

## Visual stimuli induced by self-motion and object-motion modify odour-guided flight of male moths (*Manduca sexta* L.)

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### SUMMARY

Animals rely on multimodal sensory integration for proper orientation within their environment. For example, odour-guided behaviours often require appropriate integration of concurrent visual cues. To gain a further understanding of mechanisms underlying sensory integration in odour-guided behaviour, our study examined the effects of visual stimuli induced by self-motion and object-motion on odour-guided flight in male *M. sexta*. By placing stationary objects (pillars) on either side of a female pheromone plume, moths produced self-induced visual motion during odour-guided flight. These flights showed a reduction in both ground and flight speeds and inter-turn interval when compared with flight tracks without stationary objects. Presentation of an approaching 20 cm disc, to simulate object-motion, resulted in interrupted odour-guided flight and changes in flight direction away from the pheromone source. Modifications of odour-guided flight behaviour in the presence of stationary objects suggest that visual information, in conjunction with olfactory cues, can be used to control the rate of counter-turning. We suggest that the behavioural responses to visual stimuli induced by object-motion indicate the presence of a neural circuit that relays visual information to initiate escape responses. These behavioural responses also suggest the presence of a sensory conflict requiring a trade-off between olfactory and visually driven behaviours. The mechanisms underlying olfactory and visual integration are discussed in the context of these behavioural responses.

Key words: vision, olfaction, insect, flight, behaviour, sensory integration.

### INTRODUCTION

When searching for either food or a mate an animal must successfully orient through its environment towards its desired target. During this orientation, information from the surroundings is often collected simultaneously by multiple sensory systems. This information is conveyed to the central nervous system (CNS) for processing and integration to produce an appropriate behavioural response. How the nervous system processes multimodal sensory information to produce adaptive responses is an ongoing and active area of research (see Gingras et al., 2009).

Odour-guided orientation is a well-suited model system to study processing of multimodal sensory information for the production of adaptive behaviour and occurs in many different animals, such as crabs (Zimmer-Faust et al., 1995), moths (Kennedy and Marsch, 1974; Arbas et al., 1993; David, 1986; Willis and Arbas, 1991), salmon (Johnsen and Hasler, 1980) and birds (Nevitt, 1999). In all these systems this behaviour is characterized by a zigzagging path towards the odour source. During this orientation animals acquire information about the presence of the odour through olfactory receptors and the direction of the odour flow through mechanosensory and/or visual systems. We used pheromone tracking of male *Manduca sexta* (L.) as a model system to study the effects of specific visual stimuli on an odour-driven behaviour.

Pheromone tracking by male moths and the physiological mechanisms underlying this behaviour have been well-studied (Arbas et al., 1993; Belanger and Willis, 1996; Belanger and Arbas, 1998; David, 1986; Kennedy and Marsh, 1974; Vickers and Baker, 1994; Willis and Arbas, 1991; Willis and Arbas, 1998). On sensing female pheromone, a male moth re-orientates its flight path into the

wind, followed by an upwind movement towards the source in a progressively narrowing zigzagging motion. It is currently thought that odour-guided flight is the result of the combination of two primary mechanisms: (1) an odour-modulated, orientation to the wind direction, which relies on visual input; and (2) an internal program for turning, which is activated by pheromone (Vickers, 2000; Willis, 2005). Rather than orienting towards the pheromone source using a chemical gradient (chemotaxis), upwind progress is accomplished by using visual input to make appropriate compensatory movements (optomotor anemotaxis) (Kennedy and Marsh, 1974; Marsh et al., 1978). As a moth orients through its environment, stationary objects will generate a global optic flow of motion vectors corresponding to the image displacement on the retina. These motion vectors change in a characteristic way corresponding to the moth's movement, and by combining local motion from large areas of the visual field, optic flow information can be used to guide locomotion (see Egelhaaf and Kern, 2002). At the same time wind-induced drift during flight will result in a difference between the orientation of the moth's body and the direction of flight, creating an image flow that occurs at an angle to the longitudinal axis of the moth. It is currently believed that moths use longitudinal (to body axis), and horizontal and vertical off-axis components of the image flow to compensate for wind-induced drift and to monitor their upwind progress (Vickers and Baker, 1994). The zigzagging during odour-guided flight is caused by the odour-triggered activation of an internal program of counterturns that results in regular reversals from one side to the other as the moth makes progress toward the source (Arbas et al., 1993; Schneiderman et al., 1982; Vickers, 2000; Willis and Arbas, 1998).

During flight, a male moth will not only be exposed to stationary objects that generate moving and expanding visual images over the retina (referred to as self-motion), but they can also encounter moving objects, such as attacking or approaching predators, that pose a threat. Visual stimuli elicited by moving objects (referred to as object-motion) are characterized by local retinal image shifts that may be unrelated to the global image flow. Wicklein and Strausfeld (Wicklein and Strausfeld, 2000) have shown that *M. sexta* possesses visual interneurons sensitive to object-motion. Though the behavioural responses to object-motion in *M. sexta* are unknown, visual stimuli of moving and expanding objects have been shown to trigger avoidance responses in a wide spectrum of animals ranging from locusts (Gabbiani et al., 1999; Gray et al., 2001; Judge and Rind, 1997; Rind and Simmons, 1998), crabs (Oliva et al., 2007) and flies (Holmqvist and Srinivasan, 1991; Tammero and Dickinson, 2002), to lizards (Carlile et al., 2006), monkeys (Schiff et al., 1962) and humans (Ball and Tronick, 1971). The focus of this paper is to determine the effect of visual stimuli from both object-motion and self-motion on odour-guided flight and whether these stimuli can evoke new, adaptive responses during production of a fixed, ongoing motor program triggered by olfactory input.

Although odour-guided flight in male *M. sexta* has been studied extensively, the mechanisms underlying the integration of both visual and olfactory information to adapt this behaviour remain largely unknown. In certain situations sensory cues from different modalities can result in incongruent information where each modality requires a different behavioural response. An example of this is in situations where animals are exposed to cues indicating the presence of predators while pursuing a potential mate [e.g. tympanate moths exposed to bat echolocation calls (Skals et al., 2005)]. In situations where incongruent sensory information requires a trade-off between different behavioural responses, the CNS is believed to focus on one type of information while ignoring the other. This process has been referred to as limited attention (Dukas, 2002). During odour-guided flight visual stimuli due to object motion can present the moth with incongruent sensory information, requiring the nervous system to make a trade-off between potential predation risk and mating. By studying the effects of visual stimuli caused by object-motion and self-motion on odour-guided flight we hope to gain insight in the underlying mechanisms of sensory integration.

During this study we presented male *M. sexta* with visual stimuli in the shape of stationary objects in the moth's flight path (self-motion) or projected computer generated images of a moving object (object-motion). We hypothesized that avoidance of stationary objects is part of the visual feedback used to generate odour-guided flight and would therefore not affect odour-driven components of this behaviour, such as counter-turning or the success rate at which moths will reach the pheromone source. Visual stimuli due to object-motion may, however, represent a potential predation risk and were expected to override odour-guided behaviour, affecting the moth's flight behaviour or the proportion of males reaching the source. Alternatively, neither self-motion nor object-motion will modify the moth's odour-guided behaviour, indicating a behavioural trade-off in the CNS in favour of mating over predation risk.

