RESEARCH ARTICLE

Dynamic use of optic flow during pheromone tracking by the male silkmoth, Bombyx mori

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ABSTRACT

Several insects require both olfactory and visual cues during odour-source localisation to successfully locate an odour source. In the male silkmoth, Bombyx mori, detection of the female sex pheromone triggers a programmed walking pattern, starting from a surge (straight-line walking) followed by zigzag walking. Although pheromone-triggered behaviour in silkmoths is well understood, the role of visual cues remains obscure. To address this question, we performed behavioural experiments on tethered-walking moths by recording their locomotion during stimulation with a pheromone and a visual motion pattern (optic flow). The experiments were conducted under open- and closed-loop visual stimuli. We found that the use of optic flow input was determined by the behavioural state of surge and zigzagging. Silkmoths exhibited an optomotor response, which is a behavioural visual response, by turning towards the same direction as optic flow stimuli only during surge, but not during zigzagging. In addition, modulation of the zigzag walking pattern was observed when the moths were presented with biased closed-loop visual stimuli. We found that the use of optic flow input was determined by the behavioural state of surge and zigzagging. Silkmoths exhibited an optomotor response, which is a behavioural visual response, by turning towards the same direction as optic flow stimuli only during surge, but not during zigzagging. In addition, modulation of the zigzag walking pattern was observed when the moths were presented with biased closed-loop visual stimuli (visual feedback with biased constant optic flow); however, the directional preference mechanism was different from that of the optomotor response. Based on these findings, we suggest that the optomotor response is utilised for course control during straight-line walking, whereas the absence of optomotor response during zigzagging is used to effectively perform the programmed walking pattern. Considering the neural basis of programmed behaviour, we speculate that at least two visual pathways are involved in the state-dependent use of optic flow during odour tracking behaviour in silkmoths.

KEY WORDS: Odour-source localisation, Tethered walking, Optomotor response, Multisensory integration, Behavioural state dependent, Insect

INTRODUCTION

Animals utilise sensory information from multiple modalities simultaneously to cope with highly unpredictable environments. Many studies have revealed that multisensory integration plays an important role during insect orientation and navigation (Harley et al., 2009; Giurfa, 2007; Stewart et al., 2010; Webb et al., 2004).

Studies in the fruit fly and the flying moth have demonstrated that olfactory information as well as visual information play a crucial role during odour searching by providing information about self-motion in space via visual feedback (Frye et al., 2003; Willis et al., 2011). In addition, attractive odour can modulate visual control of flight in the fruit fly Drosophila melanogaster, resulting in a straighter flight towards an odour source (Chow and Frye, 2008; Chow et al., 2011). These studies exemplify cross-modal interactions between olfaction and vision during odour source localisation.

Male moths possess an astonishing ability to track a sex pheromone plume that is highly intermittent (Willis and Baker, 1984) and successfully locate its source over long distances (Collins and Potts, 1932; Wall and Perry, 1987). The male silkmoth Bombyx mori (Linnaeus 1758) exhibits pheromone-triggered walking behaviour that is divided into three sequential phases: surge, zigzagging and the loop (Fig. 1A). The surge is elicited shortly after pheromone detection, during which the moth turns towards the direction of the higher pheromone concentration perceived by the left and right antennae (Takasaki et al., 2012). If the moth loses the pheromone cue, it initiates zigzag walking or consecutive turning from side to side. After several zigzag turns, the moth finally performs a loop by making a larger turn of >360°, resulting in rotation around its body centre. When the silkmoth detects an additional pheromone cue, this behavioural sequence restarts from the beginning (Kanzaki, 1996; Kanzaki, 1998; Kanzaki et al., 1992).

Previous neurophysiological studies on this pheromone-triggered behaviour have demonstrated that two neural circuits control behaviour during surge and zigzagging and that the loop is presumably a part of zigzagging and controlled by the same neural circuit (Kanzaki, 1996; Kanzaki, 2004; Wada and Kanzaki, 2005). Hence, from now on, we will discuss behaviour during surge and zigzagging only. Moreover, studies on the visual response of silkmoths have suggested that some descending interneurons in the ventral nerve cord show responses to moving visual stimuli (optic flow) (Olberg, 1983) and that the silkmoth uses visual information to compensate for unintentional turning (Ando et al., 2013; Minegishi et al., 2012). Although the behavioural and neurophysiological basis of the pheromone-plume tracking of the male silkmoth has been well studied (Kanzaki, 1996; Kanzaki, 2004; Wada and Kanzaki, 2005), how the silkmoth uses visual information for pheromone-plume tracking during different behavioural states (surge and zigzagging) remains unclear.

Considering the ethological implications of surge and zigzagging during pheromone-plume tracking by the silkmoth, we hypothesised that surge serves as a pheromone-source orientation mechanism by redirecting the moth towards a higher pheromone concentration, while zigzagging provides a strategy for scanning further pheromone cues in a dispersed and intermittent plume. The plausible behavioural response to a moving visual stimulus during surge is the optomotor response, steering toward the same direction as visual motion of the surroundings, resulting in compensation for involuntary deviation from the intended course (Rock and Smith, 1986; Wolf and Heisenberg, 1990). However, if the optomotor response persists through zigzag walking, it will suppress voluntary turning by the silkmoth using visual inputs for course compensation and straighter walking and, consequently, will reduce the probability of further
pheromone encounters. Therefore, we hypothesised that the role of vision during pheromone-triggered behaviour in the male silkmoth is dependent on the behavioural states of surge and zigzagging.

