

The cost of assuming the life history of a host: acoustic startle in the parasitoid fly *Ormia ochracea*

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SUMMARY

In the obligatory reproductive dependence of a parasite on its host, the parasite must trade the benefit of ‘outsourcing’ functions like reproduction for the risk of assuming hazards associated with the host. In the present study, we report behavioral adaptations of a parasitic fly, *Ormia ochracea*, that resemble those of its cricket hosts. *Ormia* females home in on the male cricket’s songs and deposit larvae, which burrow into the cricket, feed and emerge to pupate. Because male crickets call at night, gravid female *Ormia* in search of hosts are subject to bat predation, in much the same way as female crickets are when responding to male song. We show that *Ormia* has evolved the same evasive behavior as have crickets: an acoustic startle response to bat-like ultrasound that manifests clearly only during flight. Furthermore, like crickets, *Ormia* has a sharp response boundary between the frequencies of song and bat cries, resembling categorical perception first described in the context of human speech.

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Key words: acoustic parasitoid, categorical perception, convergent evolution, cricket, insect hearing, phonotaxis.

INTRODUCTION

Studies of evolution in mate recognition systems for reproduction have focused on communication within a species (Gerhardt and Huber, 2002) but the interspecific signaling that occurs between a parasite and its host holds equal evolutionary interest. In North American field crickets (Orthoptera: Gryllidae: *Gryllus*), males emit loud calling songs to attract conspecific females for mating. Females locate the male either by flying or walking to the sound source (Cade, 1979; Nolen and Hoy, 1986; Thorson et al., 1982). In many regions of the USA, local field crickets are parasitized by gravid female Tachinid flies native to North America, *Ormia ochracea* (Diptera: Tachinidae), that find their host via his mating call (Walker, 1993; Zuk et al., 1993). *Ormia* is known to be attracted to Grylline crickets that emit continuous trills (rather than discontinuous chirps) in their song. Thus, in the USA, *Ormia*’s known natural hosts are *Gryllus rubens* (in the southeastern USA, including Florida), *Gryllus integer* (in California and Arizona), *Gryllus texensis* (in Texas, Alabama and New Mexico) and *Teleogryllus oceanicus* (in Hawaii) (Cade and Otte, 2000; Cade and Tyshenko, 1990; Zuk et al., 1993). In any region, females of *Ormia* home in on the male cricket’s songs and deposit larvae, which burrow into the cricket, feed and emerge to pupate (Cade, 1975). Convergent evolution has endowed *Ormia* with specialized tympanal hearing organs that are similar to hearing organs in crickets (Robert et al., 1992; Robert et al., 1994), yet very unlike the Johnston’s organs characteristic of other auditory flies (Todi et al., 2004). These allow *Ormia* to hear and locate crickets, whose songs are far above the frequency range detectable by the more typical Johnston’s organs (Robert et al., 1996a). Indeed, *Ormia*’s auditory thresholds are lowest for carrier frequencies near those of the crickets they parasitize (Oshinsky and Hoy, 2002; Robert et al., 1992), and the primary function of these specialized tympanic ears is the acoustic localization of singing cricket hosts (Cade, 1975).

The auditory system of crickets displays similar adaptations, with behavioral and neural thresholds tuned to the carrier frequency of conspecific calling song (Moiseff et al., 1978; Zaretsky and Eibl, 1978).

When female crickets fly at night in search of male crickets they may be targeted and eaten by echolocating bats that emit ultrasonic (20–100 kHz) biosonar signals to detect and localize would-be prey. Nocturnally flying insects of five different orders perform an escape response to ultrasound; these include moths, lacewings, locusts, mantises, beetles and katydids (Forrest et al., 1995; Hoy, 1989; Moiseff and Hoy, 1983; Yager and Hoy, 1986). This escape behavior is classified as an acoustic startle response (ASR), in which rapid (i.e. tens of milliseconds), stereotyped, all-or-none movements away from the sound source and/or ‘landing’ leg-spread responses are elicited by pulses of ultrasound (Faure and Hoy, 2000; Roeder, 1967a). ASR in crickets has evolved in response to bat predation, and has co-opted the existing tympanal hearing system that primarily serves intraspecific communication (Hoy, 1989; Hoy et al., 1989). *Teleogryllus oceanicus* is an Australian field cricket (Orthoptera: Gryllidae: *Teleogryllus*) that was introduced to Hawaii and adopted as a host species by *Ormia* (Zuk et al., 1993). In *T. oceanicus*, ASR has two additional notable characteristics. First, it is dependent on behavioral context (Nolen and Hoy, 1984; Pollack et al., 1984), emerging strongly only during flight. This is evidenced by the observations that in tethered suspended crickets, a steering response to ultrasound requires flight motor activity (Nolen and Hoy, 1984), and that in walking crickets, only weak negative phonotaxis occurs to ultrasound (Pollack et al., 1984). Second, ASR-inducing frequencies are perceived categorically. Categorical perception, the existence of which has been demonstrated in many animals as well as humans, does not track precise sensory input but allows grouping of sensory information into useful response categories not present

in the raw stimuli (Kuhl, 2004; Miller et al., 2003). Although *T. oceanicus* have a very broad range of hearing (2–100 kHz) (Moiseff et al., 1978), they respond to cricket-song-like frequencies (2–6 kHz) differently than to bat-signal-like ultrasonic frequencies (20–100 kHz), demonstrating a sharp categorical boundary at about 12–16 kHz (Wyttenbach et al., 1996).