## MATERIALS AND METHODS

### Insects

Virgin adult male *M. sexta* (2–3 days old) were selected from a colony in the Department of Biology at the University of Saskatchewan in Saskatoon, Canada. Recordings were made 2–5 h into the scotophase (dark phase), when males are most sensitive to female pheromone

(Sasaki and Riddiford, 1984; Willis and Arbas, 1991; Willis and Arbas, 1998). *M. sexta* were reared on an artificial diet [modified from Bell and Joachim (Bell and Joachim, 1976)], and maintained in a 16 h:8 h light:dark cycle with a 27°C:21°C high:low temperature cycle. Adult males and females were placed in separate rooms and provided with a 20% solution of sucrose in water.

### Odour source

The odour source was prepared by applying 10 µl hexane extract of virgin female pheromone glands to a 7 mm diameter filter paper disc. Gland extracts were prepared following a technique similar to that used by Willis and Arbas (Willis and Arbas, 1991). We excised the tip of the abdomen from 2- to 3-day-old virgin females that were at least 2 h into the scotophase. After removal, the glands were placed in hexane, equivalent to 10 µl per gland, and centrifuged at low speed (100 g) for 30 s. The supernatant was separated and used as 'pheromone' for male flight experiments. The odour source was placed in the middle of the wind tunnel at the upwind end.

### Wind tunnel and data recording

During experiments, moths were placed on a platform at the downwind end of a 0.9 m × 0.9 m × 3 m Plexiglas wind tunnel, exposing them to the pheromone plume. To visualize the pheromone plume and estimate its shape and position, we applied titanium tetrachloride smoke to the same size filter-paper disc (wind speed = 1 m s<sup>-1</sup>). Wind speed was measured using a hot-wire anemometer (VWR Scientific Inc., Edmonton, AB, Canada). Throughout the wind tunnel the plume had a narrow cone shape, gradually increasing in diameter from less than 1 cm at the source to approximately 20 cm at the end of the wind tunnel (see Fig. 1). The odour plume was continuously exhausted to outside the building at the downwind end of the wind tunnel.

Moths that did not respond to pheromone within 3 min by wing-fanning and those that did not take off within a total time of 5 min were removed. Recordings were made of the odour-guided flight of a male moth in the presence and absence of a visual stimulus, with a 20 min break between each flight. To minimize variation in pheromone sensitivity, recordings only took place between 2 and 5 h into the scotophase. As a result of this time limitation, we tested the effects of visual stimuli due to self- and object-motion in two separate experiments using two different groups of animals. To reduce potential confounds due to learning, each moth was exposed to the different visual stimuli only once and the order of presentation was randomized between moths.

As the spectral sensitivity of the eye of *M. sexta* is dramatically lower beyond 640 nm (Bennett and Brown, 1985) and feeding behaviour is most robust at 440 nm (Cutler et al., 1995), we equipped the wind tunnel with infra-red lights (600–1000 nm, Philips IR100 PAR38) located at the downwind end to provide low-light conditions that would likely not affect flight behaviour. By covering the bottom of the wind tunnel with a floor pattern of randomly arranged 8 cm red discs on a white cloth, the red illumination provided a near white background while recording the moth's activity using digital cameras. Recordings of flight activity from two regular speed digital cameras (30 frames s<sup>-1</sup>) were fed into an Event and Video Control Unit (Peak Performance Technologies, Englewood, CO, USA) together with a trigger signal that could simultaneously activate computer-generated visual stimuli. Using Adobe Premiere (version 5.1, Adobe Systems, San Jose, CA, USA), video recordings of flight tracks were deinterlaced into fields (60 fields s<sup>-1</sup>) and for each flight track, fields of the two corresponding recordings were time aligned to the inserted trigger

signal, before being stored as AVI files on a computer. Both videos from each flight track were imported into WinAnalyze3D motion analysis software (Mikromak, Berlin, Germany). We obtained x, y and z coordinates of the moth's location in the wind tunnel by digitizing the position of the thorax in each video frame.

### Visual stimuli

To test the effects of incongruent visual stimuli on odour-guided flight, moths were exposed to both stationary objects and computer-generated moving objects. The visual stimuli used in this research were placed against a contrasting background. Previous research on motion sensitive neurons in *M. sexta* showed that changes in luminance do not affect their responses, as white stimuli against a dark background result in the same activation as dark stimuli against a white background (Wicklein and Strausfeld, 2000).

Stationary objects consisted of two pillars (5 cm in diameter) placed downwind of the pheromone source. Each pillar was 75 cm high and located 15 cm to either side of the midline of the wind tunnel at 65 and 80 cm downwind from the pheromone source. As the presence of a pillar will affect turbulence and result in a decreased wind speed directly downwind of the cylinders, we recorded flight tracks in the presence of black and clear (Plexiglas) pillars to distinguish between behavioural responses due to vision or changes in wind speed. Measurements of wind speeds along the wind tunnel showed that wind speeds remained unaffected between the pillars and together with visual inspection of the shape of a titanium tetrachloride plume, we are confident that the presence of the pillars did not disturb the pheromone plume.

Moving objects were simulated using computer-generated images (rendered at 85 frames s<sup>-1</sup>) of an expanding disc on a rear-projection screen (96 cm × 63 cm) on the right side of the wind tunnel (Fig. 1, inset). We used white expanding discs of 20 cm in diameter moving at a speed of 3 m s<sup>-1</sup>. For each frame the diameter of the projected image was calculated using:

$$\theta_{10} = 2 (\tan^{-1} \times (l/d)), \quad (1)$$

where  $\theta_{10}$  represent the subtense angle of the image at a fixed observer distance of 10 cm from the projection screen,  $l$  is the half-

size of the image and  $d$  the virtual distance of the object from the projection screen.

Using this formula we scaled a 1024 × 1024-pixel portable network graphics (png) file of a white disc in real-time at 85 frames s<sup>-1</sup> using VisionEgg visual stimulus generating software (A. Straw; <http://visionegg.org/>) running on a Python programming platform. Each stimulus started at an initial diameter of 4.64 mm on the screen and expanded to a final diameter of 20 cm during a 1.25 s presentation. To ensure undisturbed odour-guided flight under unstimulated conditions, the projections had a black background to reduce the overall illumination levels (<10 lux). The stimulus was characterized by its Michelson contrast ratio ( $C_M$ ), a measure commonly used to describe the luminance contrast in simple periodic patterns:

$$C_M = (L_{\max} - L_{\min}) / (L_{\max} + L_{\min}), \quad (2)$$

where  $L_{\max}$  is the maximum luminance (measured in cd m<sup>-2</sup>) of the stimulus and  $L_{\min}$  is the minimum luminance. At their maximum size our computer-generated images of a white disc (luminance = 36.4 cd m<sup>-2</sup> =  $L_{\max}$ ) against a dark background (luminance = 3.8 cd m<sup>-2</sup> =  $L_{\min}$ ), produced a Michelson contrast ratio ( $C_M$ ) of 0.81.

Projection of moving objects was triggered when moths were positioned between 50 and 75 cm away from the pheromone source, flying forward and maintaining a flight altitude at that of the pheromone plume. We used manual triggering of the stimulus projection, as the variability in flight behaviours caused automatic triggering to be unreliable. Automatic triggering reacted to any motion in the camera's field of view, irrespective of the moth's flight track, whereas the experimenter was able to assess upwind flight within the volume of stimulus space.