In flies, neural and behavioural responses to optic flow are altered with the behavioural state. In the blowfly *Calliphora vicina*, visual responses of neck motor neurons and head movements are enhanced by walking or flying (Haag et al., 2010; Huston and Krapp, 2009; Rosner et al., 2009). In *D. melanogaster*, optomotor response is enhanced during walking and flying when compared with the response during resting (Chiappe et al., 2010; Maimon et al., 2010). In addition, in the blowfly *Lucilia* spp., velocity tuning of motion-sensitive neurons is broadened towards higher velocities by flight activity (Jung et al., 2011). These observations suggest that the behavioural response and the visual response of neck motor neurons to optic flow stimuli are altered with locomotion states. In contrast to the behavioural states with drastic differences such as resting and moving in previous studies, we focused on different behavioural states comprising the sequence of pheromone tracking behaviour (surge and zigzagging) in the silkmoth, and investigated whether and how optic flow was used during each state.

We conducted behavioural experiments on tethered walking silkmoths by monitoring their behavioural responses to pheromone and optic flow stimuli during surge and zigzagging under both open- and biased closed-loop visual conditions (visual feedback with biased constant optic flow; Fig. 2). We found that optic flow utilization during pheromone-triggered behaviour was dependent on...
behavioural state: the optomotor response was induced during surge but not during zigzagging. Furthermore, the behavioural response to a biased closed-loop visual stimulus during zigzagging had a directional preference that was opposite to the optomotor response. We also discuss the ethological implications of this dynamic use of optic flow during pheromone tracking and speculate on the possible pathways for the visual and olfactory integration based on the identified olfactory-related neural mechanisms in the silkmoth brain.

RESULTS

Optic flow input elicits the optomotor response during surge

We recorded steering of tethered moths walking on an air-floated ball using an optical mouse (Fig. 1B). The moths were presented with either open-loop or biased closed-loop visual stimuli (Fig. 2B). Each moth performed one control trial and one experimental trial. In the latter case, the temporal frequencies of constant optic flow (for the open-loop experiment) or biased optic flow (for the biased closed-loop experiment) varied among moths. Continuous-pulsed pheromone was delivered to moth antennae to initiate walking and elicit repetitive surge (Fig. 2A).

Angular velocity and surge duration of the moths in the different control groups, which were presented with different optic flow velocities in the experimental trial, were not significantly different (P>0.1, Kruskal–Wallis test). Therefore, we pooled the results of all control trials together as a response to optic flow with a temporal frequency of 0 Hz (Fig. 3A,C). Note that all control trials were presented with the same visual stimulus, which was either a stationary grating pattern (for the open-loop experiment) or a closed-loop optic flow (for the biased closed-loop experiment) (Fig. 2B).

The result from the open-loop experiment shows that angular velocity changed significantly with temporal frequencies (P<0.001, Kruskal–Wallis test). The temporal-frequency tuning curve is shown in Fig. 3A. The tuning curve reached its maximum at ~2 Hz. This result shows that the velocity of optic flow stimuli modulated angular velocity during surge and the silkmoths turned in the same direction as constant optic flow, suggesting that they exhibited an optomotor response. However, we found no modulation of surge duration by optic flow stimuli (P>0.1, Kruskal–Wallis test; Fig. 3B).

Biased closed-loop optic flow also modulated moth behaviour in the same manner as the open-loop optic flow. Angular velocities of moths during the surge changed with temporal frequency of biased optic flow (P<0.001, Kruskal–Wallis test; Fig. 3C), whereas surge duration remained unchanged (P>0.05, Kruskal–Wallis test; Fig. 3D). Note that x-axes in Fig. 3A,B represent temporal frequency of constant optic flow in the open-loop condition, whereas those in Fig. 3C,D represent temporal frequency of biased optic flow in the biased closed-loop condition, as a representative value for intensity of visual stimulus. Because of the difference in stimulus conditions, we did not compare these two results quantitatively. Nevertheless, both tendencies agree that the silkmoth exhibited an optomotor response during the surge.

No behavioural modulation is elicited by open-loop optic flow stimuli during zigzagging

We recorded the steering response of silkmoths to the same open-loop visual stimuli as in the above experiment, but a single-pulsed pheromone was used instead to trigger one cycle of pheromone-triggered behaviour, starting from surge and followed by zigzagging (Fig. 2A). We evaluated the effect of optic flow stimuli on the behavioural response during zigzagging by considering the direction of constant optic flow in reference to turn direction. Results from experimental trials with optic flow stimuli moving to the left and to the right at the same temporal frequency were merged together and then divided into two groups by considering the direction of the first turn. As a result, the first group had the first and third turns in the same direction as that of constant optic flow, whereas the second
The turn angles of the first four zigzag turns were measured from different control groups showed no significant differences (P>0.05, Kruskal–Wallis test; Fig. 4B,C), whereas those in experimental trials showed lower correlation coefficients (r<0.51; Table 1). In summary, our results indicate that when silkmoths were presented with biased closed-loop optic flow as in the experimental trials, the rank of the turn angle by size increased with the order of turns (r=0.50, P<0.001, Kendall’s tau-b test), whereas those in experimental trials showed lower correlation coefficients (r<0.51; Table 1).

