The start of phonotactic walking in the fly *Ormia ochracea*: a kinematic study

Andrew C. Mason[1],*, Norman Lee[1] and Michael L. Oshinsky[2],†

[1]*Integrative Behaviour and Neuroscience Group, Department of Life Sciences, University of Toronto at Scarborough, 1265 Military Trail, Scarborough, Ontario, Canada M1C 1A4* and [2]*Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA*

*Author for correspondence (e-mail: amason@utsc.utoronto.ca)
†Present address: Department of Neurology, Thomas Jefferson University, Philadelphia, PA 19107, USA

Summary

Ormia ochracea (Diptera, Tachinidae) are acoustic parasitoids of crickets that have one of the most directionally sensitive auditory systems known. We studied dynamic characteristics of walking phonotaxis in these flies in response to variations in sound source azimuth, and compared phonotaxis of flies in freely walking conditions to tethered flies walking on a treadmill. Motor patterns at the initiation of phonotaxis are not stereotyped even for similar stimulus conditions. Flies respond to directional sound sources by walking in a tight curve that combines rotation and forward translation until they are oriented towards the source direction, then continue on a straight path. Translational velocity accelerates throughout the duration of the stimulus then decelerates following stimulus offset. In contrast, rotational velocity accelerates and then decelerates within the duration of the stimulus such that flies have completed the rotational component of the response and reached their final heading before the end of the stimulus. Rotational velocity is the only response parameter that varies systematically with sound source direction (azimuth). Differences in the amplitude of rotational velocity as a function of source azimuth determine the directional orientation of phonotactic responses. The relationship between rotational velocity and source azimuth is similar to a neural measure of auditory directionality (interaural latency). There were some differences between freely walking and tethered conditions, although both showed qualitatively similar responses. Flies accelerated more slowly and attained lower maximum velocities on the treadmill, consistent with the greater inertia of the treadmill sphere relative to the flies. Also, flies tended to continue walking longer on the treadmill following cessation of the stimulus.

Key words: *Ormia*, phonotaxis, kinematics, sound localisation.

Introduction

The ability of sensory systems to extract information from stimuli is subject to fundamental physical constraints of the stimulus and the limitations of neural coding (Bialek, 1987; Bialek et al., 1991). Sound localization represents a clear example of the trade-offs inherent in the design of sensory systems (Michelsen, 1992). The fly, *Ormia ochracea* (Diptera: Tachinidae), possesses an auditory system capable of accurate sound localization (Mason et al., 2001), but this may come at the cost of a reduction in the range of cues that are detectable by this highly specialized auditory pathway. The aspects of directional hearing that have been studied in detail in this species, namely tympanal mechanics (Robert et al., 1996, 1998; Miles et al., 1995), receptor physiology (Oshinsky and Hoy, 2002) and directional acuity and coding (Mason et al., 2001), suggest that the fly represents an excellent model system for understanding a sensorimotor pathway that links physical cues in external stimuli with sensory transduction, neural coding and orientation behaviour.

Ormia ochracea (Diptera: Tachinidae) are acoustic parasitoid flies. Adults are free-living, but their larvae develop as internal parasites of crickets. Female *Ormia* locate their cricket hosts by phonotaxis to the male calling song using an auditory system that functions solely in this context. Females first fly towards cricket song, then land and walk in preparation for larviposition as they near the source of the stimulus (Cade, 1975; Walker, 1983). Unlike intraspecific acoustic communication systems, in which the sensory system and the signals co-evolve to optimize the transfer of information (Bradbury and Vehrencamp, 1998), acoustic parasites must adapt to the characteristics of pre-existing host signals. Remarkably, the flies can localize the 5 kHz tone pulses (wavelength=6.9 cm) produced by crickets, using a pair of ears separated by less than 500 μm (Edgecomb et al., 1995). Flies reliably orient towards sound sources broadcasting the calls of host species despite the fact that their small size severely constrains the physical cues available to them for determining the direction of sound propagation (Robert and Hoy, 1998).

This auditory directional sensitivity is derived from

mechanical coupling of the two tympani, which provides sensitivity to tiny interaural time-differences arising from the minute separation of the two ears (Robert et al., 1996, 1998; Miles et al., 1995). Previous work has shown that this tympanal mechanism is combined with specializations of auditory receptors to provide exquisite sensitivity to sound direction (Mason et al., 2001; Oshinsky and Hoy, 2002). These adaptations allow female *Ormia* to localize an appropriate sound source to within 2° of azimuth (Mason et al., 2001).

A thorough analysis of behavioural responses to directional acoustic cues is required in order to determine the relationship between physical acoustic cues, mechanical cues (at the tympanal level), and neural coding of sound direction. Previous behavioural analyses have focused on both flying (Mueller and Robert, 2001) and walking (Mason et al., 2001) acoustic responses. However, the kinematics of walking phonotaxis in *Ormia ochracea* has not been described in detail. Here we compare the initial stages of phonotactic responses in flies tethered on a spherical treadmill with those of freely walking flies. We analyse the responses of flies to a single stimulus presentation, during which they reorient to the direction of the sound source. We describe the dynamic features of these responses with a view to identifying how the flies modify their walking patterns to achieve appropriately oriented responses.

Materials and methods

Study animals

All experiments were carried out using gravid female *O. ochracea* drawn from laboratory-reared populations descended from animals collected in Florida, USA. Adult flies were kept on a 12 h:12 h light:dark cycle. All tests were conducted during the first 4 h of the dark phase.

Acoustic stimuli

Acoustic stimuli were synthesized using Tucker-Davis Technologies (TDT, Gainesville, FL, USA) hardware and custom software. Stimuli for behavioural experiments consisted of a train of 5 kHz, 10 ms tone pulses with 1 ms rise/fall times, delivered at a rate of 50 pulses s^{-1} with 10 pulses/train for a total stimulus duration of 200 ms. Stimuli were amplified (Harman Kardon PM655; Château du Loir, France, or NAD S300; London, UK), passed through a computer-controlled attenuator (TDT PA4; Tokyo, Japan or PA5) and broadcast from a speaker (Sony MDR-ED228LP or Radio Shack piezo horn tweeter; Fort Worth, TX, USA). Stimulus timing and amplitude were controlled by computer. Stimulus levels were calibrated with a probe microphone (B&K Type 4182; Naerum, Denmark).