Ormia are also at risk of bat predation, as they too must fly at night to locate their cricket hosts. Although the tachinid ear evolved primarily for host localization, *Ormia*'s hearing may have been secondarily adapted for detecting bats, as indicated by its crepuscular activity and the presence of bats in its habitat. Indeed, *Ormia* shows neural and tympanic membrane sensitivity in the ultrasonic range (Oshinsky and Hoy, 2002; Robert et al., 1992; Robert et al., 1996b); however, until now, its behavioral responses to ultrasound have not been reported. From an evolutionary point of view, *Ormia* experiences similar selective pressures as its field cricket hosts: to use tympanic hearing to avoid predation while pursuing reproduction. This suggests that *Ormia*, like crickets, may demonstrate an evasive ASR to ultrasound, context-dependence of the ASR and categorical perception based on carrier frequency. The ASR in several other species has ultrasonic auditory thresholds similar to the neural response thresholds in *Ormia* (Hoy, 1989; Moiseff and Hoy, 1983; Robert et al., 1992; Yager and Hoy, 1986). *Ormia* might be expected to show evasive behaviors only during flight as predation is less likely to be a threat while on the ground under cover (Bailey and Haythornthwaite, 1998; Faure and Barclay, 1992; Fullard et al., 2005). The ability to parse cricket and bat signals into separate categories would improve the flies' ability to avoid predation while in pursuit of reproduction.

To determine whether *Ormia* exhibits evasive maneuvers in response to ultrasound, and to describe the nature of the response, four experiments were conducted. First, the attractive or repulsive nature of a wide range of carrier frequencies was measured during walking. Second, to test for an ASR during walking, positive phonotaxis to artificial cricket song was disrupted by ultrasonic and non-ultrasonic startle stimuli. Third, to test whether the ASR is dependent on behavioral context, the attractive or repulsive nature of cricket song *versus* ultrasound frequencies was measured during flight. Finally, to examine the categorical *versus* continuous nature of frequency perception, the steering responses to a wide range of carrier frequencies were measured during flight.

Our results show that ultrasonic stimuli evoke a strong evasive response only during flight, similar to the context-dependent evasive movements described in crickets. This negative phonotactic response is consistent with ASR and may indicate categorical perception. These elements of ASR also occur in at least one of *Ormia*'s field cricket hosts, *T. oceanicus*, indicating that parasite and host have solved the problem of predation in similar ways, suggestive of convergent evolution.

MATERIALS AND METHODS

Subjects

Ormia ochracea (Diptera: Tachinidae, Ormiini) flies were lab-reared from a natural population collected at sound traps in Gainesville, FL, USA [as in Walker and Wineriter (Walker and Wineriter, 1990)]. The colony was established in 1991, and the life cycle is approximately one month; thus, our experiments were conducted on ~180th generation flies. Flies were maintained on a 14 h:10 h light:dark cycle, and experiments were performed during their dusk or dark period, as *Ormia* is crepuscular. Gravid females were selected for experiments based on exhibiting a strong phonotactic walking response to an artificial chirp simulating the calling song of *G. rubens*, the preferred host of our Florida-caught flies.

Sound field calibration and auditory stimuli

Experiments were conducted in a 1.22 m × 0.91 m × 0.91 m anechoic chamber, with two speakers placed at a distance of 22 cm from the fly at ±45 deg. relative to the fly. Stimuli were generated *via* custom-written software (M.J.R. and G. Lott) in Matlab (MathWorks, Natick, MA, USA), converted to analog and attenuated (TDT System3 RP2.1 16-bit digital-to-analog converter and PA5 attenuator, Gainesville, FL, USA), amplified (Harman-Kardon HK6100, Stamford, CT, USA) and delivered through high-performance tweeters (All Electronics, Van Nuys, CA, USA). Stimuli were calibrated at the location of the fly using a Bruel & Kjaer (B&K, Norcross, GA, USA) type 4135 1/4-inch microphone (0 deg. angle of incidence) connected to a B&K model 5935 microphone power supply. The calibration system was checked against a B&K 4220 pistonphone calibrator. All signals had their main energy peak at the respective frequency, and harmonics were at least 30 dB lower. Sound pressure levels (dB SPL) throughout this manuscript are referenced to 20 μPa.

The auditory stimuli consisted of synthesized sound pulses (rise and fall time, 2 ms; this trapezoidal shape is very similar to pulses in natural cricket song) in a pattern designed to mimic the calling song of *Ormia*'s Florida host *G. rubens*, as our fly population originated in Florida. Florida *Ormia* prefers pulse rates matching those of its local host, and other *Ormia* populations are presumed to prefer pulse rates of their local hosts (Walker, 1993). We thus used 250 ms duration pulse trains with 10 ms pulses, presented at 45 pulses s⁻¹ (p.p.s.). These envelopes were applied to various carrier frequencies as appropriate for each experiment (described below).

Behavioral measurements

Flies were tested in the dark, using either tethered walking or flying paradigms.

Walking experiments

The strength of walking phonotactic responses to auditory stimuli was quantified by the response latency, duration, distance traveled and angular accuracy of phonotaxis. Flies were anaesthetized on ice for 5 min, tethered on their dorsal surface to a stiff wire with low-melting-point wax, the wire was attached to a micromanipulator and the flies placed on a spherical custom-built treadmill in a normal walking position. This treadmill was a custom-designed system ('flyball'), based around an optical mouse (MICRO Innovations PD430P, Arlington Heights, IL, USA) modified with an Agilent ADNS-2620 camera chip (Santa Clara, CA, USA) and interfacing with a custom-built microcontroller board, with a temporal resolution of 2.3 kHz (Lott et al., 2007). The fly sat on a floating table-tennis ball, and its movements were tracked by the camera chip. The device interfaced with TDT System 3 *via* custom Matlab software, which synchronized stimulus presentation with flyball movement. With this system, response latency differences of <0.5 ms were detectable.