### Flight track analysis

The parameters used to describe flight behaviour are the same as those used by Willis and Arbas (Willis and Arbas, 1991; Willis and Arbas, 1998). From the coordinates describing the moth's position over time we obtained movement vectors from one coordinate to the next that describe the moth's change in position over successive frames. Along each vector we directly calculated the ground speed and track angle

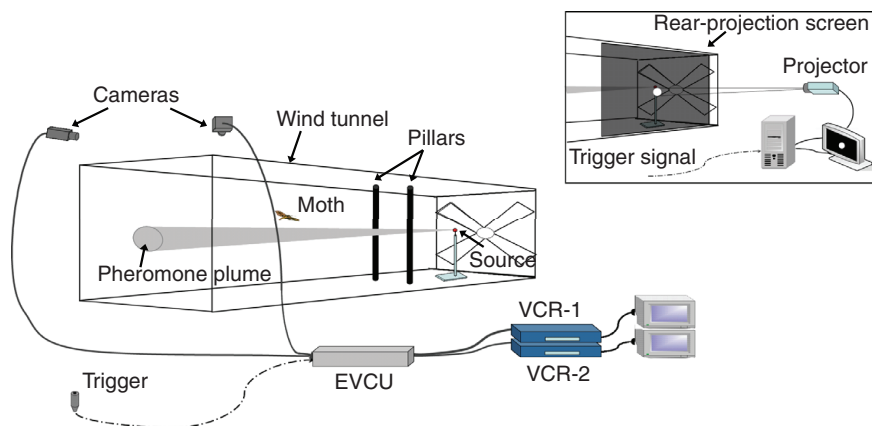


Fig. 1. Experimental setup to record flight behaviour. The wind tunnel (0.9 m × 0.9 m × 3 m) was equipped with two standard rate digital cameras (30 frames s<sup>-1</sup>) to record the entire flight track. To synchronize both video recordings to the event and video control unit (EVCU) was used to insert a time marker in both recordings. Recordings of flight behaviour were stored on VHS tape. As stationary objects we used two black or two clear pillars placed downwind of the pheromone source. Each pillar was 75 cm high and located 15 cm to either side of the midline of the wind tunnel and 65 or 80 cm from the plane of the pheromone source. The pillars did not disturb the shape of the pheromone plume. (Inset) Moving objects were presented as computer generated images of a 20 cm expanding white disc on a black background moving at 3 m s<sup>-1</sup>. These images were projected (at 85 frames s<sup>-1</sup>) on a rear-projection screen placed against the side of the wind tunnel and could be activated by the trigger signal.

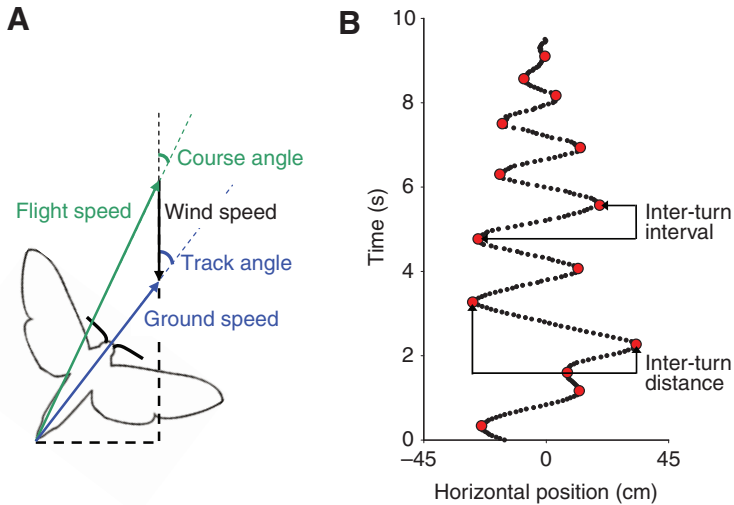


Fig. 2. Track parameters used to describe flight behaviour. (A) By determining each shift in location between consecutive frames, movement vectors were obtained from which a vector describing ground speed and track angle could be calculated. Flight speed and course angle were calculated, by taking the wind direction and speed into account. (B) When plotting the lateral displacement over time, the apices of each turn were identified to measure the inter-turn distance and inter-turn interval.

(flight direction relative to the wind). Using the triangles-of-velocities method (Marsh et al., 1978) as described by Cardé and Knols (Cardé and Knols, 2000), a vector representing the flight speed was calculated by taking the wind direction and speed into account (Fig. 2A). The course angle was calculated as the angle between the wind direction and flight speed vector, whereas the drift angle corresponded to the difference between track and course angle (drift angle = track angle – course angle). Inter-turn distances in each flight track were measured as the greatest lateral displacement between consecutive counterturns, whereas inter-turn intervals were measured as the time between the apices of consecutive counterturns. By plotting the lateral displacement over time we obtained a simplified view of turns and straight legs for each flight from which the apices for each turn could be determined (Fig. 2B). To quantify flight patterns, track parameters such as flight and ground speed, track angle, course angle and drift angle were obtained for each vector, describing the movement of the moth from one frame to the next.

To examine the effects of stationary objects, we used the flight parameters obtained in a region from 90 cm to 65 cm downwind of the pheromone source. Within this region, visual images of the pillars during upwind flight subtended at least 3 deg. of the moth's visual field, which is the spatial resolution of the moth's eye (Sprayberry and Daniel, 2007). As presentation of a moving object was manually triggered, a moth's position within the wind tunnel varied strongly between flight tracks. To account for this variability, we used flight parameters over a span of 3 s before and 3 s after the onset of stimulus presentation (see Willis and Arbas, 1991) to examine the effects of moving objects. For both experiments the effects of the presented stimuli on flight parameters were determined using the general linear model procedures for multivariate data in SPSS v. 14 (SPSS, Chicago, IL, USA).

To test for changes in overall flight direction, we calculated frequency distribution histograms of absolute track and course angles, with an angle of 0 deg. corresponding to upwind flight and 180 deg. to down wind flight. The frequency distribution histograms were created by categorizing angles in 10 deg. bins. Differences between the distributions were assessed using a two-sample Kolmogorov–Smirnov test.

## RESULTS

### Odour-guided flight

We analyzed flight track data that satisfied two criteria: (1) successful upwind orientation towards the pheromone source during

odour-guided flight in the absence of stationary or moving visual stimuli, (2) regular zigzagging tracks with the altitude mainly confined to that of the pheromone plume, as reported by Willis and Arbas (Willis and Arbas, 1991; Willis and Arbas, 1998). An example of a flight track representative of odour-guided flight in the absence of stationary or moving visual stimuli can be seen in Fig. 3. These flights lasted, on average,  $8.26 \pm 0.60$  s ( $N=24$ ). The average ground and flight speeds were  $1.09 \pm 0.06$   $\text{ms}^{-1}$  and  $1.72 \pm 0.06$   $\text{ms}^{-1}$ , respectively, whereas the inter-turn interval was  $0.708 \pm 0.04$  s and inter-turn duration was  $22.52 \pm 1.83$  cm. Reported values for track parameters are average values for the entire flight track (mean  $\pm$  s.e.m.).