In this experiment, we delivered biased closed-loop visual stimuli and single-pulsed pheromone (Fig. 2), and then recorded moth locomotion in the same manner as in earlier experiments. Similarly, the results were regrouped considering direction of optic flow in reference to direction of the first zigzag turn. In this experiment, 72 samples had their first turn in the same direction as biased optic flow and 51 samples had their first turn in the opposite direction. The binomial test suggested that turn direction for the first turn was not modulated by direction of biased optic flow (P>0.5). The turn angle of each zigzag turn measured from different control groups showed no significant differences (P>0.05, Kruskal–Wallis test); therefore, we pooled the results from all control groups (Fig. 5A). Note that all control trials were similarly presented with feedback optic flow alone.

The size of the turn angle of each turn was not significantly changed when we varied the temporal frequency of the moving pattern (P>0.05, Kruskal–Wallis test; Fig. 5B,C). However, the tendency to change the size of the turn angle during sequential turns in the experimental trial was slightly different from that in the control trial. Specifically, in the control trial the turn angle tended to increase turn by turn, whereas in the experimental trials the turn angle was larger when the moth turned in the direction opposite to that of biased optic flow. To focus on the walking pattern, we further investigated this issue by ranking each zigzag turn of each individual by size of the turn angle. In the control trials, the rank of the turn angle by size increased with the order of turns (r=0.50, P<0.001, Kendall’s tau-b test), whereas those in experimental trials showed lower correlation coefficients (r<0.51; Table 1).

In summary, our results indicate that when silkmoths were presented with biased closed-loop optic flow as in the experimental trials, the rank of the turn angle by size increased with the order of turns (r=0.50, P<0.001, Kendall’s tau-b test), whereas those in experimental trials showed lower correlation coefficients (r<0.51; Table 1).
trials, the size of the turn angle was less correlated with the order of the turn than that in control trials when they were presented with optic flow feedback alone. This suggests that modulation of the zigzagging pattern by the introduced biased optic flow was apparent and that the turn angle increased when the silkmoth turned to the direction opposite to biased optic flow. However, change in the biased optic flow velocity did not significantly modulate the behavioural response in terms of the size of the turn angle of each zigzag turn.

**Relationship between turn angle, turn duration and angular velocity during zigzagging**

The turn angle is a product of turn duration and angular velocity; therefore, we investigated which parameter was more responsible for turn angle. The relationships between these three parameters of turning (turn angle, duration and angular velocity) during four zigzag turns are illustrated in Fig. 6. All data in these plots were drawn from all four turns measured during the experiments with single-pulsed pheromone and open- or biased closed-loop visual stimuli. The solid lines were fitted by linear regression. All regressions were significant (P<0.001). However, the fitted relationship between turn angle and duration in both the open- and biased closed-loop visual conditions yielded higher R^2^ values than those from the fitted relationship between the turn angle and angular velocity. These results indicate that the turn angle was more linearly dependent on turn duration than angular velocity under both open- and biased closed-loop visual stimuli.

**DISCUSSION**

In this study, we investigated how optic flow input modulated the pheromone-triggered behavioural response in male silkmoths by observing the behavioural response to combinations of pheromone and optic flow stimuli under both open- and biased closed-loop conditions. In the following discussion, we discuss the behavioural state dependent use of optic flow observed here, speculate on its ethological implications and propose possible neural pathways of the visual information based on the identified olfactory pathways of the silkmoth.

**The role of vision during pheromone searching behaviour**

Our results suggest that the silkmoths exhibited an optomotor response during surge but not zigzagging. During the surge, optic flow information from the surrounding environment modulated moth behaviour by eliciting an optomotor response such that the moth turned to the same direction as optic flow stimuli and changed its angular velocity of turning with optic flow velocity. This optomotor response was observed under both the open- and biased closed-loop visual stimuli. The duration of the surge was not modulated by the presence of optic flow stimuli. In contrast, no modulation of behaviour was found during zigzagging in the experiment with single-pulsed pheromone and open-loop optic flow. Although the behavioural response under biased closed-loop visual stimuli suggested modulation of the zigzagging pattern induced by biased optic flow, its function and mechanism contrasted with the optomotor response. Specifically, during zigzagging, moths tended to make a larger turn when turning towards the direction opposite to biased optic flow, whereas they turned to the same direction as constant/biased optic flow during the surge. Hence, we conclude that optic flow utilization during the pheromone-triggered behavioural response in male silkmoths is behavioural-state dependent.

It is arguable whether changes in the visual field by head turning are responsible for the absence of the optomotor response in the experiments with single-pulsed pheromone under both open- and biased closed-loop visual stimuli conditions. Nevertheless, results from our preliminary experiment on head-fixed moths using single-pulsed pheromone and open-loop visual stimuli agreed with the results presented earlier in this study (supplementary material Fig. S1), and, consequently, we ruled out this assumption. Moreover, we decided to conduct an experiment on normal moths instead to maintain natural-like locomotion and avoid defects from the head-fixed method, e.g. misalignment between the head and body or any damage to the moths that might occur during fixation.