Experiment 1: carrier frequency effect on walking phonotaxis

Flies were positioned 22 cm from speakers situated at +45 deg. and -45 deg. Artificial cricket chirps (see Sound field calibration and auditory stimuli above) were presented singly from either the left or right speaker at sinusoidal carrier frequencies of 3, 5, 7, 15, 20 or 24 kHz. The natural carrier frequency of both *G. rubens* and *T. oceanicus* song is ~4.8 kHz, and ultrasonic frequencies (≥20 kHz) are in the range emitted by echolocating bats (Cade and Tyshenko, 1990; Moiseff et al., 1978; Schnitzler and Kalko, 2001), although higher frequencies may be necessary to detect an insect of *Ormia*'s

size (see Discussion). As each carrier frequency has a different phonotactic threshold, all frequencies were presented from 50 to 100 dB SPL in 10 dB increments. Stimuli were randomized across frequency, speaker and amplitude, and each stimulus was presented 5–10 times from each speaker to generate mean responses for each fly. These means were then used to calculate group means, which are reported in the Results. To compare across frequencies, one amplitude level per frequency (among the several collected) from each fly was included in the group mean. This amplitude level either was 10 dB above each fly's phonotactic threshold (i.e. a different amplitude for each frequency within each fly) or was equal to each fly's threshold +10 dB for the least sensitive frequency (i.e. the same amplitude for all frequencies within each fly). If flies were no longer performing at the end of the experiment, thresholds were determined from the range of amplitudes presented across frequency in the main experiment ($N=6$ flies). As stimuli were presented in 10 dB increments, these thresholds have a certain range of uncertainty. Therefore, if flies continued to perform, thresholds were measured directly for all frequencies tested using an adaptive staircase procedure with 3 dB increments ($N=5$ flies) (Levitt, 1971). Any individual animal's intrinsic directional bias was eliminated by averaging equal numbers of responses to left and right speakers within each fly before computing group means.

Experiment 2: acoustic startle test during walking phonotaxis

Flies were positioned 22 cm from two speakers situated at 0 deg., one located 9 cm above the other. Artificial cricket chirps (5 kHz carrier frequency, 600 ms duration, 75 dB SPL) were presented from the lower speaker. Each chirp was interrupted at 50 ms, which was after phonotactic onset, with a 100 ms single-pulse startle stimulus from the upper speaker, after which the chirp resumed up to its 600 ms duration. The startle stimulus was either a pure tone of 5 kHz at 100 dB SPL, a pure tone of 24 kHz at 100 dB SPL or a period of silence. Thresholds are slightly lower for 100 ms pulses of 4.5 kHz (~72 dB SPL) versus 24 kHz (~87 dB SPL; see Results).

Flight experiments

For measurement of flight phonotaxis, *Ormia* were tethered as described above and positioned just above the treadmill (thus maintaining auditory calibration accuracy) and allowed to fly freely in response to stimuli. Flies were positioned 22 cm from speakers situated at +45 deg. and -45 deg. Only flies that demonstrated strong spontaneous flying upon recovery from anesthesia were tested. Phonotactic threshold to different carrier frequencies was measured in three flies using an adaptive staircase procedure with 3 dB increments (Levitt, 1971), and these values were applied to the remainder of animals tested (measurement was not possible in all animals as each would fly for only a limited duration). To score steering responses (see Data analysis below), video recordings were made using a Sony Digital Handicam (Sony, Tokyo, Japan) and recorded onto digital videotape (mini DV). A red LED was positioned behind and out of sight of the fly yet within the camera frame. It flashed in synchrony with each pulse of artificial cricket song, to allow latency measurements during ultrasound, when the signal was inaudible to humans. Flashing the LED alone did not elicit a steering response in the flies.

Experiment 3: steering and evidence for ASR during flight

Artificial cricket chirp stimuli were presented at a calling song frequency (5 kHz) and an ultrasound frequency (24 kHz). The order of presentation was semi-randomized across the two frequencies and the two speakers. To measure latencies of steering responses,

high-speed (1000 Hz) video was recorded using a Red Lake (San Diego, CA, USA) camera video system.

Experiment 4: evidence for categorical perception of calling song versus ultrasonic frequencies

Artificial cricket chirp stimuli were presented at carrier frequencies of 4.5, 5, 5.5, 7, 8, 9, 10, 11, 12, 13, 15 and 24 kHz in semi-randomized frequency order, with several (3–7) repetitions of each frequency at threshold +10 dB. Data were scored from videotape as described below.

Data analysis

Walking experiments

Custom-written Matlab software computed four measurements of walking phonotaxis: latency, duration, distance and angular accuracy. Overall analyses of variance (ANOVAs) were performed on each of these measurements. For significant ANOVAs, planned comparisons were then performed as described in the Results (Keppel, 1991). For example, with the assumption that *Ormia* respond most robustly to the frequency of cricket song (~5 kHz for *G. rubens* and *T. oceanicus*), planned *t*-tests compared 5 kHz responses with those at other carrier frequencies (3, 7, 15, 20 and 24 kHz), and Bonferroni corrections for multiple comparisons were applied. Angular errors (i.e. deviations from 0 deg.) were normalized to absolute values in order to measure absolute error rather than left versus right deviations. Equal numbers of +45 deg. and -45 deg. trials were presented for each fly to eliminate any directional bias (i.e. some flies reliably deviated slightly in one direction).

Flight experiments

Flight phonotaxis was scored blind from videotape. Responses were scored as (1) steering toward, (2) steering away, (3) no response, (4) landing response (i.e. leg splaying), (5) twitch or (6) unclear response (i.e. non-directional leg movement). The latter four non-directional steering responses were grouped as 'other', and the latter three were grouped as 'ambiguous'. Response types for each frequency were tallied within each fly and percentages of each response type were calculated. All data are reported as means \pm s.e.m.

RESULTS

Experiment 1: carrier frequency effect on walking phonotaxis

Walking phonotaxis of *Ormia* was measured using a high-speed spherical treadmill system (Fig. 1A) (Lott et al., 2007) in response to acoustic stimuli presented in a cricket chirp pattern but at carrier frequencies varying from below the natural calling song frequency (~5 kHz) to ultrasonic frequencies (24 kHz; see Materials and methods). This ensured that response differences were due to frequency rather than call pattern. All frequencies were presented at amplitudes 10 dB above phonotactic threshold, which was lower for calling song than ultrasonic frequencies (5 kHz threshold: 60.5 ± 2.8 dB SPL; 24 kHz threshold: 75.0 ± 2.3 dB SPL, $N=11$ flies; $t(10)=7.4$, $P<0.0001$). This made certain that responses would not be due to differences in perceived intensity.