Behavioural recordings

We measured phonotactic walking responses with both freely walking (closed loop) and tethered flies (open loop). Responses of freely walking flies were recorded in two ways, using standard and high-speed video (see below). Standard-speed video recordings were used to capture complete phonotactic responses to single stimulus presentations from which we extracted trajectory and velocity information for comparison with open loop recordings (tethered flies, see below). High-speed recordings were used to obtain a detailed description of the walking patterns flies displayed during phonotactic responses.

High-speed recordings

We made high-speed video recordings at 1000 frames s^{-1} (Redlake Motionscope HR2000; San Diego, CA, USA), using a macro lens (Nikon Micro-Nikkor 55 mm; Tokyo, Japan) at a distance of 1 m. This provided a viewable area of approximately 5 cm^2 with a resolution of 240×210 pixels. The movement of individual limbs was clear and the animal could walk 3–4 steps before moving out of the viewable area. Variation in the sound field within the viewable area of the arena was ±2 dB.

We centred untethered flies on a platform between two speakers separated by 40 cm. A fibre optic ring light (5 cm diameter) placed 6.5 cm above the animal illuminated the immediate area around the fly. With the room lights turned off (trial conditions) the speaker locations were dark. The video camera was mounted above the ring-light. The arena centre and radial angles relative to the speakers were marked on a video monitor on which the flies were displayed during experiments. By placing the flies on a blank sheet of paper in the arena, we could rotate or position them to allow precise placement relative to the fixed speakers. In most cases, gravid females were quiescent in the absence of acoustic stimulation. Under these conditions, we could control the angle of the fly relative to the speakers to within 10°. Occasionally flies took flight immediately upon being placed in the arena. These individuals were not tested further.

In these experiments, we positioned flies with their body axis perpendicular to the direction of the speakers and randomly presented stimuli from either of the two speakers (i.e. right or left side of the arena). The direction in which flies were facing along this perpendicular axis was randomized between trials (i.e. front or back of the arena). We recorded details of limb and body movements during the initiation of phonotactic turns. These measurements were derived from frame-by-frame analysis of high-speed video recordings. We recorded responses to single presentations for speaker positions of 0° (straight ahead), 90° (perpendicular to midline axis) and 180° (directly behind).

Standard video recordings

Phonotactic responses were recorded with a video camera (Panasonic WV-GP460; Matsushita Electric Industrial Co., Osaka, Japan) mounted above the arena and VCR (Hitachi DA4; Tokyo, Japan). The analog video data were digitized at 15 or 30 frames s^{-1} using Adobe Premiere 6.0 and Cinepak compression codec (Radius). Digitized video clips were imported into motion analysis software (Midas 2.0, Xcitex; Cambridge, MA, USA) to extract distance, velocity and direction of movement frame by frame. Setup and stimulus

presentation were similar to high speed recordings, except that flies were 40 cm from the speakers.

Responses of tethered flies

We measured the responses of tethered flies on a spherical treadmill (Mason et al., 2001) that transduced the locomotor responses of flies fixed in position relative to the sound source. Flies were attached to a wire with low-melting-point wax applied to the dorsal surface of the thorax. Using a micromanipulator under red light, we then placed mounted flies in a normal walking position on a spherical treadmill consisting of an optically actuated computer pointing device (Logitech Marble Mouse; Fremont, CA, USA) that was modified to hold a lightweight (2.5 g) hollow plastic sphere floating on an air stream. A random dot pattern on the sphere activated the optical sensor when the fly's walking movements rotated the ball. The fly's virtual trajectory was recorded by computer (40 Hz sampling rate), using custom software. The treadmill was located at the centre of rotation of a speaker that was attached to a moveable arm at a distance of 12 cm from the position of the fly. The speaker could be rotated through 40° azimuth on either side of the midline axis of the fly and positioned with an accuracy of 0.5°. An additional pair of fixed speakers was located at ±90°. Stimulus levels were monitored during experiments with a probe microphone (B&K Type 4182) positioned within 0.5 cm of the fly's tympani. The treadmill was calibrated by rotating the sphere by a measured distance (1 cm) in the *x*- and *y*-axes (representing lateral and forward/backward movements, respectively). Data were captured as coordinates representing cumulative displacement in these two axes relative to the position at stimulus onset. The spatial resolution of the system was 0.1 mm. From these data we calculated the walking paths of flies in equivalent real-world distances and directions. References below to locations and distances during the course of open loop phonotactic responses are derived from these virtual paths.

Data analysis

We recorded phonotactic responses from 19 flies on the treadmill. Flies did not always continue to respond long enough to allow measurements at all stimulus angles. Sample sizes for different datasets are given in figure legends. At least 10 responses were recorded for each stimulus condition. Data from repeated responses for individual flies were averaged and individual averages pooled across flies. Unless otherwise indicated, data are presented as means ± S.E.M. For comparisons of different stimulus conditions, flies' angular headings were calculated in two ways: (1) the overall response angle and (2) the instantaneous angular heading (Fig. 1). (1) The overall response angle was determined by calculating two lines, one determined by the starting position of the fly and a point defined by the location of the fly at the midpoint of the response (i.e. the position of the fly when it was half way between its starting position and its final position at the end of the response) and the other by a line following the midline axis of the fly before stimulus presentation. This was taken as the
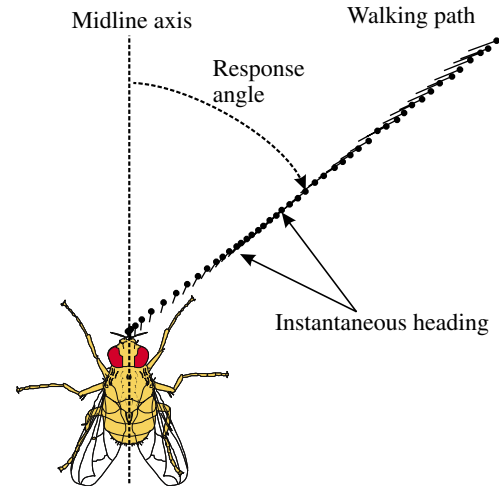

Fig. 1. Calculation of two measures of the directional orientation of *O. ochracea* phonotactic responses. Symbols indicate the location and angular heading of a fly at each time-step along its walking trajectory. The arc represents the overall orientation of the response as an angle measured from the midline axis of the fly and a point on the walking trajectory halfway between the starting and end points of the response.

overall orientation for that response. (2) The instantaneous angular heading of the fly was calculated by converting the location of the fly, relative to its starting point, to polar coordinates at each time-step. We also calculated the instantaneous speed and rotational velocity of flies over the course of phonotactic responses. Statistical analyses were carried out using Matlab (version 6.5) and R (version 1.9) software.