### Responses to stationary objects

The effects of visual stimuli induced by self-motion were determined by comparing the flight tracks of 15 males flying in the presence or absence of stationary pillars. For each trial moths were able to reach the odour source, but in the presence of clear or black pillars six out of the 15 moths turned back upon approaching the objects and then proceeded to reach the pheromone source. Compared with unrestricted flights, moths exposed to either black or clear pillars typically showed a reduction in flight speed and changes in elevation in front of the pillars before narrowing their track width (Fig. 4).

Detailed analysis of flight track parameters showed significant increases (MANOVA,  $P < 0.05$ ) in elevation, azimuthal position, course angles and track angles upon approaching both clear and black pillars. The presence of stationary objects also resulted in a significant decrease in flight speeds, inter-turn distance and inter-turn duration measured across a part of the wind tunnel from 90 cm to 65 cm from the pheromone source (Table 1). At any point within this region black pillars would have subtended at least 4.4 deg. of the visual field and thus been visible to the moth. The changes in course angles and track angles, azimuthal position, flight speeds and inter-turn distance were not significantly different in the presence of either clear or black pillars. There was, however, a significant increase in elevation in the presence of black pillars, whereas inter-turn intervals significantly decreased (Fig. 5).

### Responses to moving object

The effects of moving objects on flight behaviour were determined by comparing the change in flight tracks of nine males exposed to visual images of a moving object in the shape of expanding discs



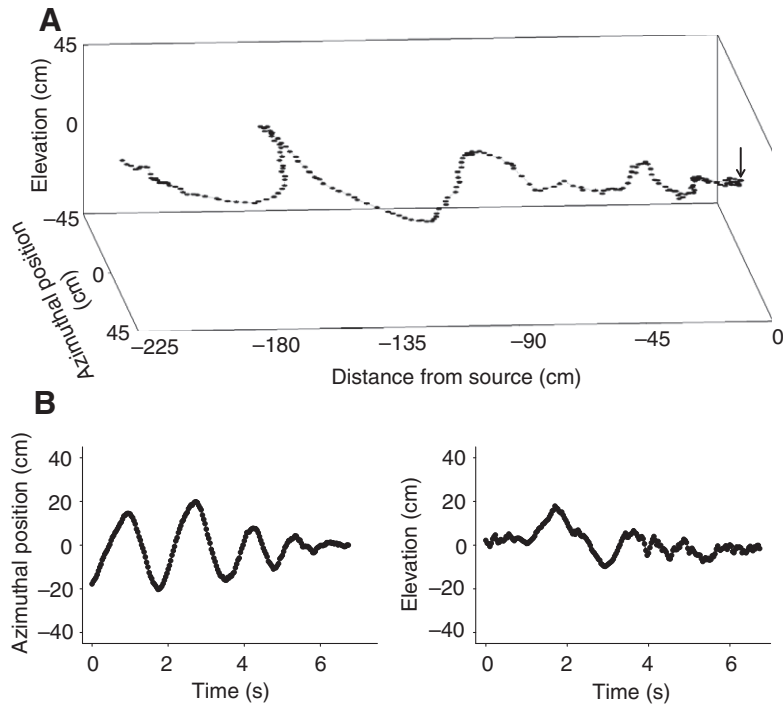


Fig. 3. Pheromone-guided flight. Plot of a representative three-dimensional flight track towards a pheromone source (arrow; A) and corresponding changes in azimuthal position and elevation over time (B) for odour-guided flight in the absence of stationary or moving visual stimuli. (B) Plotting the moths position over time, relative to pheromone source, shows that flight tracks had a zigzagging pattern in the azimuthal plane, levelling off as the moth approached the pheromone source. During the flight tracks elevation was maintained within the pheromone plume (positioned at 0).

projected onto the right wall of the wind tunnel 50 cm from the odour source. As presentation of images was manually triggered, the distance between a moth and the point of projection on the screen at the start of a presentation varied between 60 and 15 cm. Throughout the presentation this distance generally decreased as moths made upwind progress. Fig. 6 shows the variation in distance from stimulus between moths and corresponding variability in the subtense angles of each image on an individual moth's retina. There did not appear to be any relation between final subtense angles and the various behavioural responses that were observed.

Responses to images of a moving object were classified after visual inspection of flight data. The onset of a behavioural response was determined by observing an abrupt shift in continuous upwind progress toward the odour source. These shifts were not observed during flights with no visual stimuli. During stimulus presentation there was typically an increase in the distance between the moth and pheromone source. Responses to images of a moving object varied from no response to temporary or permanent displacement out of the odour plume. All moths initially displayed characteristically zigzagging odour-guided flight up to the point of

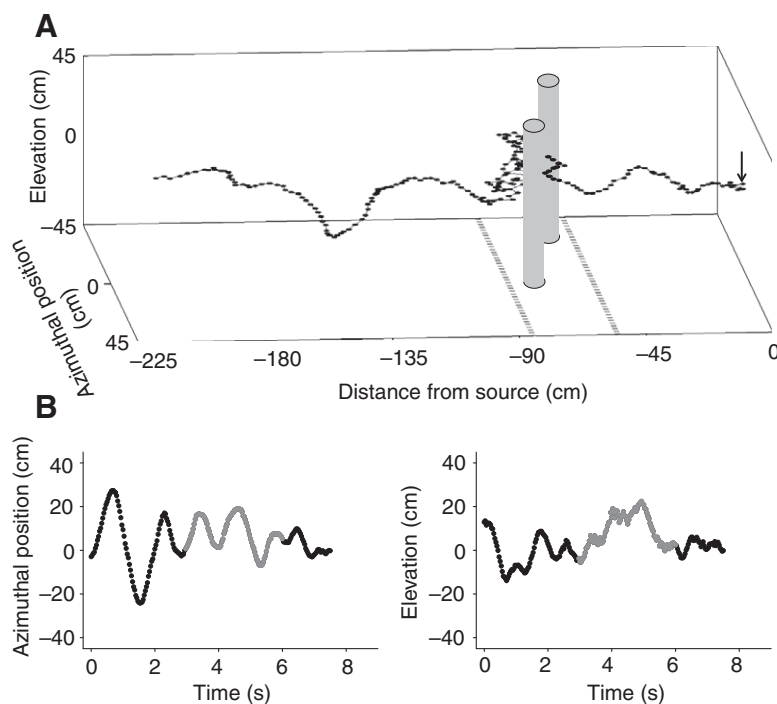


Fig. 4. Pheromone-guided flight in the presence of stationary objects. Plot of representative three-dimensional flight tracks for odour-guided flight in the presence of stationary objects (A) and corresponding changes in azimuthal position and elevation over time (B). (A) The arrow represents the pheromone source, the grey bars and dashed lines represent, respectively, the pillars and adjacent region corresponding to that part of the wind tunnel from 10 cm before the downwind pillar to the position of the upwind pillar. (B) The lighter shaded parts of the plots represent the time moths were between 90 cm and 65 cm downwind from the pheromone source, the region where the pillars were visible to the moth (see Materials and methods). Flight tracks of moths exposed to stationary objects generally showed a reduction in flight speed and changes in elevation in front of the pillars before narrowing their track width.