Surprisingly, walking *Ormia* exhibited a clear positive phonotactic response to all carrier frequencies, including ultrasonic frequencies that were expected to be aversive. There were no differences in positive phonotaxis across frequencies (Fig. 1B–E), as measured by onset latency, duration of walking, angular accuracy or distance traveled (see Table 1; $N=11$ flies). When stimuli of varying frequencies were presented at identical amplitudes rather than at equivalent levels above threshold, the only measurement that differed across frequencies was distance (Table 1). With the

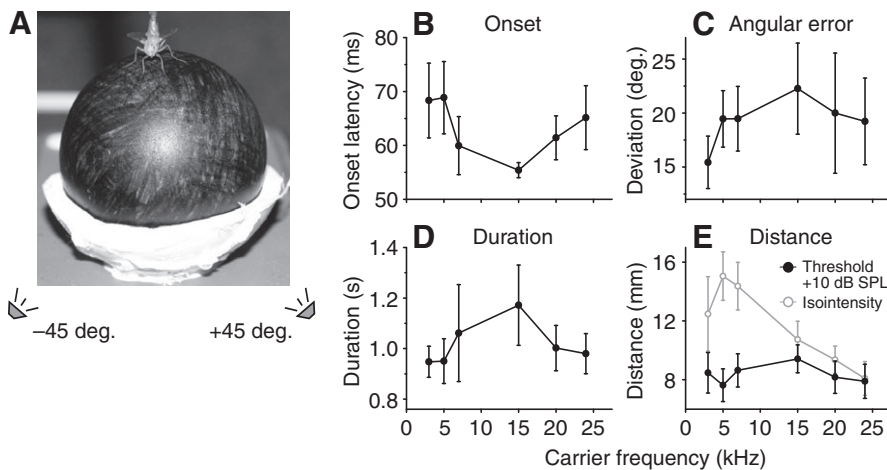


Fig. 1. During walking, *Ormia* performed equivalent positive phonotaxis across a wide frequency range. (A) The walking phonotaxis of a female *Ormia* was measured while auditory signals were presented from speakers located to the front-right (+45 deg.) and front-left (-45 deg.) of the fly. Artificial cricket chirps varying in carrier frequency from 3 to 24 kHz were presented at either threshold +10 dB (black circles) or at isointensity of 90 dB SPL (white circles). Neither (B) onset latencies, (C) angular deviation, (D) duration of running nor (E) distance traveled showed any differences across frequencies for stimuli presented at threshold +10 dB (see Table 1). At isointensity presentations (E, white circles), distance was maximal for 5 kHz *versus* ultrasonic frequencies, an effect that is presumably due to perceived intensity.

assumption that *Ormia* responds most robustly to the frequency of cricket calling song (~5 kHz), planned comparisons were performed comparing 5 kHz responses with those at each other carrier frequency (3, 7, 15, 20 and 24 kHz). These showed that flies ran further in response to calling frequencies than to ultrasonic frequencies (Fig. 1E; see Table 1; $N=11$ flies). Because this difference emerged only with isoamplitude stimuli, in which calling frequencies were presented further above the threshold than ultrasonic frequencies, it is possible that flies were responding to differences in perceived intensity rather than in frequency. Thus, carrier frequencies ranging from cricket-song through bat-bio-sonar levels elicit indistinguishable positive phonotaxis during walking.

Experiment 2: acoustic startle test during walking phonotaxis

To directly test for the existence of an ASR during walking, artificial cricket song at 4.5 kHz was presented from a speaker directly in front of the animal (at 0 deg.) to induce phonotaxis, and the signal was interrupted after phonotactic onset with a single-pulse startle stimulus, played from a speaker above the first. The startle stimulus was either a control (no startle stimulus), a pure tone of 4.5 kHz (calling song frequency for 100 ms at 100 dB SPL), a pure tone of 24 kHz (ultrasound for 100 ms at 100 dB SPL) or a period of silence (100 ms) in the ongoing cricket song. Neither the latency to walk nor the angular accuracy were affected by any of the startle stimuli but there was a main effect of distance traveled and walking duration across startle conditions (Fig. 2; Table 2; $N=15$ flies). Thus, ASR in the walking context was indicated by a disruption of positive phonotaxis, measured by a shortening of distance traveled or duration walked. With the assumption that an ASR should be largest to the ultrasonic stimulus, we performed planned comparisons comparing the 24 kHz response with the other stimulus responses for these two measurements.

Our data suggest that *Ormia* exhibits an ASR to ultrasound that is not very strongly expressed during walking. The distance traveled was significantly shorter with an ultrasonic startle stimulus than in any other condition (Fig. 2D; Table 2; $N=15$ flies). This result is particularly credible because calling song is normally a stronger stimulus than ultrasound: a short (100 ms) calling song pulse elicits walking from a stationary state at a lower amplitude than does a short ultrasonic pulse (4.5 kHz threshold: 72.3 ± 3.5 dB SPL; 24 kHz threshold: 86.7 ± 4.0 dB SPL, $N=6$ flies). However, the calling song stimulus was also effective in evoking an ASR: as measured by walking duration, there was no difference in the magnitude of the ASR evoked by ultrasound *versus* 4.5 kHz (Fig. 2C; Table 2; $N=15$ flies). The ASR that is expressed during walking is thus not limited to ultrasonic frequencies.

Experiment 3: steering and evidence for ASR during flight

As a first step, directional steering needed to be characterized, as it has not previously been described in *Ormia*. Flies were tethered and suspended to allow flight, and their steering responses were measured to artificial cricket chirps at carrier frequencies of either 5 or 24 kHz, from speakers positioned to the left and right of the fly. Fig. 3A–D shows still-frames from a videotaped experiment, depicting head-on views of a fly; videos are included in supplemental materials (see Movies 1 and 2 in supplementary material). Prior to a 5 kHz stimulus, the female fly's left hindleg was extended (Fig. 3A; in the absence of an acoustic stimulus *Ormia* may fly with hindlegs tucked or differentially extended). In Fig. 3B, the fly steered toward the cricket-like stimulus emerging from the speaker to the right of the fly (to the left when looking head-on at the fly). Steering was indicated clearly by the extension of the ipsilateral (right) hindleg (toward the speaker in this case). This was accompanied by a tilting of the head in the same direction. These movements are