Results

Freely walking flies

Turn kinematics

We recorded kinematic details of 92 phonotactic responses in 15 females. For a sound source located at 0°, initiation of movement was highly stereotyped (Fig. 2A). Flies extend both prothoracic legs and lunge forward when these legs contact the substrate. This is followed by forward movement of the mesothoracic leg of one side and the metathoracic leg of the other side to initiate a typical insect tripod gait (Wilson, 1966).

For a sound source direction of 90°, the motor patterns involved in the initiation of a phonotactic turn were not stereotyped. We observed three different patterns (Fig. 2B–D). The most common (*N*=42 turns) was for a turn to be initiated with the ipsilateral prothoracic leg (Fig. 2B). Nearly as frequently (*N*=30 turns), turns were initiated with a movement of the contralateral prothoracic leg (Fig. 2C). The remaining turns (*N*=18) were initiated with movement of the contralateral mesothoracic leg (Fig. 2D). For speakers located to the rear, the flies turned through 180° to orient towards the sound source. Turns were initiated to the right and left with equal frequency. Variations in the motor patterns associated with the
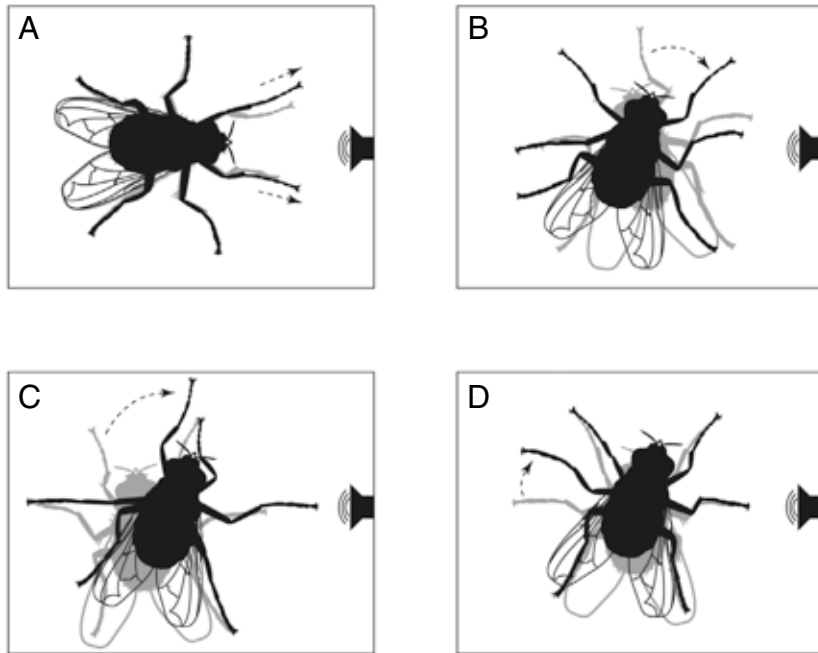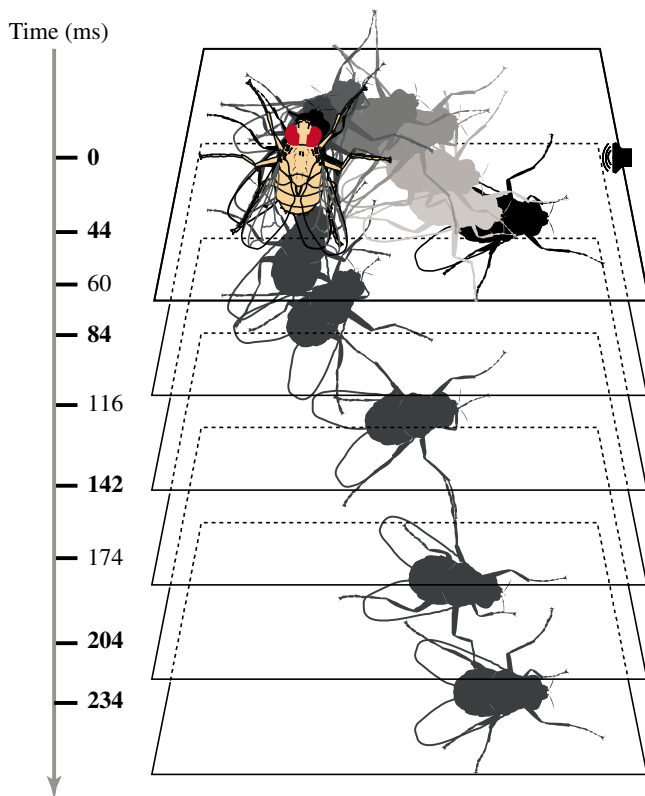
Fig. 2. Initial movements of flies at the onset of walking phonotaxis are not stereotyped. Flies lunge forward with both prothoracic legs in response to a sound source directly ahead (A). In response to a lateral sound source, flies may initiate phonotaxis by stepping with either the ipsilateral (B) or contralateral (C) prothoracic legs, or with the contralateral mesothoracic leg (D).

that direction. In this example, the initial movement in response to sound onset was a movement of the contralateral mesothoracic leg that occurred with very short latency (28 ms). The fly had turned through 25° and translated forward a full body length in only 84 ms, and was oriented to within 10° of the sound source within 142 ms.

Walking speed and distance

Phonotactic responses to the presentation of single stimuli were analysed using standard video recordings (Fig. 4). In these recordings the sound source was located either directly ahead of the fly (0°) or laterally (90° right). We calculated the path, distance and walking speed of flies over a 500 ms interval from stimulus onset. This was sufficient duration for the flies to reach their final heading. Walking paths were very consistently oriented in the direction of the speaker (Fig. 4A,B). Flies accelerated over the duration of the stimulus and decelerated (usually to a complete stop) following stimulus offset (Fig. 4C–F).