Table 1. Grand means ( $\pm$ s.e.m.) of parameters of odour-guided flight with and without stationary objects

Flight parameter	Control	Clear	Black
Azimuthal position (cm)	<b>7.46<math>\pm</math>0.38</b>	<b>8.65<math>\pm</math>0.22</b>	<b>8.39<math>\pm</math>0.17</b>
Elevation (cm)	<b>6.05<math>\pm</math>0.30</b>	<b>7.60<math>\pm</math>0.19</b>	<b>9.47<math>\pm</math>0.20*</b>
Inter-turn interval (s)	<b>0.65<math>\pm</math>0.05</b>	<b>0.61<math>\pm</math>0.03</b>	<b>0.55<math>\pm</math>0.03*</b>
Inter-turn distance (cm)	<b>23.04<math>\pm</math>2.59</b>	<b>17.14<math>\pm</math>1.46</b>	<b>14.70<math>\pm</math>0.99</b>
True ground speed (m s <sup>-1</sup> )	1.08 $\pm$ 0.05	1.06 $\pm$ 0.02	1.06 $\pm$ 0.02
True flight speed (m s <sup>-1</sup> )	<b>1.77<math>\pm</math>0.05</b>	<b>1.51<math>\pm</math>0.02</b>	<b>1.48<math>\pm</math>0.02</b>
Ground speed – horizontal (m s <sup>-1</sup> )	0.89 $\pm$ 0.04	0.84 $\pm$ 0.02	0.84 $\pm$ 0.02
Flight speed – horizontal (m s <sup>-1</sup> )	<b>1.61<math>\pm</math>0.06</b>	<b>1.30<math>\pm</math>0.03</b>	<b>1.27<math>\pm</math>0.02</b>
Ground speed – vertical (m s <sup>-1</sup> )	1.00 $\pm$ 0.05	0.98 $\pm$ 0.03	0.99 $\pm$ 0.02
Flight speed – vertical (m s <sup>-1</sup> )	<b>1.72<math>\pm</math>0.05</b>	<b>1.46<math>\pm</math>0.03</b>	<b>1.42<math>\pm</math>0.02</b>
Track angle – horizontal (deg.)	<b>114.77<math>\pm</math>4.39</b>	<b>132.48<math>\pm</math>2.56</b>	<b>132.30<math>\pm</math>2.24</b>
Course angle – horizontal (deg.)	<b>41.00<math>\pm</math>5.03</b>	<b>75.42<math>\pm</math>2.62</b>	<b>77.91<math>\pm</math>2.20</b>
Drift angle – horizontal (deg.)	97.08 $\pm$ 3.36	91.90 $\pm$ 1.42	87.03 $\pm$ 1.21
Track angle – vertical (deg.)	<b>54.45<math>\pm</math>5.44</b>	<b>90.29<math>\pm</math>2.64</b>	<b>94.68<math>\pm</math>2.16</b>
Course angle – vertical (deg.)	<b>41.96<math>\pm</math>4.46</b>	<b>73.45<math>\pm</math>2.36</b>	<b>79.71<math>\pm</math>2.05</b>
Drift angle – vertical (deg.)	15.95 $\pm$ 3.78	19.36 $\pm$ 1.80	15.92 $\pm$ 1.45

Grand means are based on all data points within a part of the wind tunnel from 10 cm before to right after location of the stimulus for 15 animals.

\*Significant difference for black pillars.

Differences in parameters were compared using the general linear model procedures for multivariate data in SPSS v. 14 (SPSS, Chicago, IL, USA) and considered significant at  $P < 0.05$ . Bold indicates significant differences.

stimulus presentation. For one moth, presentation of a moving object did not lead to any discernable response. The remaining eight moths flew away from the pheromone source during or soon after stimulus presentation. The onset of these changes began  $0.18 \pm 0.16$  s (mean  $\pm$  s.d.) after the projected images subtended more than 3 deg. on the moth's retina. Based on the moth's position relative to the last frame of stimulus presentation, the estimated time of projected collision ranged from 0.1 to 0.3 s. The onset of behavioural responses relative to time of collision was  $-0.26 \pm 0.27$  s (mean  $\pm$  s.d.). The changes in flight track during the first 3 s after stimulus presentation were accompanied by an increase in inter-turn distance and inter-turn duration, similar to behavioural responses to odour loss for *M. sexta* as reported by Willis and Arbas (Willis and Arbas, 1991). After the initial change in flight track six moths were able to resume their odour-guided flight 1–3 s after stimulus presentation (see Fig. 7).

Statistical comparison of flight track parameters measured over 3 s before and 3 s after stimulus presentation, showed a significant increase in inter-turn interval, track and course angles as well as flight speed and elevation (Table 2). Behavioural responses to the

projected images suggest that visual stimuli induced by object-motion can override odour-guided flight and trigger changes in both flight direction and the timing of counterturns. When comparing absolute track and course angle values before and after stimulus presentation, we observed a clear shift towards higher angles (away from the source, Fig. 8). This increase in angle together with an increase in ground speed, but not in flight speed, is attributed to an overall change in flight direction away from the source.

## DISCUSSION

Our study examined, for the first time, the effects of visual stimuli induced by self-motion and object-motion on odour-guided flight in male *M. sexta* and is the first in a series of studies with the aim of understanding the neural mechanisms underlying multimodal sensory integration in this system. Here we demonstrated that visual stimuli can modify and override odour-guided flight in male *M. sexta*.

By placing stationary objects (pillars) on both sides of the odour plume, moths were exposed to visual stimuli induced by self-motion

Table 2. Grand means ( $\pm$ s.e.m.) of parameters of odour-guided flight 3 s before and after the presentation of a moving object

Flight parameter*	Before	After
Azimuthal position (cm)	9.949 $\pm$ 0.856	10.892 $\pm$ 1.733
Elevation (cm)	<b>7.465<math>\pm</math>0.927</b>	<b>11.374<math>\pm</math>1.812</b>
Inter-turn interval (s)	<b>0.84<math>\pm</math>0.12</b>	<b>1.061<math>\pm</math>0.166</b>
Inter-turn distance (cm)	23.451 $\pm$ 3.221	32.578 $\pm$ 5.039
True ground speed (m s <sup>-1</sup> )	<b>0.859<math>\pm</math>0.0793</b>	<b>1.193<math>\pm</math>0.102</b>
True flight speed (m s <sup>-1</sup> )	1.507 $\pm$ 0.0519	1.587 $\pm$ 0.0745
Ground speed – horizontal (m s <sup>-1</sup> )	<b>0.669<math>\pm</math>0.0678</b>	<b>0.971<math>\pm</math>0.0856</b>
Flight speed – horizontal (m s <sup>-1</sup> )	1.376 $\pm$ 0.0393	1.374 $\pm$ 0.0581
Track angle – horizontal (deg.)	<b>61.455<math>\pm</math>4.112</b>	<b>83.627<math>\pm</math>2.503</b>
Course angle – horizontal (deg.)	<b>49.735<math>\pm</math>3.696</b>	<b>72.324<math>\pm</math>2.663</b>
Drift angle – horizontal (deg.)	0.104 $\pm$ 1.962	-1.447 $\pm$ 0.892
Ground speed – vertical (m s <sup>-1</sup> )	<b>0.746<math>\pm</math>0.0627</b>	<b>1.09<math>\pm</math>0.0829</b>
Flight speed – vertical (m s <sup>-1</sup> )	1.444 $\pm$ 0.0407	1.514 $\pm$ 0.0566
Track angle – vertical (deg.)	<b>62.553<math>\pm</math>2.654</b>	<b>84.305<math>\pm</math>2.191</b>
Course angle – vertical (deg.)	<b>48.894<math>\pm</math>3.176</b>	<b>69.672<math>\pm</math>2.562</b>
Drift angle – vertical (deg.)	-15.712 $\pm$ 1.997	-16.601 $\pm$ 1.703

\*Grand means are based on  $N=9$ , taken over 3 s before and after the onset of stimulus.