Table 1. Effect of carrier frequency on walking phonotaxis

Amplitude	<i>N</i>	Onset latency	Duration of walking	Angular accuracy	Distance traveled
Threshold +10 dB SPL	11	$F(5,60)=0.93$, $P=0.47$	$F(5,60)=0.50$, $P=0.77$	$F(5,60)=0.34$, $P=0.89$	$F(5,60)=0.30$, $P=0.91$
Isointensity	11	$F(5,60)=2.1$, $P=0.08$	$F(5,60)=0.9$, $P=0.45$	$F(5,60)=0.5$, $P=0.77$	$F(5,60)=3.7$, $P=0.005^*$

*Planned comparisons for distance traveled during isointensity stimuli:
5 kHz *versus*:

3 kHz	7 kHz	15 kHz	20 kHz	24 kHz
$t(10)=1.5$, $P=0.08$	$t(10)=1.3$, $P=0.11$	$t(10)=2.9$, $P=0.009$	$t(10)=3.1$, $P=0.006$	$t(10)=4.0$, $P=0.001$

*Significant ANOVA (on distance traveled) allowing planned individual comparisons. Significant values are in bold ($P<0.05$).

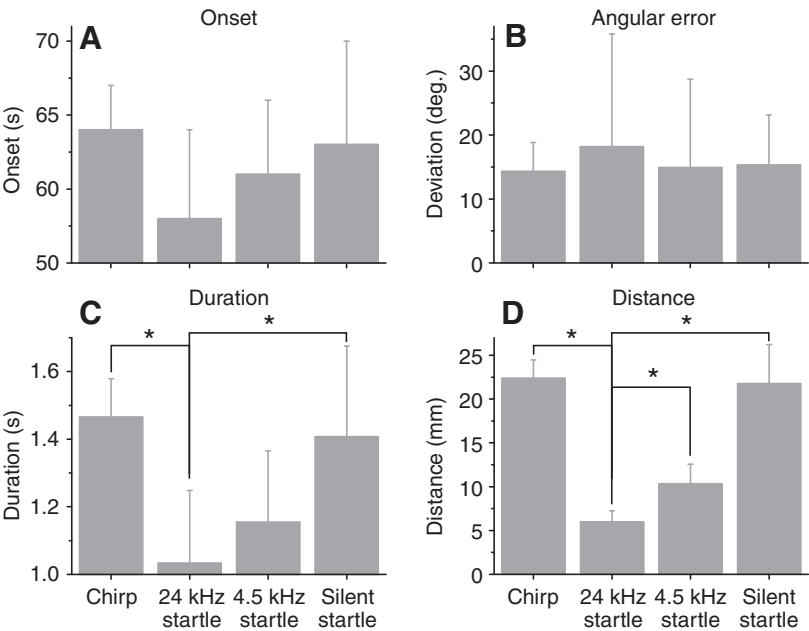


Fig. 2. During walking, ultrasonic frequencies elicited a mild acoustic startle response (ASR) that was somewhat stronger than that elicited by calling song frequencies. During ongoing positive walking phonotaxis (elicited by a 600 ms duration artificial chirp at 5 kHz), the (A) onset latencies and (B) angular deviation of phonotaxis were not altered by presentation of a brief, 100 dB SPL ‘startle’ stimulus intended to induce an ASR. Indicative of an ASR, the 24 kHz stimulus reduced the (C) duration run and (D) distance run significantly more than did a silent startle stimulus. However, in terms of duration, a 4.5 kHz stimulus elicited a startle response equivalent to the 24 kHz stimulus, indicating that the effect was induced by a broad frequency range.

similar to steering responses in several other insect species, including flies, crickets, locusts and moths (Götz et al., 1979; Govind, 1972; Moiseff et al., 1978; Robert, 1989; Roeder, 1967b). By these two indicators, flies exhibited positive flight phonotaxis to calling song frequencies.

Bat-like ultrasonic frequencies were tested next. A pulse train identical in temporal pattern but containing a 24 kHz carrier frequency abruptly elicited a steering response away from the speaker (negative phonotaxis). Fig. 3C depicts the same female, flying prior to the stimulus with its right hindleg extended; acoustic presentation of the 24 kHz pulse train from the right caused extension of the contralateral (left) hindleg and tilting of the head to the contralateral side, indicating steering away from the speaker (Fig. 3D). This negative flying phonotaxis occurred at shorter response latencies than positive flying phonotaxis, measured either by onset latency or latency to maximum leg extension [Fig. 3E black bars: onset of leg movement for positive phonotaxis: 58.5 ± 2.9 ms; for negative phonotaxis: 21.6 ± 0.4 ms, $t(5) = 13.66$, $P < 0.0001$; $N = 6$; gray bars: latency to maximum leg extension for positive phonotaxis: 253.5 ± 20.8 ms; for negative phonotaxis: 183.3 ± 21.2 ms, $t(4) = 2.91$, $P = 0.02$; $N = 5$]. The threshold for leg extension to calling song was lower than that to ultrasound [59.4 ± 1.5 dB SPL versus 87.5 ± 0.9 dB

SPL, $t(7) = 15.0$, $P < 0.0001$, $N = 8$]. Additionally, in four out of eight flies tested, an increase in amplitude of the ultrasound resulted in what appeared to be a ‘landing’ response (characteristic of ASR in other insects), where the flies splayed all legs outward [Fig. 3F; threshold for negative steering: 84.3 ± 0.8 dB SPL, threshold for ‘landing’: 95.0 ± 2.0 dB SPL, $t(3) = 4.3$, $P = 0.008$; $N = 4$]. These movements match characteristics of the ASR described in other insects.