Responses to a lateral sound source were somewhat slower and more variable than for a source at 0°. There was a trend for flies to walk farther for a sound source at 0° than 90°, but this difference was not significant. Flies covered a distance of 1.63±0.11 cm in response to a single 200 ms chirp at 0° and 1.15±0.33 cm for a source at 90° (t=1.22, d.f.=13.894, P=0.242, Fig. 4C,D). Peak walking speed was faster for a 0° source (7.84±1.22 cm s^{-1}), compared with a 90° source (2.87±0.91 cm s^{-1}) (t=2.1787, d.f.=14.412, P=0.046, Fig. 4E,F). Although they walked with lower maximum speeds, flies also tended to continue walking longer following the end of a stimulus at 90° (although this difference was not significant). For this reason, the difference in total distance is less evident than the difference in walking speed for 0° *vs* 90° source locations.

We also recorded the responses of flies for a range of stimulus levels (42–96 dB SPL) and measured the total distance flies walked in response to a single stimulus presentation. Surprisingly, the total distance of phonotaxis did

initiation of 180° turns were similar to 90° turns but they were not analyzed in detail.

Fig. 3 shows a typical turn sequence in response to a 90° sound source. Flies did not show separate orientation and locomotion responses. Rather, turning and forward translation begin simultaneously such that the fly walks in a tight curve until it faces the sound source and then continues to walk in

Time (ms)

0
44
60
84
116
142
174
204
234

Fig. 3. Frame-by-frame sequence of walking phonotaxis in response to a lateral sound source. Time is indicated on the vertical axis (progressing downward). Top layer shows complete sequence and lower layers repeat selected frames for clarity (times of selected frames indicated in bold). The fly initiates forward translation and rotation simultaneously and walks in a tight arc until oriented towards the speaker.

Fig. 4. Average responses of freely walking flies for speaker azimuth 0° (A–C, *N*=5 flies, 10 runs/fly) and 90° (D–F, *N*=7 flies, 10 runs/fly). (A,D) Mean walking path; (B,E) cumulative distance *vs* time from stimulus onset; (C,F) instantaneous velocity *vs* time from stimulus onset. Bold horizontal lines in B,C,E,F indicate stimulus duration.

not increase monotonically with stimulus level. At low stimulus levels (<66 dB SPL), the distance of phonotaxis increased with increasing stimulus level. At higher stimulus levels, however, the distance walked by flies in response to a single stimulus chirp tended to decrease, such that flies showed strongest responses (in terms of distance travelled) for intermediate levels (approximately 70 dB SPL, Fig. 5).