Differences in parameters were compared using the general linear model procedures for multivariate data in SPSS v. 14 (SPSS, Chicago, IL, USA) and considered significant at  $P < 0.05$ . Bold indicates significant differences.

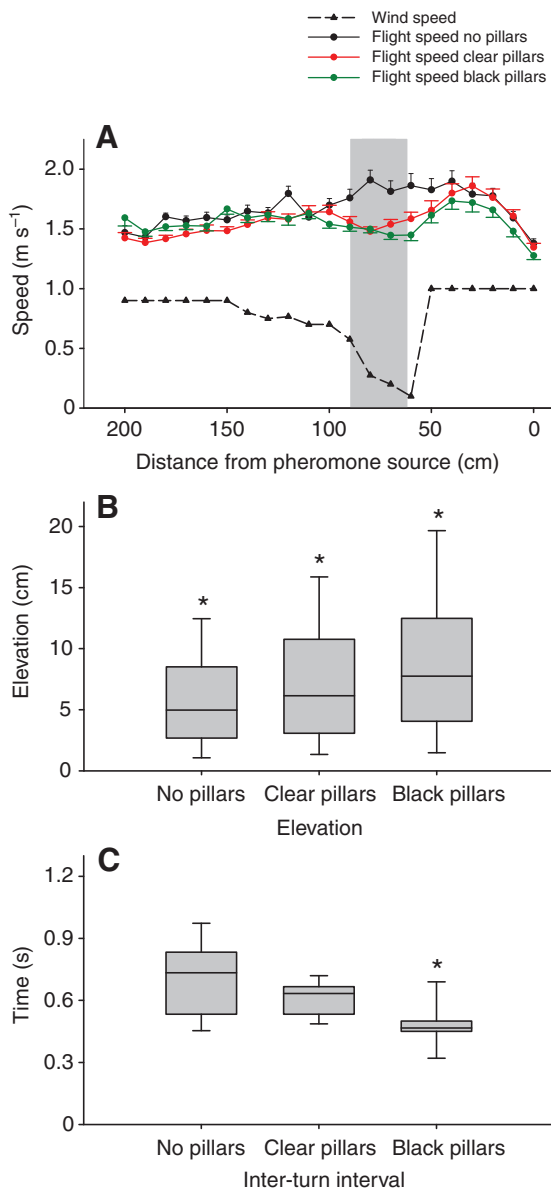


Fig. 5. Effects of stationary objects on flight speed, elevation and inter-turn intervals. (A) Plot showing the average flight speed (mean  $\pm$  s.e.m.) of 15 animals along the length of the wind tunnel. The grey shaded region indicates the region in which the pillars were visible to the moth. For flights in the absence of stationary objects flight speed slowly increased over the length of the wind tunnel from 1.5 to 1.9 m s<sup>-1</sup>, before rapidly slowing down to approximately 1.3 m s<sup>-1</sup> in the last 20 cm before reaching the pheromone source. Wind speeds, measured directly downwind of the pillars, showed a noticeable reduction in the region from 150 cm to 50 cm downwind of the pheromone plume. Corresponding to this reduction in wind speed we observed a reduction in flight between 100 cm and 50 cm downwind of the pheromone plume for both clear and black pillars. (B,C) Box plots showing the average elevation and inter-turn intervals within the region in which the pillars were visible. Both elevation and inter-turn intervals were significantly different in the presence of black pillars compared to flight tracks without pillars or with clear pillars. Asterisks indicate a significant difference (Kruskal–Wallis one-way ANOVA,  $P < 0.05$ ).

during odour-guided flight. As the presence of the two pillars resulted in a reduction of wind speed directly downwind of the pillars, we exposed moths to both clear and black pillars to distinguish between visual and wind-induced responses. Since

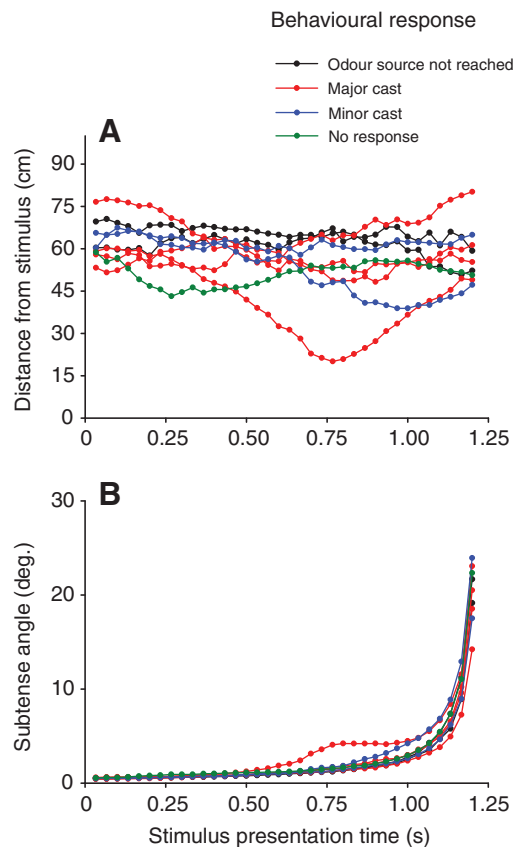


Fig. 6. Graphs showing the variability in distance from the stimulus, subtense angles and the corresponding behavioural responses. (A) As moths were exposed to images of a moving object they experienced variability in the distance from the stimulus and the corresponding subtense angles of the images (B). The distance between a moth and stimulus at the start of a presentation varied between 60 and 15 cm. As stimulus presentation progressed this distance generally decreased, producing corresponding variability in subtense angles of the disc. The lines in each graph are colour-coded to indicate the type of behavioural response that was displayed. We identified four different responses, as described in the Results, varying from no response to temporary or permanent displacement out of the odour plume. There does not appear to be any relationship between the type of response displayed and changes in subtense angles of the projected images.

moths are good learners (Daly and Smith, 2000; Kelber, 1996) we cannot rule out that learning affected their behavioural responses. However, by presenting each stimulus in random order and only once for each moth our stimulus presentation protocol was designed to reduce potential learning effects. We also cannot rule out that moths could see the clear pillars. However naïve moths mainly use chromatic contrasts in flower recognition, giving more weight to the chromatic aspect of colour in detection compared to achromatic contrasts (Kelber et al., 2003; Kelber, 2005). The black pillars we used provided the moths with both chromatic and achromatic visual cues, with the contrast between black pillars and background, expressed as a Michelson contrast ratio, increasing from 0.07 at 50 cm to 0.80 at 5 cm in front of the pillars. Over the same distance contrast levels between background and clear pillars only increased from 0.06 to 0.14. With the clear pillars mainly providing achromatic visual cues and having lower contrast levels, we believe that the visual stimuli from clear pillars are strongly reduced compared with those of black pillars. Given these constraints we are therefore