**Ethological implications of odour-search strategies**

Localisation of an odour source by male moths has been widely investigated over many decades. Flying male moths commonly...
utilise a combination of upward surge and self-steering countercurrenting for sex-pheromone plume tracking (Baker, 1990; Vickers and Baker, 1996; Willis and Baker, 1984). Moths show a reiterative upward surge when tracking a pheromone plume with high-frequency dispersion, which results in straighter flight (Mafra-Neto and Cardé, 1994; Vickers and Baker, 1996). Its function is to reorient towards the pheromone source (Murlis, 1992). However, loss of the pheromone plume triggers intermediate countercurrenting during upward flight, which frequently occurs when manoeuvring through a meandering plume (Mafra-Neto and Cardé, 1994; Murlis, 1992; Vickers and Baker, 1996; Willis and Baker, 1994). This countercurrenting, which includes both zigzagging and casting, is a self-steering mechanism and its function is to regain contact with the wind-borne odour (Murlis, 1992; Kennedy, 1983). Flying moths as well as walking moths, including silkmoths, show a similar odour tracking behaviour (Kanzaki et al., 1992). The ethological implication for this behaviour is presumably similar to that of the flying moth. Moreover, this zigzag-like odour tracking strategy has also been observed in fish and birds (DeBose and Nevitt, 2008).

Our results suggest that silkmoths performed the optomotor response during surge, a reflexive behaviour, which allows course compensation for unintentional turning through the use of visual feedback, and has been observed mostly in insects and fish (Borst and Bahde, 1987; Borst et al., 2010; Lönnendonker and Scharstein, 1991; Rock and Smith, 1986). The ability for locomotory compensation in the silkworm has also been reported in earlier studies on insect–machine hybrid systems (Ando et al., 2013; Minegishi et al., 2012). However, we did not observe any modulation of behaviour during zigzagging by constant optic flow stimuli (Fig. 4). Because of differences in functions and mechanism between the surge and zigzagging, we speculate that the optomotor response was only evoked during surge and not zigzagging because a course correction was not absolutely necessary to perform a local search during zigzagging, unlike goal-oriented straight walking during surge. In contrast, the optomotor response during zigzagging would interfere with internally controlled voluntary locomotion by suppressing turning in an attempt to induce straighter walking. Our results only demonstrate that the optomotor response was valid during the surge; nevertheless, an interaction between positive chemotaxis and the optomotor response could also have occurred. A similar problem was discussed in a study on crickets (Webb et al., 2004), in which the inhibitory interaction between two modalities was proposed. Further behavioural analyses with fine temporal control of odour stimuli will be needed to reveal the detailed mechanisms during surge.

**Behavioural state dependency of visual feedback during the behavioural sequence**

Several studies have demonstrated the necessity for visual cues while localising an odour source (Frye et al., 2003; Willis et al., 2011) and have suggested an interaction between visual and olfactory pathways (Stewart et al., 2010). Our results show that silkmoths used visual feedback for course compensation during surge behaviour, which supports the idea that visual information is required during pheromone-source localisation. Furthermore, studies in flies have also demonstrated the effects of locomotor activity on the visual response (Maimon et al., 2010; Rosner et al., 2010) and enhancement of optomotor flight control by olfactory input (Chow and Frye, 2008; Chow et al., 2011). These studies suggest evidence for modulation of the visual response by locomotion state (e.g.
resting, walking and flying) and by behavioural state (e.g. odour source localisation and collision avoidance). In this study, we investigated how the use of visual input is modulated by the sequential states of pheromone-triggered behavioural response of the male silkmoth, which consists of surge and zigzagging. These two states involve the same locomotor activity (walking) and behavioural state (odour tracking). Our novel finding is that the visual response not only depends on locomotion state and behavioural state but is also altered by the sequential state of behaviour (surge and zigzagging).

Differences between responses to open-loop and biased closed-loop visual stimulation

Modulation of the zigzag walking pattern was only observed in experiments with biased closed-loop visual stimuli (Fig. 5). The biased closed-loop optic flow during zigzagging consisted of repeating periods of (1) fast changes in optic flow velocity at the onset of each turn (onset optic flow) followed by (2) relatively constant optic flow during turn (steady-state optic flow), whereas the open-loop visual stimulus only contained steady-state optic flow. Furthermore, in the biased closed-loop experiment, feedback optic flow was accelerated by biased optic flow velocity when silkmoths turn to the direction opposite to the biased optic flow, or decelerated when they turned to the same direction as the biased optic flow (supplementary material Fig. S2).

Results from the experiment with biased closed-loop visual stimuli during zigzagging suggest that silkmoths made large turns during turning to the direction opposite to the biased optic flow in which flow velocity was accelerated (Fig. 5, shaded areas), whereas they made small turns during turning to the same direction as the biased optic flow in which the flow velocity was decelerated. Focusing on the steady-state optic flow, the difference of optic flow velocity (i.e. temporal frequency) during each turn was presumably responsible for modulation of the zigzagging pattern. However, optic flow velocity and the strength of visual stimulus perceived by silkmoths were not linearly correlated. Assuming that the magnitude of turn angular velocity in the temporal frequency turning curve (Fig. 3A) represents the strength of visual stimuli, temporal frequency ranging from ±1.5 to ±2.5 Hz is considered the maximum visual stimulus intensity. Our analysis shows that temporal frequencies of accelerated or decelerated optic flow during the biased closed-loop experiment were mostly out of this range (supplementary material Fig. S3). Therefore, although the difference in optic flow velocity during each turn represents a difference of stimulus strength to some degree, there is a possibility that there was not much difference in stimulus strength perceived by the moths between accelerated and decelerated optic flow. In addition, the behavioural response to open-loop visual stimuli during zigzagging indicated no significant modulation of the zigzagging pattern and no significant difference in the size of the turn angle between moths that were presented with constant optic flow stimuli with different temporal frequencies including 1.67 Hz (Fig. 4), which is in the range of maximum visual stimulus intensity. These results suggest that modulation of the zigzagging pattern was not elicited solely by a difference in velocity of the steady-state optic flow.