Tethered flies
Walking speed and distance

The treadmill sphere used to record tethered phonotaxis was relatively massive (2.5 g) compared with the flies themselves (mean mass ± S.D., 19.74±4.7 mg, *N*=10). Thus the force required by flies to rotate the sphere was considerably greater than that required to translate or rotate themselves and we expected some differences in the dynamics of tethered phonotaxis relative to freely walking conditions. Nevertheless, phonotactic responses recorded in tethered flies were qualitatively similar to freely walking conditions in the following characteristics. Flies typically responded with a short latency, oriented to the source location (Fig. 6A,B), accelerated through the duration of the stimulus and then decelerated following the end of the stimulus (Fig. 6C–F). Behavioural latencies were 93±3.8 ms for tethered flies *vs* 49±3.7 ms for freely walking (mean ± S.E.M.). But it should be noted that these two measures are not strictly comparable. For freely walking flies, we measured latency to the initiation of walking (i.e. first movement of any leg) with a resolution of 1 ms, whereas tethered latencies are for approximately 0.1 mm displacement of the treadmill sphere with a resolution of 25 ms. Flies covered a (virtual) distance of 1.39±0.08 cm in response to a single 200 ms chirp at 0° and 1.28±0.16 cm for a source at 90° (*t*=0.9691, d.f.=8.91, *P*=0.358, Fig. 4C,D). There were no significant differences in walking speed among responses to different angles (Fig. 4E,F; see below for statistical comparisons). Peak walking speed was slower on the treadmill, and since the time course of
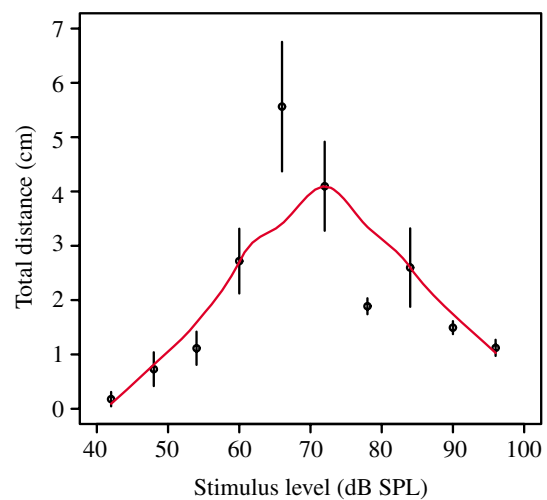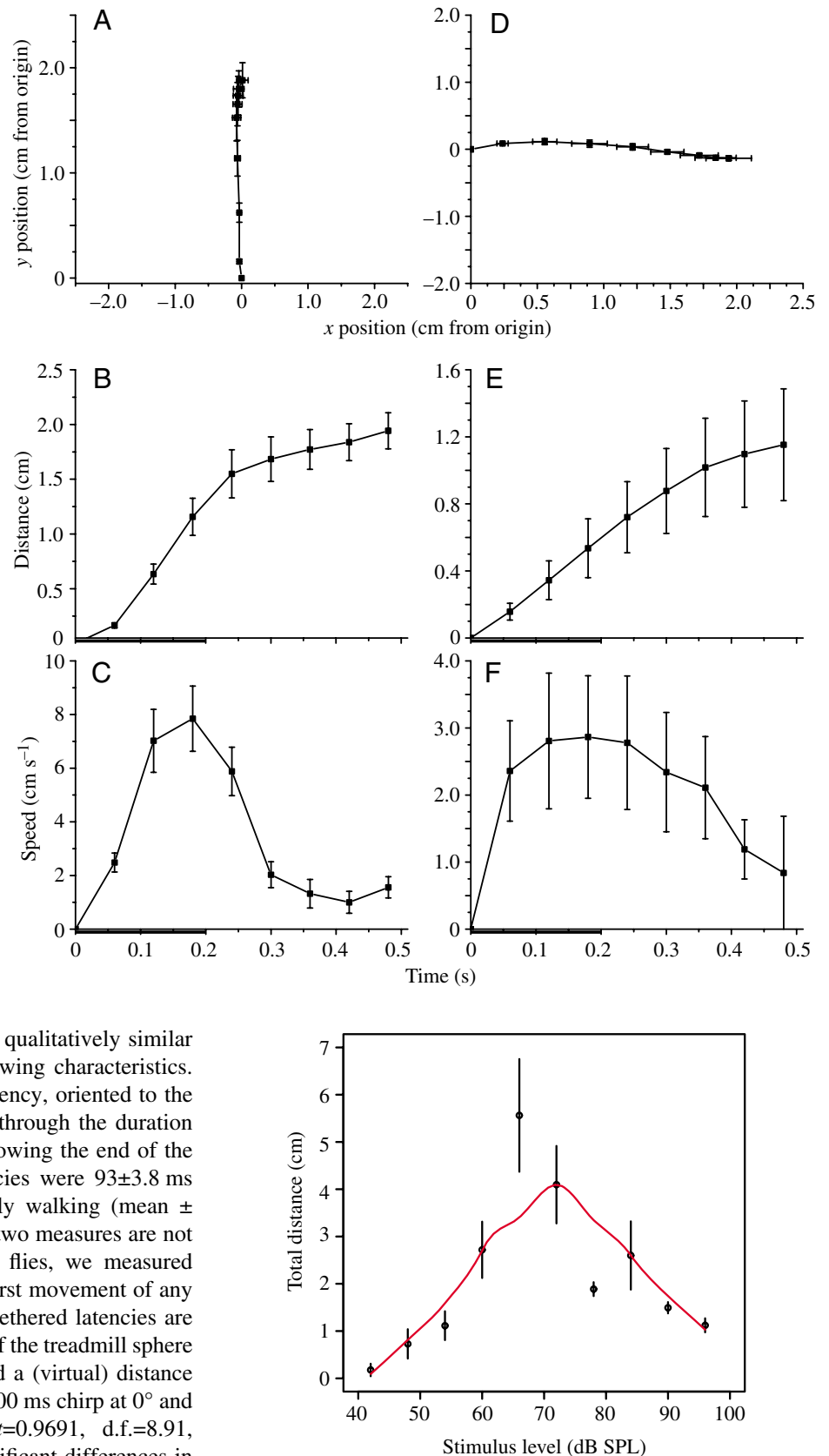
Fig. 5. Total walking distance in response to a single stimulus presentation with best-fit line (loess smoothing). Value are means ± S.E.M., *N*=16 flies, 5 runs/fly.
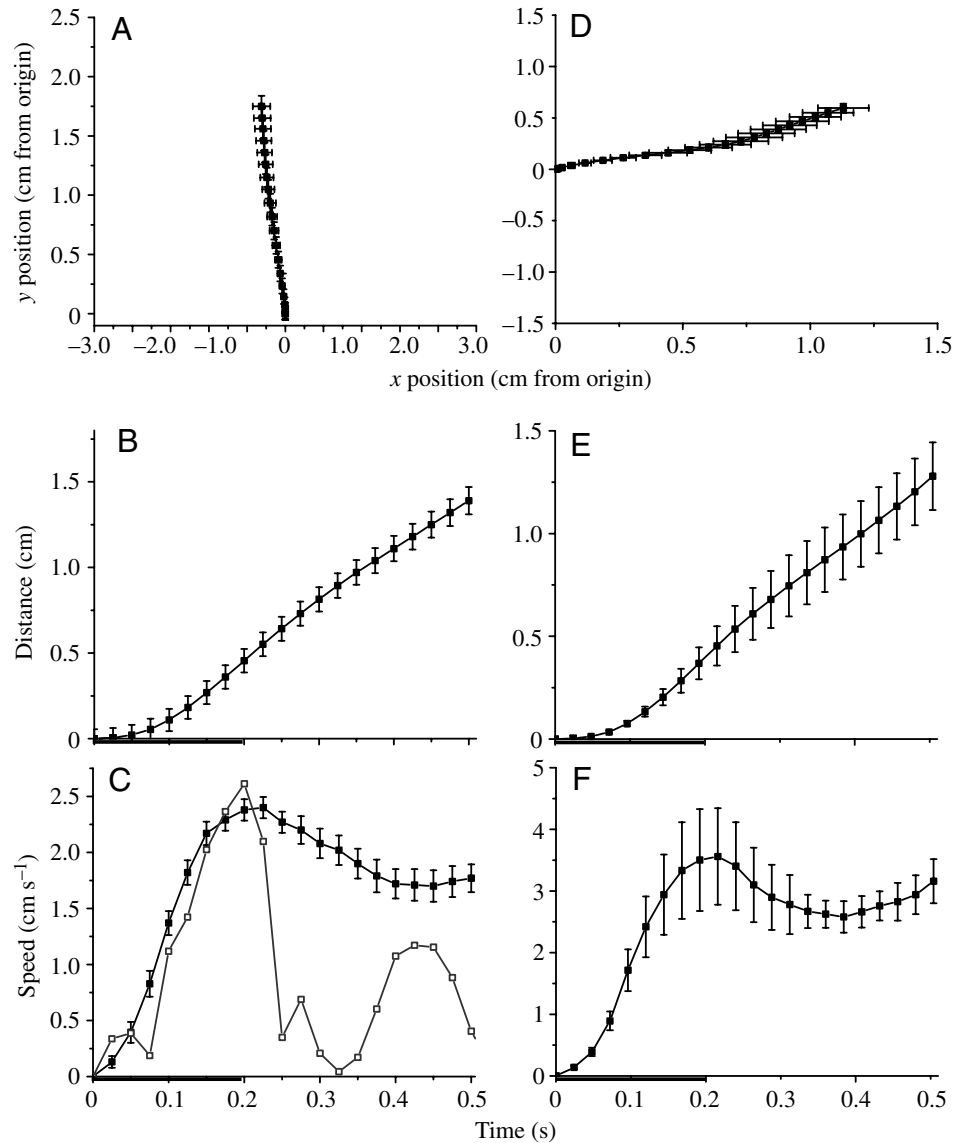
Fig. 6. Average responses of tethered flies walking on a treadmill for speaker azimuth 0° (A–C, *N*=18 flies, 10 runs/fly) and 90° (B–F, *N*=7 flies, 10 runs/fly). (A,D) Mean walking path; (B,E) cumulative distance *vs* time from stimulus onset; (C,F) instantaneous velocity *vs* time from stimulus onset. Open symbols and gray line in C show a single response in which the fly decelerated to a complete stop following stimulus offset. Bold horizontal lines in B,C,E,F indicate stimulus duration.

acceleration/deceleration during stimulus presentation was similar in both conditions, this meant that tethered flies covered shorter distances than freely walking flies during the duration of the stimulus. Tethered flies tended to continue walking beyond the duration of the stimulus-locked response, however, therefore the total distances walked by tethered flies over the recording interval were similar to freely walking flies at the same stimulus level (Figs 4B,E, 6B,E). There was a deceleration after stimulus offset, but this was quite variable even among different responses of the same fly. Although some individual responses demonstrated that flies were capable of stopping on the treadmill with timing similar to freely walking responses, in most cases tethered flies did not come to a stop by the end of 0.5 s recording interval (Fig. 6C,F).