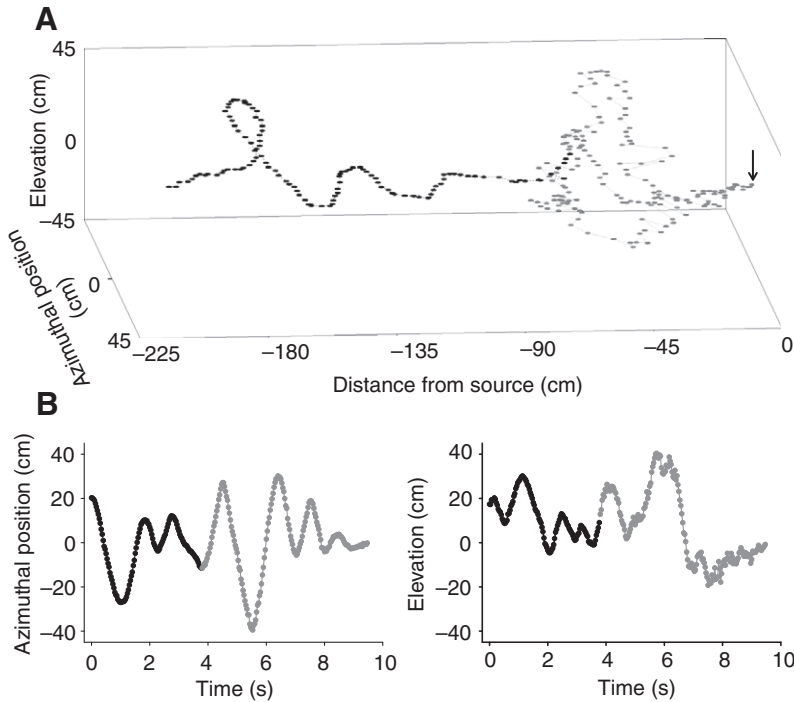


Fig. 7. Pheromone-guided flight in the presence of moving objects. Plot of a representative three-dimensional flight track in the presence of a moving object (20 cm expanding disc) and the corresponding changes in azimuthal position and elevation over time. (A) The arrow represents the position of the pheromone source, the grey dots identify the moth's position during stimulus presentation. Immediately following stimulus presentation the flight track shows sharp turns and no zigzagging pattern. (B) Before stimulus presentation the flight track was similar to odour-guided flight in the absence of objects and was a smooth zigzagging pattern in azimuthal plane, while maintaining elevation close to the pheromone plume. After stimulus presentation the track generally widened and the inter-turn interval increased (from  $0.84 \pm 0.12$  s before to  $1.016 \pm 0.166$  s after). Simultaneously the vertical distance from the source increased significantly (from  $7.465 \pm 0.927$  cm before to  $11.374 \pm 1.812$  cm after).

confident that we could reliably use clear pillars as a procedural control to identify visually induced behavioural responses. Detailed analysis of odour-guided flight in the presence of both clear and black pillars showed that both elevation and inter-turn interval were significantly different in the presence of black pillars and can therefore be attributed to visual object detection (Fig. 5). The clearly noticeable reduction in flight speed cannot however be attributed to visual detection, as both clear and black pillars resulted in a similar reduction, corresponding to changes in wind speed (Figs 4 and 5;

Table 1). Moreover, the presence of stationary objects did not prevent moths from reaching the pheromone source.

Exposure to visual stimuli induced by object-motion, an expanding 20 cm disc, had a profound effect on odour-guided flight as eight out of nine moths turned away from the pheromone source and two moths were unable to resume odour-guided flight. During each stimulus presentation the subtense angles of images on a moth's retina varied depending on the moth's location, but there does not appear to be any relationship between the subtense angles of the

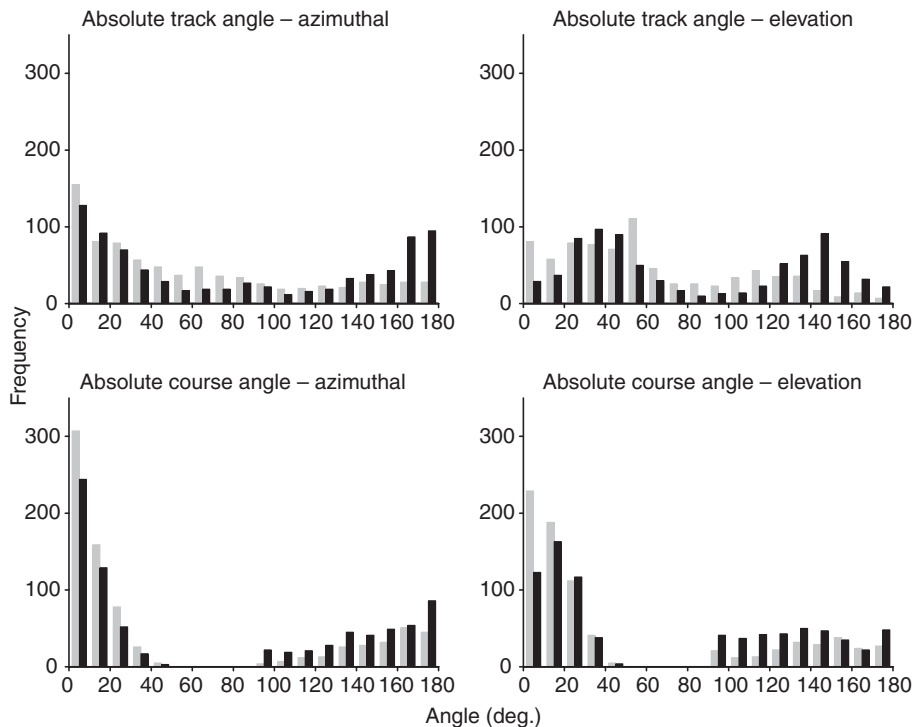


Fig. 8. Effect of moving objects on track and course angles. To illustrate the overall changes in flight direction, frequency distribution histograms of absolute track and course angles were calculated using data from all animals ( $N=9$ ) 3 s before (grey bars) and 3 s after (black bars) presentation of a moving object. Turn angles were measured as absolute, where an angle of 0 deg. corresponds to upwind flight and 180 deg. to downwind flight. For all angles, stimulus presentation creates a significant change in frequency distribution (two-sample Kolmogorov–Smirnov,  $\alpha=0.05$ ). After stimulus presentation both track and course angles shifted to higher values in the azimuthal and elevation planes, indicating flight away from the source ( $>90$  deg.).



presented images and the various behavioural responses (Fig. 6). We assume that the behavioural responses are due to the presented visual stimuli, as the onset of the responses occurred  $0.18 \pm 0.16$  s (mean  $\pm$  s.d.) after the projected images subtended more than 3 deg. on the moth's retina and  $-0.26 \pm 0.27$  s (mean  $\pm$  s.d.) before the estimated time of collision. Detailed analysis of behavioural responses to object-motion revealed a modification of flight direction, away from the source in both azimuthal and elevation planes, as indicated by the increase in track and flight angles and vertical distance from source (Table 2; Figs 7 and 8). Counter-turning behaviour was also modified as the inter-turn interval significantly increased after stimulus presentation.