Experiment 4: evidence for categorical perception of calling song versus ultrasonic frequencies

To test for categorical labeling, we used a paradigm originally designed for human listeners (and later, crickets): presenting stimuli along a continuum and measuring whether a categorical response emerges and the width/sharpness of the categorical boundary. We presented tethered flying *Ormia* females with pulse trains of 300 ms duration, ranging in carrier frequency from 4.5 to 23.5 kHz, from left or right speakers (+45 deg. or -45 deg.), at amplitudes +10 dB above threshold. Fig. 4A indicates that *Ormia* labeled frequencies near that of cricket calling song (5 kHz) as ‘attractive’ while labeling ultrasonic frequencies near those of bat echolocation signals (25–80 kHz) as ‘aversive’. This behavior is similar to the

Table 2. Acoustic startle test during walking phonotaxis

N	Onset latency	Duration of walking	Angular accuracy	Distance traveled
15	$F(3,20) = 0.5$, $P = 0.72$	$F(3,20) = 3.7$, $P = 0.02^*$	$F(3,20) = 0.2$, $P = 0.88$	$F(3,20) = 3.8$, $P = 0.01^\dagger$
*Planned comparisons for duration of walking: 24 kHz versus:				
	Chirp alone	5 kHz startle	Silent startle	
	$t(14) = 3.4$, $P = 0.002$	$t(14) = 1.2$, $P = 0.13$	$t(14) = 2.8$, $P = 0.007$	
† Planned comparisons for distance traveled: 24 kHz versus:				
	Chirp alone	5 kHz startle	Silent startle	
	$t(14) = 3.6$, $P = 0.002$	$t(14) = 2.4$, $P = 0.01$	$t(14) = 3.0$, $P = 0.005$	

*Significant ANOVA (on duration of walking) allowing planned individual comparisons.

† Significant ANOVA (on distance traveled) allowing planned individual comparisons.

Significant values are in bold ($P < 0.05$).

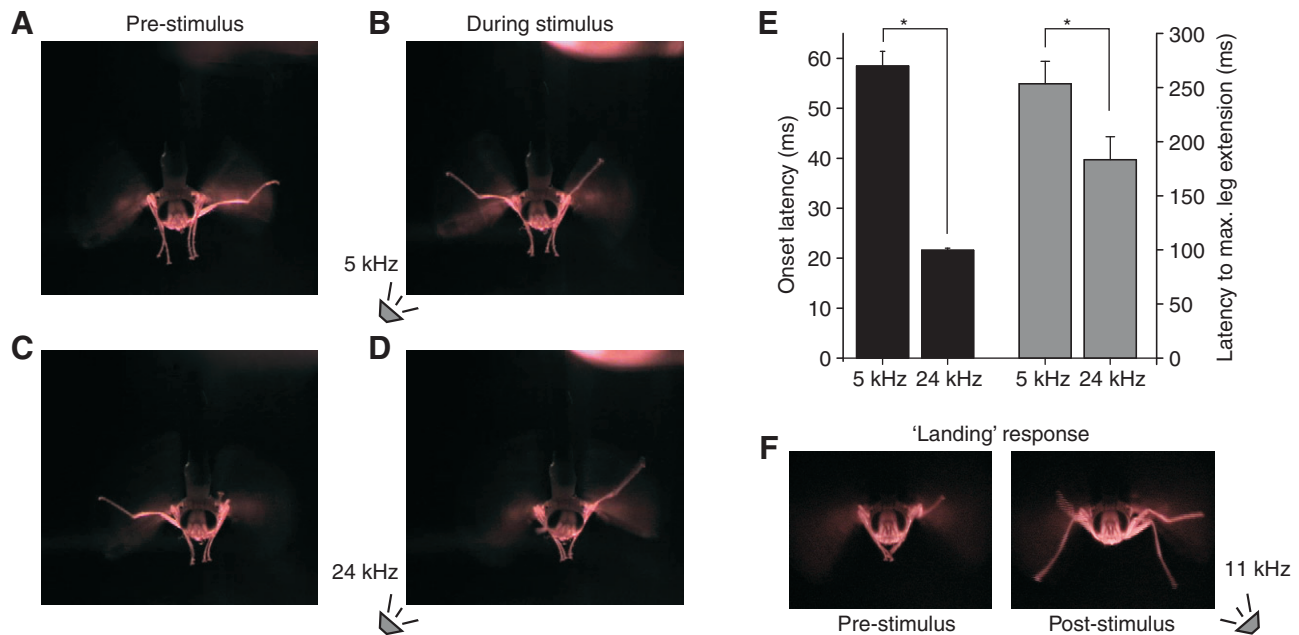


Fig. 3. Flying phonotaxis was described and measured in *Ormia* based on stereotyped steering movements, showing that *Ormia* flies towards calling song frequencies but away from ultrasound frequencies. Still frames depict head-on views of a flying *Ormia* (also see Movies 1 and 2 in supplementary material). (A) A female gravid *Ormia* flew with its left leg slightly extended. (B) Upon presentation of an artificial cricket chirp at 5 kHz from its right, it extended its right leg and tilted its head toward the right, indicating a positive flying phonotaxis. (C) Before stimulus presentation, the same *Ormia* flew with its right leg slightly extended. (D) Upon presentation of an artificial cricket chirp at 24 kHz from its right, it extended its left leg and tilted its head toward the left, indicating a negative flying phonotaxis (right panel). The bright spot above is an LED (behind and not visible to the fly during data collection) that was synched with the auditory stimulus. (E) Phonotactic latencies were faster for responses to ultrasound *versus* calling song frequencies, measured either by movement onset latency (left) or latency to maximum leg extension (right). (F) A 'landing' response, an aspect of the acoustic startle response (ASR), was elicited by an 11 kHz chirp at 100 dB SPL.

categorical labeling seen in *T. oceanicus* (Fig. 4B). Flies steered toward frequencies ranging from 4.5 to 7 kHz very reliably (>80%), and steered away from frequencies ranging from 12 to 24 kHz nearly as reliably (>60%), with a relatively sharp transition at 8–11 kHz. The transitional range was narrower (visible as a steeper slope) for positive steering than for negative steering. (All positive steering responses were significantly different from 50% except the response to 8 kHz, and all negative steering responses differed significantly from 50% except responses to 10, 11, 12 and 13 kHz; paired *t*-tests, *P* values <0.05; *N*=8.) This transition range also included a larger number of non-steering responses (scored as 'other') than in the calling song or ultrasound ranges, indicating greater ambiguity in the categorical boundary area. In Fig. 4C, these non-steering responses are broken down into non-responses and ambiguous responses (including landing, twitch and unclear leg movements). When no sound was presented, ambiguous responses did not occur: the flies remained still. The relative percentages of these two response types were equivalent across the frequencies tested [one-way ANOVA, interaction of frequency by response type: $F(11,1)=1.2$, $P=0.3$]. Thus, when a stimulus did not strongly elicit positive or negative steering (i.e. when a stimulus was in the mid-frequency range), *Ormia* was equally likely to either respond ambiguously or not respond at all.