The other plausible factor is the onset optic flow. In the experiment with biased closed-loop visual stimuli, the silkmoths experienced two different types of fast changes in optic flow velocity: from slow to fast at the onset of turns to the direction opposite to the biased optic flow, and from fast to slow at the onset of turns in the same direction as the biased flow (supplementary material Fig. S2). Although we do not have any evidence that the onset of optic flow elicits particular neuronal responses in the visual system of silkmoths, studies on the motion-sensitive interneurons in blowflies show that activity responses rise sharply during the initial transient state after the onset of optic flow stimuli, and then decrease and stabilise to smaller steady-state values (Jung et al., 2011; Kalb et al., 2008; Suver et al., 2012). Therefore, we speculate that this kind of transient visual response occurred at the onset of each turn, and might be one of the potential factors that are responsible for the modulation of zigzagging observed only in our biased closed-loop experiment (Fig. 7).

Although the ethological implication of this mechanism is still unknown, our results suggest that multiple neural pathways are involved in olfactory and visual integration and that the internal programmed behaviour underlying zigzagging could be modulated by visual-motion information.

Speculation of the neural mechanisms and pathways underlying dynamic use of optic flow during the pheromone-triggered behavioural response

Earlier studies on the neural mechanism underlying pheromone-triggered behavioural responses have demonstrated that surge and zigzagging are controlled by two different neural circuits (Kanzaki, 2004; Wada and Kanzaki, 2005). Neural activity during surge shows a phasic response in a descending motor pathway, whereas zigzagging shows so-called ‘flip-flop’ activity, which is a long-lasting tonic response generated by the premotor centre of the silkmoth’s brain (Iwano et al., 2010; Wada and Kanzaki, 2005).

The existence of two different neural pathways underlying the different behavioural states of surge and zigzagging supports our finding on behavioural-state-dependent use of optic flow. A previous
study demonstrated that when presented with pulsed pheromone alone, the silkmoth determines its surge direction by chemotaxis, i.e. turning towards the direction of higher pheromone concentration (Takasaki et al., 2012). The optomotor response observed in our experiments is also involved in directional control during surge; therefore, it was convincing that there was an interaction between visual and surge-related olfactory pathways. In contrast, we did not find the optomotor response to constant optic flow alone during zigzagging behaviour (Fig. 4). One possible explanation is that the visual response is suppressed by efference copy or corollary discharge during zigzagging (Zaretsky and Rowell, 1979). These mechanisms are related with the internal copy of the motor command for distinguishing self-generated sensory feedback and externally generated sensory information. This has been demonstrated in several animals and in visual and non-visual sensory pathways (Murphey and Palka, 1974; Poulet and Hedwig, 2007; von Holst and Mittelstaedt, 1971). However, visual modulation observed during zigzagging in the experiment with single-pulsed pheromone and biased closed-loop visual stimuli (Fig. 5) suggested an mechanism inconsistent with that occurring during surge. During surge, optic flow stimuli modulated turning angular velocity in correspondence with optic flow velocity, whereas the turn duration was modulated during zigzagging, and this modulation was possibly elicited by the onset of optic flow stimuli. Note that the linear relationship between turn angle and turn duration (Fig. 6) suggests that turn angle of each zigzag turn was determined by turn duration rather than angular velocity. We speculate that the absence of an optomotor response during zigzagging is due to different interactions between the visual-motion and olfactory processing pathways for surge and zigzagging. This idea is supported by evidence that separate neural circuits are responsible for controlling behaviour during these two behavioural states (Kanzaki, 2004; Wada and Kanzaki, 2005).

Although the speculation about the neural mechanism underlying dynamic use of optic flow is purely based on our results from behavioural studies, the idea of visual response gating by the sequential states of pheromone-triggered behaviour is promising. This finding could lead to further understanding of the neural mechanisms underlying this behaviour and guide our future studies at the physiological level.

MATERIALS AND METHODS
Preparation and setup
Male silkmoths, B. mori (Lepidoptera: Bombycidae), were either purchased as pupae from Aseptic Sericulture System Laboratory (Kyoto, Japan) or were reared from eggs to adults in our laboratory on artificial diets at 25–27°C and 50–60% relative humidity under a 16:8 h light:dark photoperiod. Adult male silkmoths were used in the experiment within 2–6 days of eclosion. The scales on the dorsal thorax of the moth were removed and a piece of paper (5×10 mm) was attached to the thorax with adhesive glue (G17, Konishi, Osaka, Japan). This piece of paper was used as a holder to position the silkmoth on top of a 75-mm-diameter polystyrene ball. The ball was supported by a plastic funnel with an electric fan (MBDC12B4, Nidec Servo Corp., Kiryu, Japan) attached at the bottom for air flow to keep the ball floating in the air to reduce its friction when rotated by the moth (Fig. 1B). Locomotion of the moth was recorded with an 800 dpi optical mouse (M-BG2UR, Elecom, Osaka, Japan) positioned behind the polystyrene ball. Silkmoths were allowed to move their head freely during locomotion. The mean (±s.d.) head turning angle was 32.4±3.1 deg from the middle of the screen.