### Self-motion

In our experiments the presence of stationary objects resulted in an increase in counter-turning frequency and decrease in track width upon approach. Previous work (Marsh et al., 1978; Willis and Arbas, 1991; Willis and Arbas, 1998; Willis and Baker, 1994) showed that both the frequency of counterturning and ground speed are modulated as the moths fly up the pheromone plume to the source. Counter-turning is thought to be driven by an internal program of track reversals, the frequency of which increases with odour concentration (Arbas et al., 1993; Mafra-Neto and Cardé, 1994; Kuenen and Baker, 1983). Our results further suggest that vision also affects counter-turning through modulation of complex sensorimotor pathways.

The processing of visual information from object-motion could underlie the behavioural responses to pillars that we recorded. In insects, visual information used to guide locomotion is extracted from the retinal array and ultimately relayed to motor centres. As an insect moves forward through its environment, stationary objects will move over the retina from front to back, creating a pattern of image flow. This image flow depends on the direction and speed at which the insect moves through its environment (self-motion) and is characterized by global changes in the retinal image. Visual information corresponding to these global changes is acquired by pooling motion information over large areas of the visual field and plays a role in controlling the course and velocity of locomotion in insects (for reviews, see Borst and Haag, 2002; Egelhaaf and Kern, 2002). Aspects of the visual information from stationary objects come from the angular velocity at which the edges of their image move over the retina. Bees are known to keep the angular velocity of the image of their environment constant to control their flight speed in a moment-to-moment fashion, resulting in a reduction of their speed when the distance from objects in their environment decreases (Srinivasan et al., 1996; Srinivasan et al., 1999; Srinivasan et al., 2001). Contrary to bees, however, our data showed that the reduction in velocity upon approaching stationary objects was similar for both clear and black pillars and therefore more likely to be an effect of wind speed rather than angular velocity. That angular velocity has little effect on flight speed is also supported by research done by Willis and Arbas (Willis and Arbas, 1998) in which flight speeds in *M. sexta* were shown to change throughout odour-guided flight. Moreover, our data support the finding that mechanosensory information is important for odour-guided flight of *M. sexta* (see Sane et al., 2007).

Similar to other insects, visual flow is believed to stabilize odour-guided flight of moths. Off-axis components in optic flow have been shown to play an important role in controlling for wind-induced drift (Marsh et al., 1978; Vickers and Baker, 1994; Willis and Arbas, 1991; Willis and Cardé, 1990). When a moth experiences wind-induced displacement during flight, the image flow occurs at an

angle to the longitudinal body axis. By using longitudinal, horizontal and vertical off-axis components of the image flow moths are thought to compensate for wind-induced drift and monitor their upwind progress. Several studies have indicated the presence of wide-field visual neurons responsive to movement in vertical or horizontal directions, which could play a role processing components of the image flow and mediate upwind flight responses (Milde, 1993; Olberg and Willis, 1990; Rind, 1983). In *Lymantria dispar* some of these wide-field visual neurons also showed amplified optomotor responses in the presence of female pheromone (Olberg and Willis, 1990). When approaching stationary objects the moth's image flow will undergo strong changes in the off-axis components. We therefore suggest that descending wide-field visual neurons used in controlling wind-induced drift may also play a role in mediating avoidance responses to stationary objects. Future research will focus on whether such neurons are present in *M. sexta* and have pheromone-modulated responses.

### Object-motion

Relative to background visual flow, retinal image size of an approaching object increases more than that of a stationary object in the flight path. Approaching objects may be indicative of danger and thus present the moth with incongruent sensory information during odour guided flight, requiring the nervous system to make a trade-off between potential predation risk and mating. This trade-off, where one stimulus is ignored so as to attend the other has been referred to as limited attention (Dukas, 2002) and is thought to be the result of the limited amount of information a nervous system can process in a given time. Studies with tympanate moths where males were simultaneously exposed to female pheromones and artificial bat cries, suggests these moths make a behavioural trade-off depending on the relative intensity from the two sensory modalities (Acharya and McNeil, 1998; Skals et al., 2005; Svensson et al., 2004).

When presenting moths with an approaching object (images of an approaching disc), a trade-off between visually driven escape behaviour and olfactory driven mating behaviour is required. In the presence of this incongruent visual information moths showed a modification of counter-turning behaviour similar to odour loss (increase in inter-turn distance and inter-turn duration [as reported by Willis and Arbas (Willis and Arbas, 1991)], as well as interruption of odour-guided flight in some animals. These behavioural responses suggest that visual information induced by object-motion overrides the olfactory input to initiate an avoidance response, and seem to support the concept of limited attention where olfactory information is ignored to attend visual information. As our current research focussed on whether visual stimuli from object-motion and self-motion could modify odour-guided flight we did not test for stimulus intensity effects. Future research should therefore be focussed on studying odour-guided flight at various intensities of visual stimuli (e.g. different size objects) in a manner similar to that reported by Skals et al. (Skals et al., 2005).

The mechanisms by which sensory information from olfaction and vision are integrated in moths to modify odour-guided flight are still poorly understood. Besides the use of visual flow field information for steering, our data suggests that visual information of object motion can override odour-driven counter-turning. Contrary to the global changes in retinal images of objects caused by self-motion, object motion is characterized by local changes. As an animal encounters an approaching object, such as a predator, it will experience an increase in retinal image size of the object independent of background motion. These visual stimuli probably

represent a more acute danger to an animal than those from stationary objects, and often trigger avoidance responses. Neurons responding to images of an approaching object have been found in both invertebrates and vertebrates, ranging from locusts (Gabbiani et al., 1999; Judge and Rind, 1997; Rind and Simmons, 1998), crabs (Oliva et al., 2007) and flies (Holmqvist and Srinivasan, 1991; Tammero and Dickinson, 2002), to lizards (Carlile et al., 2006), monkeys (Schiff et al., 1962) and humans (Ball and Tronick, 1971). Though there have been studies on motion-sensitive neurons and their descending pathways in moths, little is known about pathways that transmit visual information about approaching objects to the thoracic flight circuitry. Previous work has shown the presence of descending visual interneurons responsive to horizontal motion over a large area of the moth's visual field (Rind, 1983) as well as the presence of two classes of wide-field neurons in the optic lobes of *M. sexta* that respond to object motion (Wicklein and Strausfeld, 2000). The presence of these neurons combined with the visually triggered interruption of odour-guided flight supports the possibility of a neural circuit similar to the locust 'descending contralateral movement detector' that relays visual information about object motion to initiate escape responses (Burrows, 1996; Gray et al., 2001; Rind and Simmons, 1997; Santer et al., 2005). We suggest that sensory integration for collision avoidance during odour-guided flight operates on a neural network responsible for turning, where pathways for visual and olfactory input are integrated to modify the output of the network. Depending on relative stimulus intensities, one sensory modality could directly influence the effect of the other sensory modality with respect to modulation of flight steering muscle activity. Alternatively, as in fruit flies (Frye and Dickinson, 2004) both modalities could affect different parts of the flight musculature, with visual feedback affecting the direct flight muscles, allowing for quick changes, while the olfactory pathway drives the turning program. In this case, presence of pheromone will not affect the neuronal responses to visual stimuli.

To gain a greater understanding of how olfactory and visual inputs are integrated to generate an appropriate behavioural response, future experiments will use multi-channel electrode arrays to locate descending interneurons relaying visual information about object motion to the thorax. The experiments will attempt to characterize response properties of these interneurons and will explore putative modulatory effects of other sensory modalities on descending visual input.

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