Orientation

The pattern of changes in angular heading as flies oriented to the direction of the sound source was similar in freely walking and treadmill responses (Fig. 7A,B). Flies walked a curved path until oriented towards the speaker, and then continued in that direction. The initial stages of this orientation were somewhat compressed in treadmill responses, however, due to the slower walking speeds. In addition, walking beyond the duration of the stimulus in treadmill responses was less consistently oriented towards the sound source (Fig. 7B).

A more detailed summary of the time course of phonotactic orientation from treadmill responses is presented in plots of angular heading and rotational velocity *vs* time (Fig. 7C,D). Two phases of directional orientation can be distinguished in plots of instantaneous angular heading as a function of time following stimulus onset (Fig. 7C). Initially, flies' headings changed very rapidly, indicating that they were turning very sharply from their original position. Subsequently, the flies' angular heading remained more constant at an angle that varied with the location of the speaker. In other words, flies responded to a phonotactic stimulus with a rapid turn followed by a
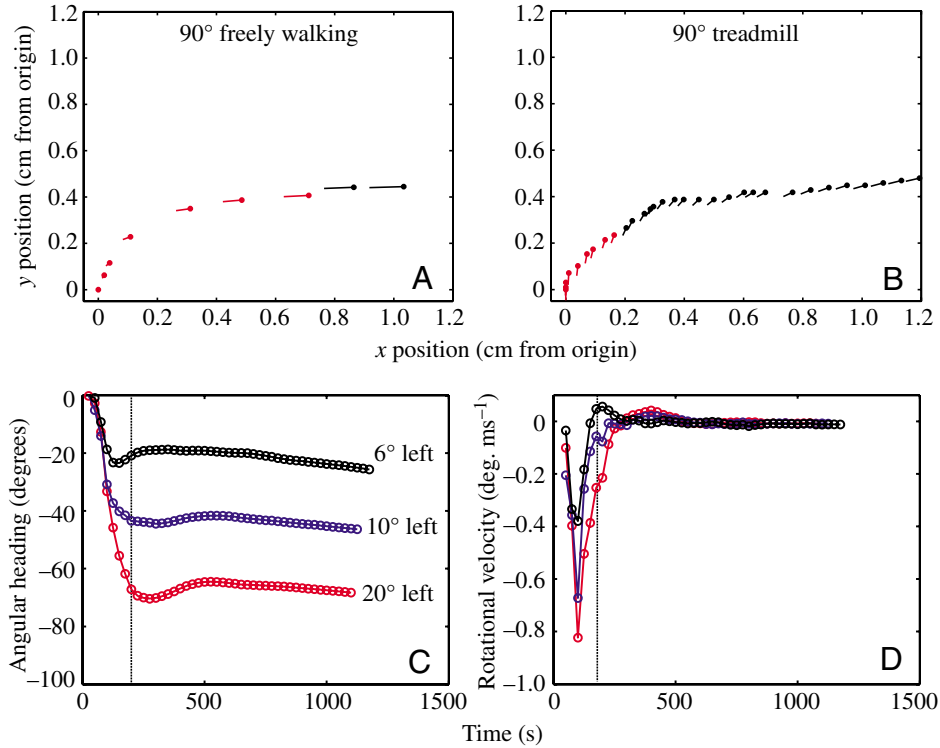
Fig. 7. Orientation of walking phonotaxis. Walking path for single responses of a freely walking (A) and tethered fly (B) to a speaker at 90°. Symbols indicate instantaneous heading at each time-step, with those in red indicating the duration of the stimulus. (C) Instantaneous angular heading *vs* time from stimulus onset for three different stimulus angles in a tethered fly. Plots are averages of 10 runs each. Vertical line indicates end of stimulus. (D) Rotational velocity *vs* time from stimulus onset for the same responses as shown in C. Peak rotational velocity occurs midway through the stimulus duration regardless of turn angle, and rotation decelerates before stimulus offset.

sustained run in a direction proportional to the speaker azimuth but with a tendency to overshoot the actual source azimuth. This overshoot results from the fact that, for treadmill responses, flies are tethered so their phonotactic responses do not result in a change in their position relative to the speaker. Therefore, unlike freely walking conditions in which the flies stop turning when they are oriented to the speaker, tethered flies continue to receive the same directional acoustic cues throughout the duration of the stimulus. This means that flies continue to rotate the treadmill beyond the point that would represent a correct orientation to the source azimuth. Responses to angles greater than approximately 30° tend to saturate at turn angles near 90° (Fig. 8B; see also Mason et al., 2001).
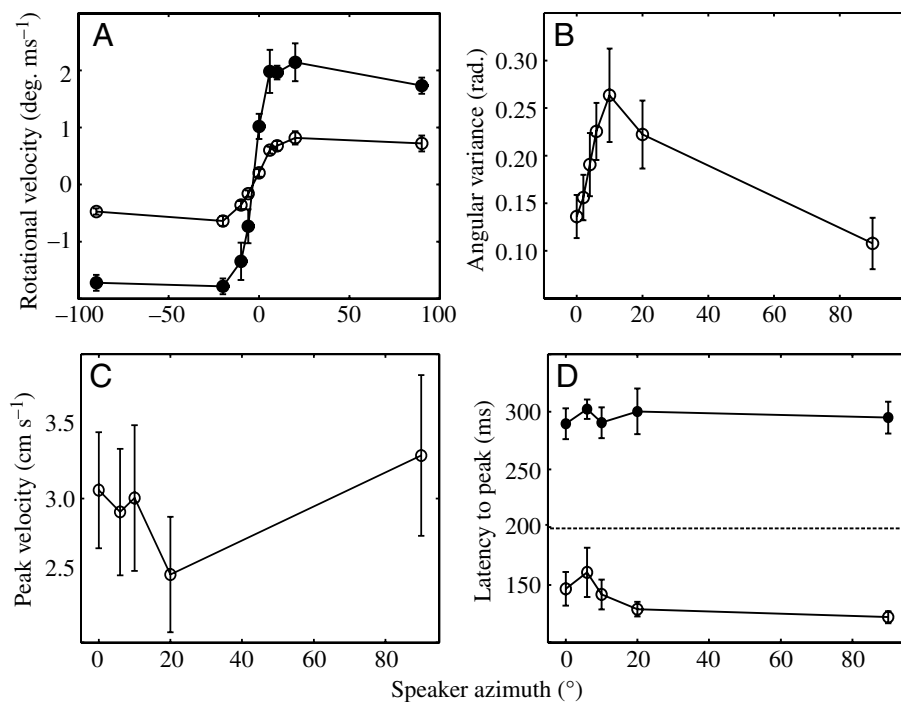