Locomotion tracking
The locomotion of silkmoths was detected with an optical mouse (Fig. 1B). We acquired the output of the optical mouse every 8 ms, which consisted of vertical and horizontal displacement detected during each sampling interval. The turn angle moths performed during each sampling interval (δn) as follows:

\[ \delta_n = \frac{x_n}{R}, \]  

where \( x_n \) is horizontal displacement detected by the optical mouse during the \((n–1)\)th and \(n\)th samples, which is equivalent to the displacement of the surface of the polystyrene ball rotating along the azimuth axis, and \( R \) is the radius of the polystyrene ball. The accumulated turn angle \( \Sigma \delta_n \) represents the turn angle of the silkmoth in reference to the starting position at the time \( n \times \) sampling interval.

Pheromone delivery
Synthetic bombykol, \((E,Z)\)-10,12-hexadecadien-1-ol, the principal pheromone component of \( B. \ mori \), was delivered from stimulant cartridges (Pasteur pipette, 1 mm tip diameter, tip bent using a pocket torch lighter) to both antennae of the moth simultaneously. The tips of the cartridges were positioned ~5 mm in front of both antennae. Each cartridge contained a piece of filter paper bearing 100 ng of synthetic bombykol. Airflow generated by a compressor (NUP-1, As One, Osaka, Japan) was filtered through the cotton, charcoal and water units and then adjusted to 11 min\(^{-1}\) by flow metres (RK1600R, Kohfloc, Kyoto, Japan) before being delivered to the stimulant cartridges. Solenoid valves were used to control the duration and interval of stimuli and were controlled by an ARM7 32-bit microcontroller education board (JX-2148, Inex, Bangkok, Thailand), which allowed manipulation of pheromone delivery via a computer (Fig. 1C).

Two different types of pheromone stimulation were used: a single-pulsed stimulus (1.0 s in duration) and continuous-pulsed stimulus (a train of 20 pulses, duration of each pulse was 0.25 s with an inter-pulse interval of 0.25 s) (Fig. 2A).

Visual stimulation
Visual stimulation was built on a Codeblocks IDE for C++ platform (see http://www.codeblocks.org) on a Linux kernel with Xenomai patches for real-time control (see http://www.xenomai.org). A horizontally moving vertical back-and-white grating pattern was rendered at a frame rate of 60 Hz and was projected onto a 300-mm diameter cylindrical screen by a projector (EB-X6, Epson, Tokyo, Japan). The Michelson contrast of the pattern was >87%, and mean luminance was ~1200 cd m\(^{-2}\) across the screen. The projected pattern covered 420 mm in width and 245 mm in height of screen area (~160 and 40 deg of the horizontal and vertical visual field, respectively). The horizontal distortion due to cylindrical screen’s curvature was compensated during grating pattern rendering using the following formula:

\[ x' = \frac{L}{\pi} \left( 1 - \frac{\cos(\theta) \cos(\theta + \phi)}{1 - \sin(\theta) \sin(\theta + \phi)} \right), \]

\[ \phi = \frac{x(\pi - 2\theta)}{L}, \]

where \( x \) and \( x' \) are pixel positions of the image in the horizontal axis before and after distortion correction, respectively; \( L \) is the width of the image in pixels; and \( \theta \) is a projection angle in radians, which was calculated as follows:

\[ \theta = \tan^{-1} \left( \frac{W/D}{2D} \right), \]

where \( W/D \) is the throw ratio of the projector, \( W \) is the width of a projected image onto the flat surface and \( D \) is the distance of the projector from the screen. Spatial wavelengths of the grating pattern were chosen from 31, 52 and 62 mm to adjust the temporal frequency of optic flow stimuli. Two types of visual stimulation, open- and biased closed-loop, were used to investigate the significance of closed-loop visual feedback that occurs during natural locomotion. For the biased closed-loop visual stimulus, locomotion of the moth detected with the optical mouse was used to create feedback optic flow (Fig. 2B). Only rotation about the yaw axis during the previous rendering cycle was considered. The gain of feedback optic flow was equal to one, and the spatial wavelength of grating pattern was fixed at 62 mm.
Experimental procedures
Neurophysiological studies suggest that the generation of the pheromone-triggered walking pattern is controlled by two different identified neural circuits, activities of which correspond to surge and zigzagging (Kanzaki, 2004). Therefore, we focused on the behavioural response during these two states.

Experiment 1: behaviour during surge
To investigate the behavioural response during surge, we delivered continuous-pulsed pheromone stimulus to activate consecutive surges (Fig. 2A). Stimulation with a 0.5 Hz pheromone stimulus or higher frequency results in a reset of the walking pattern before the moth generates a zigzag turn (Kanzaki et al., 1992). This allowed observation of surge behaviour for a long period. Experiment 1 is further separated into two experiments: (1) the experiment with open-loop visual stimulus and (2) the experiment with biased closed-loop visual stimulus. The optic flow stimulus was started 1 s before onset of the pheromone stimulus to avoid the effect from visual stimulus onset, and persisted throughout the recording period for 10 s. We recorded locomotion of moths in response to optic flow stimuli with different temporal frequencies defined as the number of cycles of black-and-white grating pattern per second measured at any specific position on the screen. In the experiment with open-loop visual stimulus, the temporal frequency of the moving pattern was 3.33 to 3.33 Hz (signs indicate optic flow direction, minus for right and plus for left). In the experiment with biased closed-loop visual stimulus, the temporal frequency of biased optic flow was limited to ~2.50 to 2.50 Hz to prevent the exact optic flow’s velocity, which was composed of feedback and biased components, from exceeding the limitation of our visual-stimuli rendering system and to retain a smooth-moving grating pattern throughout the experiments.