DISCUSSION

We have described steering responses during flight in a tachinid fly *O. ochracea* that involve hindleg extension and head tilt in the direction of the turn. Using this indication of positive or negative flight phonotaxis, we have demonstrated that *Ormia* exhibits a clear evasive response to ultrasonic frequencies, manifest as negative

phonotaxis. This occurs strongly only during flight, and is thus behaviorally context-dependent. This evasive maneuver is consistent with an ASR, and suggests that *Ormia* may be perceiving frequency categorically. Our results indicate that the auditory behavior of *Ormia* can be mapped remarkably well onto that of *Ormia*'s cricket host *T. oceanicus*: positive phonotaxis to 4–6 kHz frequencies (i.e. those of cricket calling songs), negative phonotaxis to 15 kHz and above [i.e. those frequencies used by bats in their echolocation calls (Schnitzler and Kalko, 2001)], frequency-dependent decisions to steer toward or away from a sound and context-dependent escape movements. Thus, the auditory behavior of both *Ormia* and its hosts have adapted to the necessities of their environment, which requires assuming the risk of nocturnal bat predation in order to achieve reproductive success (Cade, 1975; Moiseff et al., 1978).

The similarity of the ASRs described in the present study implies possible convergent evolution between a parasite and its cricket host. However, these experiments compared *Ormia* flies native to one region (Florida) with crickets introduced to another (Hawaii). *Ormia* throughout the USA and Hawaii adapts to parasitize the local field cricket population, demonstrating behavioral preferences for its chosen host's song (Gray et al., 2007). These songs vary widely in temporal pattern but all have a carrier frequency of 4–5 kHz. *Ormia* across a wide geographical range is therefore under evolutionary pressures to behaviorally distinguish this frequency range from that of ultrasonic bat signals. Thus, *Ormia* introduced to Hawaii would be expected to exhibit the same adaptations as its Florida counterparts, although this remains to be demonstrated. It is also likely that *Ormia*'s Florida host *G. rubens*, like many cricket species, would exhibit frequency-dependent positive and negative phonotaxis (Nolen and Hoy, 1986) but categorization of the

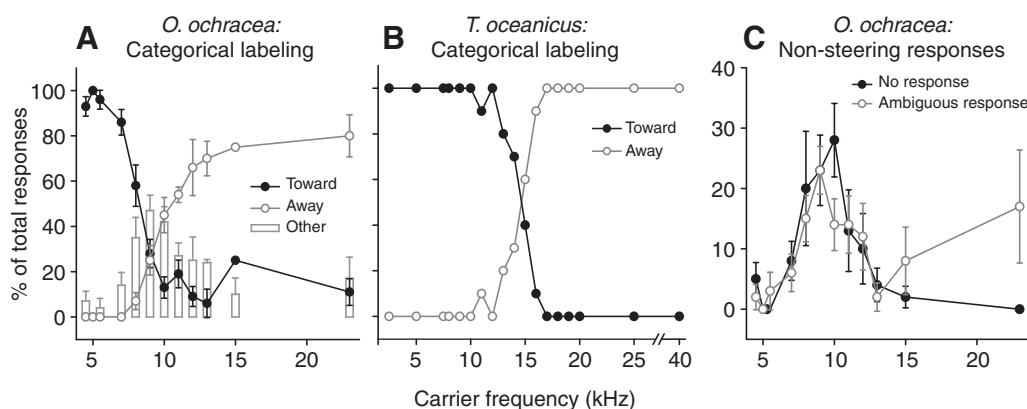


Fig. 4. Categorical labeling across a wide range of carrier frequencies. (A) *Ormia* performed positive flying phonotaxis reliably toward cricket chirps at frequencies lower than 9 kHz (black circles), and reliably performed negative phonotaxis away from cricket chirps at frequencies above 10 kHz (gray circles), indicating categorical labeling with a sharp boundary. Non-steering responses ('other') were maximal at the categorical boundary (white bars). (B) Flying crickets steered reliably toward low (cricket calling song-range) frequencies and away from ultrasonic (bat echolocation-range) frequencies, with a sharp boundary at 15 kHz (data replotted from Wytenbach et al., 1996). No ambiguous, non-steering responses were detected. (C) Non-steering responses for *Ormia* were broken down, indicating that the percentages of ambiguous responses versus complete lack of responses were equivalent across the entire frequency range.

frequency range and context-dependence of the ASR need to be tested in this species. The evolutionary timescale involved supports the possibility of convergent evolution: tachinid flies, all of which are parasites of insects and other arthropods, predate bats by ~50 million years (Stireman et al., 2006). *Ormia*'s dependence upon crickets was probably established prior to bat predation, putting pressures for evasive behaviors in both species under a similar evolutionary time frame. A convincing case for convergent evolution would involve a phylogenetic approach, examining close relatives of *Ormia* that have evolved under different pressures and thus lack the ASR and its characteristics. Appropriate relatives must possess the specialized tympanic ear (as the phenotype is auditory in nature); all such tachinids evolved this ear *via* convergent evolution with their Orthopteran hosts (Robert et al., 1999). Therefore, the only variation in pressure must be the presence or absence of bats as predators. *T. oceanicus*, the cricket host described here, is a resident of Tahiti, an island that has been absent of bats since its emergence 0.25–1.75 million years ago, although it is not known whether crickets inhabiting this island are parasitized by any tachinid. Moths endemic to the island have a reduced ASR at both behavioral and neural levels (Fullard et al., 2007), raising the possibility that the crickets, and their putative parasitoids, may have followed suit.