Fig. 8. Characteristics of phonotactic responses as a function of source azimuth. (A) Peak (filled circles) and average (open circles) rotational velocity vary systematically with source azimuth, but saturate at large angles. (B) Angular variance of response orientation varies with source azimuth. Orientation is most variable at intermediate angles. (C) Peak translational velocity is highly variable and does not show a consistent pattern of variation with source azimuth. (D) Neither latency to peak translational velocity (filled circles) nor latency to peak rotational velocity (open circles) differs significantly for different stimulus angles. Horizontal line indicates end of stimulus duration. Peak translational velocity occurs after the end of the stimulus, whereas peak rotational velocity occurs earlier than stimulus offset. Data are means ± S.E.M. (*N*=7 flies, 10 runs/angle/fly).

We calculated instantaneous rotational velocity from the derivative of angular heading (Fig. 7D). Rotational velocity increased rapidly at stimulus onset, but reached a peak and began to decrease before the end of the stimulus, such that flies usually reached their final heading before the end of the stimulus. This is surprising given that, as described above, the perceived directionality of the stimulus remains constant throughout its duration in these open-loop experiments.

We obtained several measures of the speed and orientation of phonotaxis for comparison of responses to different source azimuths. From the records of instantaneous rotational velocity (Fig. 7D) we extracted two measures: peak rotational velocity and average rotational velocity (measured over the stimulus duration) (Fig. 8A). We also measured the angular variance in the overall orientation of responses (Fig. 8B), the peak walking velocity attained during the response (Fig. 8C), and the latencies to both peak walking velocity and peak rotational velocity (Fig. 8D). For statistical tests, we compared responses for three angles (0°, 10° and 20°) spanning a range over which turn angles varied with source azimuth but did not saturate (see below). For these analyses, responses for left- and rightward angles of the same magnitude were pooled.

Rotational velocity (both peak and average) varied systematically and significantly with source azimuth for angles near the midline, but this pattern saturated at larger angles (average rotational velocity: one-way ANOVA, $F_{2,18}$=17.26, $P<0.0001$, peak rotational velocity: $F_{2,18}$=4.94, $P<0.02$, Fig. 8A). The variability of phonotactic orientation showed a similar dependence on source azimuth (Rao's test for equality of dispersions, S_R=14.71, d.f.=6, $P=0.022$). Angular variance increased with source azimuth for angles near the midline, but declined at larger angles (Fig. 8B). This decline in angular variance reflects a truncation of the distribution of response angles as the flies' turning response saturated at large angles. Walking velocity was highly variable and a comparison of 0°, 10° and 20° showed no significant difference (one-way ANOVA, $F_{2,18}$=0.49, $P=0.62$, Fig. 7C). Neither latency to peak walking velocity nor latency to peak rotational velocity varied significantly with source azimuth (latency to peak rotation: one way ANOVA, $F_{2,18}$=0.52, $P=0.60$, latency to peak velocity: $F_{2,18}$=0.13, $P=0.88$, Fig. 8D).

Discussion

Our results establish several quantitative features of walking phonotactic responses in *Ormia ochracea*. Responses are rapid (i.e. occur with short latency) but not stereotyped. Different motor patterns initiate movement even for similar stimulus conditions. The dynamics of freely walking and tethered phonotaxis are qualitatively similar. Flies begin walking at stimulus onset in a tightly curved trajectory. There is an initial period of the response during which the rotational velocity of the fly is high and this is followed by a more sustained run in one direction. Both rotation and translation vary systematically over the duration of the stimulus. Flies accelerate both rotational and forward velocity during the stimulus.

Translational velocity declines after stimulus offset; rotational velocity declines earlier. Walking distance is not proportional to stimulus level, but instead shows a maximum at intermediate intensities.

There were some differences between freely walking and tethered (treadmill) phonotaxis. Peak walking velocity is lower in tethered phonotaxis, consistent with the excess inertia of the treadmill sphere. Also, tethered flies tended to continue walking beyond stimulus offset (though with a marked deceleration), whereas freely walking flies tended to stop. It is unclear what causes the greater tendency for flies to continue walking on the treadmill. Two differences between freely walking and treadmill responses may account for this. (1) The greater inertia of the treadmill sphere (relative to the mass of the fly) may limit the flies' ability to stop its rotation. This seems unlikely because flies are able to impose rapid accelerations and directional changes on the treadmill sphere, and in some instances did bring it to a rapid stop. (2) The open-loop nature of the stimulus may amplify flies' locomotor responses. Under freely walking (closed loop) conditions, flies receive sensory feedback, signalling their orientation with respect to the stimulus location. On the treadmill, the initial directionality of the stimulus is maintained throughout the response (analogous to a moving target). This could delay or reduce the tendency of flies to decelerate as they approach the target direction.

We measured several dynamic features of phonotactic responses for a range of sound source azimuths: two latency measures (latency to peak rotational velocity, latency to peak walking velocity), two measures of rotational velocity (peak instantaneous rotational velocity, average rotational velocity from response onset to peak), and peak instantaneous walking velocity. Of these, only rotational velocity showed systematic variation with sound source azimuth. Furthermore, the relationship between rotational velocity and source azimuth (Fig. 8A) closely matched previous measurements of directionality in *O. ochracea* walking phonotaxis – both the overall orientation of phonotactic responses and interaural latency differences (a measure of auditory directionality) showed a pattern of response magnitude increasing with stimulus angle but saturating at the largest angles (Mason et al., 2001).