Experiment 2: behaviour during zigzagging
To investigate the behavioural response during zigzagging, we delivered a single-pulsed pheromone stimulus. We only focused on the behavioural response during the first four turns because zigzag walking in silkmoths has a tendency to show a gradual increase in the turn angle during these turns (Kanzaki et al., 1992; Kanzaki, 1998), which is a useful index of normal moth behaviour. Similarly, Experiment 2 was further separated into two experiments: (1) the experiment with open-loop visual stimulus and (2) the experiment with biased closed-loop visual stimulus. According to a previous study and our observations, head turning and the neck motor response to the optic flow stimulus requires several seconds to reach a maximum response (Minegishi et al., 2012). Therefore, we started presenting optic flow stimuli 5 s before the onset of the pheromone pulse to ensure that visual stimuli had already affected the behavioural response before locomotion started. Optic flow stimuli persisted throughout the recording period, which lasted for 40 s. We recorded locomotion of the moth generated in response to both open- and biased closed-loop visual stimulation. Visual stimulus conditions in these experiments were the same as those in Experiment 1.

In each experiment, we collected data from one control trial and one experimental trial in each moth for statistical comparison of the behavioural responses under different stimulus conditions. Each moth was used in only one or two experiments (Experiment 1 and/or 2 with the same visual stimuli condition) with an approximate 3-h rest interval between them to allow recovery from pheromone habitation. In the latter case, experiments with single-pulsed pheromone stimulation were always performed prior to the continuous-pulsed pheromone stimulation to avoid reduction of physical activity in response to pheromone after pre-exposed to continuous-pulsed pheromone (Kuenen and Baker, 1981). The silkmoth does not initiate walking in response to visual stimuli alone; therefore, we did not conduct any experiments without the pheromone stimulus.

Data analysis
In Experiment 1, we measured the accumulated turn angle of each moth for 3 s, recorded 6 s after the onset of the continuous-pulsed pheromone stimulus (Fig. 2A). This allowed walking speed to reach a steady-state (supplementary material Fig. S4). Then, we calculated angular velocity by dividing the cumulative turn angle by 3 s. The duration of the surge was drawn from the results of Experiment 2.

In Experiment 2, we divided the locomotion of the moth triggered by the single-pulsed pheromone stimulus into surge and successive zigzag turns based on an empirical criterion (an example of moth performance during one trial is shown in supplementary material Fig. S5). The initiation of zigzagging was recognised when the moth generated a turn with an angular velocity >15 deg s⁻¹ lasting longer than 0.2 s with an accumulated turn angle of >5 deg. The first constraint was defined as the threshold for separating the surge from zigzag, and the last two constraints were defined as thresholds for eliminating noise from small oscillations caused by stepping of the moth. During zigzagging, each turn was separated when the moth changed its turn direction, with duration and turn angle larger than the same thresholds for noise elimination as described earlier. After separation, we measured the total accumulated turn angle and turn duration during each turn and then calculated angular velocity during each turn by dividing accumulative turn angle by turn duration.

Statistical analysis
We used the Mann–Whitney–Wilcoxon test to compare turning parameters between the two independent groups and the Kruskal–Wallis rank-sum test for multiple comparisons. Directional preference of the first zigzag turn was examined by the exact binomial test.

Because the silkmoth has a tendency to increase turn angle gradually during the first three or four turns of zigzag walking (Kanzaki et al., 1992; Kanzaki, 1998), we examined the correlation between order of turns and rank of turn by size of the turn angle to investigate the effects of visual stimuli on turning characteristics. The size of the turn angles from the first to fourth zigzag turns in each moth were ranked in increasing order (smallest, small, large and largest), and then we computed the correlation coefficient (r) between the order of turn and their ranking and conducted a significance test for Kendall’s tau-b. The relationship between the turn angle, turn duration and angular velocity during zigzagging was analysed by simple linear regression analysis. All statistical analyses were performed using the R software environment for statistical computing and graphics (see http://www.r-project.org).

Acknowledgements
We are grateful to Prof. Dr Shigeru Matsuyma (Graduate School of Life and Environmental Sciences, University of Tsukuba) for providing the purified bombykol.

Competing interests
The authors declare no competing financial interests.

Author contributions
P.P., N.A. and R.K. conceived and designed the experiments. P.P. developed the experimental apparatus, performed the behavioural experiments and analysed the data. P.P. and N.A. drafted and R.K. revised the manuscript.

Funding
This study was supported by the Japan Society for the Promotion of Science [22700197 to N.A., 24370031 to R.K.].

