, N. & Kanzaki, R. A simple behaviour provides accuracy and flexibility in odour plume tracking – the robotic control of sensory-motor coupling in silkmoths. J. Exp. Biol. 218, 3845-3854, doi:10.1242/jeb.124834 (2015).

-Line 84: "Because of the lack of the models, it has been difficult to estimate regarding the performance of such future insect-mimetic robots." - confusing, please revise.

Response: We have revised this sentence to make clear the problem presentation as “Therefore it is still unknown how such a biological system actually works as a controller of a robotic platform.”

-Line 97: change "…of the future inset-mimetic…" to "…of the future insect-mimetic…"

Response: This word has been deleted in the revision.

-Line 140: change "The adhesive does not…" to "The adhesive should not…"

Response: We have changed.

-Line 144: what does "Hold the tethered moth at a stand…" mean?

Response: This is a step to keep the moth tethered before putting it inside the cockpit. We have revised as “Keep the moth tethered before placing it inside the cockpit of the robot. Hold the attachment at a stand and …” in step section 2.6.

-Line 155: change "Design the hardware…" to "Design of the hardware…"

Response: We have not changed because "Design the hardware…" is based on the format of protocol section in JoVE.

-Line 168: change "…the middle legs is at…" to "…the middle legs are at…"
-Line 257: change Figure 7D to Figure 8D
-Line 263: change "significances" to "significance"
-Line 293: change "…robot continued to circling…" to "…robot continued circling…"
-Line 333: change "Red allows…" to "Red arrows…"
-Line 456: Cite: Duistermars BJ, Chow DM, Frye MA. Flies require bilateral sensory input to track odor gradients in flight. Curr. Biol. 2009 Aug 11; 19 (15):1301-7

Response: We have corrected these errors and added the citation.

-Figure 8: please indicate gap width, inversion of tubes and motor output with cartoon vehicles in B-D (as in A).

Response: We have added cartoon vehicles in Figure 11B-D (originally Figure 8).

Additional Comments to Authors:
N/A