Previous analyses of phonotaxis in *O. ochracea* have examined flying phonotaxis (Mueller and Robert, 2001). This earlier study did not systematically examine the effects of source azimuth on responses, but used two source locations approximately 6° on either side of the midline. A further difference is that Mueller and Robert (2001) recorded free-flight responses in which flies were allowed to complete their approach to the sound source. Our results are derived from tethered (open loop) responses, or freely walking responses that correspond only to the onset and initial orientation of the response for a wider range of source azimuths. Nevertheless, some comparisons are justified.

In flying phonotaxis, three phases of the response were identified: take-off, cruising and landing (Mueller and Robert,

2001). Our results are most comparable to the take-off and cruising phases. During the take-off phase, flies gain altitude and orient towards the sound source. In the cruising phase, flies travel in the direction of the source at a more or less constant altitude, and then make a spiral descent in the landing phase. Similarly, our results for walking phonotaxis show an initial orientation phase in which accelerating forward translation is combined with accelerating rotation. This is followed by forward translation in a consistent direction that is proportional to the location (azimuth) of the sound source.

Mueller and Robert (2001) also observed that when the acoustic stimulus is discontinued during a phonotactic flight, flies are still able to complete their approach to the sound source. The accuracy of approach to the speaker location under these conditions decreases with earlier interruption of the stimulus. The authors conclude that flies obtain, during the early phases of the response, a measure of direction and distance to the sound source that they retain beyond the duration of the stimulus. Walking phonotaxis appears to be more strictly gated by the duration of the stimulus. This may simply result from the fact that a pause in forward movement during flight would be much more costly than a pause during walking and flies are therefore more committed to continued locomotion during flight. Nevertheless, in walking phonotaxis flies do continue for varying durations beyond the end of a stimulus – particularly in treadmill responses – and maintain the directional heading they establish during the stimulus (see Fig. 7C). If continued walking on the treadmill is due to inertial effects, then the sphere would tend to continue rotating in the same direction and consistent orientation of walking paths beyond the stimulus duration would be an artefact. However, another feature of walking responses is consistent with the possibility that flies obtain localisation information to be used independently of an ongoing stimulus. As discussed above, only rotational velocity varies systematically with source azimuth, and this parameter determines the final directional heading. Flies do not, however, simply rotate throughout the duration of the stimulus. Rather, rotational velocity peaks midway through and decreases during the latter part of the acoustic stimulus, so that flies have reached their final heading and stopped rotating at the end of the stimulus (or shortly thereafter; Figs 7D, 8D). This contrasts with translational velocity, which tends to accelerate throughout the stimulus and decelerate following stimulus offset. These details suggest that flies obtain directional information early in the stimulus. Possibly, walking interferes with the accuracy of directional hearing and flies must derive most of their directional information in the interval between detecting the stimulus and beginning to walk. Furthermore, our results suggest a simple mechanism for distance estimation by flies. For intermediate to high stimulus levels, phonotactic walking distance declines with increased stimulus amplitude. Assuming that crickets tend to call with similar amplitudes, stimulus level should predict source proximity. Flies may therefore reduce the distance they cover with each stimulus as they approach the source more closely. The weaker responses at low stimulus levels may

reflect a transition between sources at greater distances that elicit flying phonotaxis and closer sources that elicit walking phonotaxis.

Our results allow some inferences about the processing of localisation cues by the flies' auditory system that can be tested with neurophysiological measurements. First, flies derive a measure of the directionality (not simply laterality) of a sound source. This is consistent with previous results showing that flies discriminate source locations on the same side of the midline that differ by only a small angle. In other words, the flies obtain a graded signal of binaural disparity that encodes source azimuth. Second, directionality of responses is determined by the rotational component of phonotaxis. Flies don't run faster or longer, or turn longer for larger angles, they simply turn faster. Therefore the rotational component of phonotactic responses reflects the underlying binaural disparity cue that encodes sound direction. The pattern of variation in rotational velocity with source azimuth is strikingly similar to the pattern of interaural latency difference in auditory receptors (a putative neural code for auditory directionality; Mason et al., 2001). This allows for more precise testing of candidate directional codes in neural responses. Finally, these results also suggest possible mechanisms for multiple stimulus characteristics to be encoded in the sparse responses of auditory receptors (Mason et al., 2001; Oshinsky and Hoy, 2002). Flies are sensitive to differences in stimulus amplitude (Wagner, 1996; Ramsauer and Robert, 2000). Source azimuth could thus be encoded as interaural latency differences that ultimately affect the rotational component of phonotactic responses. Stimulus amplitude may be independently encoded by variation in response amplitude to affect speed and/or duration of phonotactic responses.

Such a mechanism would not exclude the possibility of binaural differences in response amplitude also playing a role in directional coding, as suggested by Oshinsky and Hoy (2002), but this may be significant only for large angles. Our analyses demonstrate that, even for the largest stimulus angles, flies have oriented to the sound source azimuth within 300 ms and then continue to walk with little meander (see also Mason et al., 2001). For most of the phonotactic approach, therefore, flies are maintaining a course with only a small error angle relative to the sound source. Because of the physiology of *Ormia* auditory receptors (phasic, sound-onset responses with intensity-dependent variation in spike latency, but not in spike rate or number), interaural differences in response amplitude are based on differential recruitment of receptors in the two ears. Such direction-dependent differences in receptor recruitment are due to amplitude differences in tympanal vibration (Robert et al., 1996) combined with threshold variation among receptors (Oshinsky and Hoy, 2002). In other words, interaural differences in response amplitude are due to differences in the number of receptors in each ear that are stimulated above threshold, with each receptor making an all-or-nothing contribution to response amplitude. In contrast, interaural latency differences are derived from pooling intensity-dependent variation in spike latencies of individual

receptors – potentially a more fine-grained measure of acoustic directional cues. Measurements of auditory directional responses have not been made for the smallest angles of sound incidence that *Ormia* have been shown to discriminate behaviourally. Physiological measurements of interaural latency and amplitude differences at small source angles are required to test these hypotheses.

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