Supplementary material
Supplementary material available online at http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.090266/-/DC1

References


Fig. S1. Results from the experiment with a head-fixed moth. (A) Head-fixed preparation. The head and prothorax of the silkmoth were glued together to fix head movement. The same open-loop experimental procedure for normal moths was conducted on head-fixed moths. (B) The behavioural response during the surge of a head-fixed moth to constant optic-flow. Similar to a normal moth, the head-fixed moth elicited an optomotor response during the surge. (C) The behavioural response of the head-fixed moth during zigzag in response to 1.67 Hz constant optic-flow after being stimulated with a single-pulsed pheromone. Results from both experimental groups (C, middle and right) showed a consecutive increase in turn angle, which was similar to the response of the control group. Although the results in the experimental group whose first turn was to the direction opposite to constant optic flow (1st-Opposite) showed a slightly decreasing turn angle in the fourth turn, it did not explicitly imply that the behavioural response was different from normal moths or the control group, as variations in fourth turns are normally large. Furthermore, we did not find any modulation in the experimental group whose first turn was to the same direction as constant optic flow (1st-Same). Therefore, we assumed that there was no visual modulation during zigzag in the head-fixed moth and that the behavioural response was considerably similar to that of a normal moth. In A and B, dots represent median of samples, lower and upper limits on error bars represent 25th and 75th percentiles, respectively. Kendall’s tau-b correlation coefficient between the order of the turn and the rank of the turn angle by size in the control, 1st-Same and 1st-Opposite groups were 0.81***, 0.40*** and 0.35**, respectively (**P < 0.01; ***P < 0.001), suggesting that the size of the turn angle significantly increased with the order of the turn in both the control and experimental trials, although the correlation coefficients measured in the experimental trials were lower than that measured in the experiment with normal moths (Table 1).
Fig. S2. Schematic plots for optic flow velocity during experiments with open- and closed-loop visual stimuli. (A) Open-loop visual stimulus for control trials (cont.). A stationary grating pattern was presented (red line, optic-flow velocity is zero). A positive value represents turning to the left, and a negative value represents turning to the right. (B) Open-loop visual stimuli for experimental trials (exp.). Optic flow with a constant temporal frequency was presented. (C) Closed-loop visual stimuli for control trials. Feedback optic flow with a unity gain, comprising transient (yellow) and relatively constant optic flow (green), was presented. (D) Biased closed-loop visual stimuli for experimental trials. Due to the biased optic flow (to the left), optic flow velocity became slower when the moth turned to the same direction as biased optic flow (to the left) and became faster when it turned to the opposite direction (to the right). There were two states of steady-state optic flow: fast and slow optic flow (OF), and there were two states of onset optic flow: slow-to-fast and fast-to-slow.
Fig. S3. Temporal frequency of optic-flow in experiments with biased closed-loop visual stimuli. (A) Temporal frequency tuning curve of turn angular velocity during surge in the experiment with open-loop visual stimuli (adopted from Fig. 3A). Assuming that the magnitude of angular velocity in the tuning curve corresponds to the strength of visual input to silkmoths, its maximum is achieved by optic flow stimuli with temporal frequencies ranging from ±1.5 to ±2.5 Hz (blue-shaded area). Positive or negative values indicate optic flow direction, negative for right and positive for left. (B) The temporal frequency of optic flow stimuli during each turn in the experiments with biased closed-loop visual stimuli. Positive values indicate the opposite direction to corresponding turns and negative values indicate the same direction as turns. In the control condition, which was only comprised of feedback optic flow, its temporal frequency mostly fell into the blue-shaded area corresponding to the peak of the tuning curve in A. In contrast, temporal frequencies of accelerated or decelerated biased optic flow due to self-motion (1st-Same and 1st-Opposite) fell outside the blue-shaded area, except for the experimental groups with 0.83 Hz biased optic flow, suggesting that the accelerated and decelerated optic flow were not perceived as strong visual inputs to the moths. 1st-Same, experimental groups whose first turn is to the same direction as biased optic flow; 1st-Opposite, experimental groups whose first turn is to the direction opposite to biased flow. Dots represent median of samples, lower and upper limits on error bars represent 25th and 75th percentiles, respectively.
Fig. S4. Relationship between angular velocity and duration of continuous-pulsed pheromone stimuli (N = 15) measured in the biased closed-loop experiment. Pheromone pulse train onset was at 0 s and lasted for 10 s. Temporal frequency of biased optic flow (OF) was set to 2.50 Hz (move to the left) for all samples. Dots represent median of samples, lower and upper limits on error bars represent 25th and 75th percentiles, respectively. Silkmoth activity was triggered by the pheromone stimulus and angular velocities of turning increased gradually until they reached a steady-state at approximately 6 s. Absolute turn angle during 6–9 s (grey shade) were used in our analysis to calculate angular velocity of silkmoths during the surge.
Fig. S5. One trial of silkmoth’s locomotion and head angle. The silkmoth’s behaviour was triggered by single-pulsed pheromone stimulus with a 1 s duration and onset at 0 s. The visual stimulus was a stationary pattern. A short-lived surge was followed by successive zigzag turns. (A) Plots of turn angle and head angle of a silkmoth during walking. Black line represents the turn angle of the moth detected by the optical mouse. The blue dotted line represents the head angle deviated from the centre (B) manually measured from a movie recorded by a video camera (GZ-MG275, Victor Co. of Japan, Kanagawa, Japan), which was positioned over the experimental apparatus. Head angle was measured every 200 ms. (C) Plots of turn angle and angular velocity of the silkmoth. In A and C, positive turn angle represents turning to the left, and negative value represents turning to the right.
Movie 1. The experiments with single-pulsed pheromone stimuli under four different visual stimuli conditions.