Positive walking phonotaxis in *Ormia* was cut short upon interruption with ultrasonic pulses. While indicative of a startle behavior, this response was non-directional, and a similar walking cessation occurred to a pulse of calling song (Fig. 2B,D). Thus, like crickets, who exhibit a weak negative walking phonotaxis to ultrasound (Pollack et al., 1984), *Ormia* shows only a weak and non-directional ASR in a walking context. *Ormia* exhibited negative phonotaxis in response to ultrasound only during flight (Figs 1C, Fig. 4A). In the flight context, this negative phonotaxis is similar in nature to evasive maneuvering described in other insects and can be classified as an ASR. Specifically, the response is shorter in latency than that to calling song, is directionally oriented away from the loudspeaker and is frequency sensitive. Furthermore, the 'landing' behavior at higher amplitudes suggests the response is graded with increased amplitude (Faure and Hoy, 2000; Hoy, 1989; Moiseff et al., 1978; Nolen and Hoy, 1986; Roeder, 1967a). This similarity to evasive maneuvering and ASR in other insects, along

with the nocturnal activity of *Ormia* and its coexistence with bats, suggests that *Ormia*'s negative phonotactic response during flight is an adaptive strategy for predator avoidance, although field studies documenting bat predation of flying *Ormia* are necessary to confirm this idea. One caveat is that theoretically the wavelength of an echolocation signal should be less than half the size of the object to be detected (Metzner, 2008), requiring an 80 kHz signal to resolve the body size of *Ormia* (~7×3 mm). However, aerial hawking bats using 20–30 kHz echolocation signals often prey upon Dipterans of sizes comparable with *Ormia* (e.g. Catto et al., 1994; Rydell, 1992; Sullivan et al., 1993). Detectable echoes from insects of these sizes are produced reliably at shorter distances (<4 m), and the increased resolution is likely to be due to reflected 'glint' artifacts from insect wingbeats (Schnitzler and Kalko, 2001; Waters et al., 1995). While there are no studies that have investigated directional hearing in *Ormia* above 12 kHz (behavioral) or 25 kHz (neural), *Ormia* should be able to detect frequencies of at least up to 100 Hz (Robert et al., 1992). Even so, it is important to verify that negative phonotaxis in *Ormia* is similar for higher frequency signals.

A subtle point of difference in the evasive response between host and parasite is in the nature of the walking ASR. In relation to predator avoidance, a walking ASR has little adaptive value to either crickets or to flies that have landed in search of cricket hosts. Stationary male crickets (and walking females attracted by their calls) call primarily under cover of grass or brush, reducing the risk of predation from gleaning bats (which rely primarily on prey-emitted sound) and eliminating vulnerability from aerial hawking bats (Bailey and Haythornthwaite, 1998; Hofstede et al., 2008). Therefore, predator-related pressures are unlikely to have influenced walking responses to ultrasound, and would have no differential influence across species. However, in relation to mate or host finding, the details (and differences) of a walking ASR are much more relevant. Walking phonotaxis to ultrasound is weakly negative in crickets (Pollack et al., 1984), yet weakly positive in *Ormia*. We suggest that this is because the two species are under different evolutionary pressures in a walking context. Positive phonotaxis in crickets is driven by sexual selection: females use the parameters of male calling song as a reliable indicator of mate quality (Wagner, 1996; Wagner and Harper,

2003), such that indiscriminate positive walking phonotaxis could result in low-quality mating. *Ormia* females have no such pressure to be discriminating during walking, in fact quite the opposite: the cricket host need only survive for 10 days until *Ormia* larvae develop and emerge, rendering the genetic quality of the host irrelevant. Importantly, when *Ormia* females walk toward a host, they have already effectively localized that host based on acoustic preferences during flight. Sound localization in *Ormia* consists first of a flight phase in which flies show preferences for elements specific to their cricket host's song (Ramsauer and Robert, 2000; Walker, 1993); this phase produces a flight trajectory culminating in accurate localization within 10 cm of the target sound (Mueller and Robert, 2001). In the second phase, flies walk the remaining short distance to the host, during which (as our data indicate) they do not discriminate based on frequency. By this point, any strong signal is most likely to be that from the cricket host, lessening the importance of discrimination during walking compared with quick localization of the host. By this logic, *Ormia* should exhibit better discrimination during flight than walking, which we have shown holds true for frequency-based discrimination.

Our experiments indicate that *Ormia* may perceive the continuum of frequency categorically (Fig. 4A). Categorical perception, which has been demonstrated in crickets, birds and non-human primates as well as humans, can structure the acoustic environment to improve sound recognition and facilitate quick accurate responses in life-and-death situations (Ehret, 1987), a benefit to both flying crickets and flying *Ormia* who must quickly discriminate between reproductive target and predator. Categorical perception in *T. oceanicus* is revealed by bimodal responses to a continuous frequency range, where at a sharp transition point they flip from steering toward low frequencies to steering away from high frequencies, with no distinction within members of a category (Wytenbach et al., 1996). Thus, crickets fulfill both requirements for categorical perception: (1) stimuli from a continuum (i.e. frequency) are labeled as separate classes, and (2) stimuli across categories are well discriminated whereas stimuli within categories are not well discriminated (Ehret, 1987). In the present study we have shown that *Ormia* fulfills the first requirement, flying toward low frequencies and away from high frequencies with a sharp categorical boundary between 8 and 10 kHz, indicating that frequencies near that of cricket calling song (5 kHz) are labeled as attractive while frequencies nearer those of bat echolocation signals (25–80 kHz) are labeled as aversive. However, the increase in ambiguous responses around this category boundary may be an indication of difficulty in discriminating in this range. Unfortunately it was not possible in *Ormia* to directly test whether stimuli within a category are poorly discriminated, which would be compelling additional evidence for categorical perception. The higher number of ambiguous responses around the boundary may speak to the neural mechanisms driving positive *versus* negative phonotaxis (see below).

Neural data from *Ormia* and other species may speak to mechanisms that subserve context-dependent gating and categorical perception in *Ormia*. The mechanism underlying context-dependent gating has been revealed in crickets and locusts. Aversive steering to ultrasound is mediated by a single neuron in crickets, which reaches threshold during ultrasound stimulation but is inhibited by calling song frequencies, and is thought to be gated by flight motor oscillatory input (Hoy and Nolen, 1987; Nolen and Hoy, 1987). Such a mechanism also exists in the locust nervous system, where flight-generated oscillatory activity onto thoracic motoneurons gates the transmission of convergent sensory input, such that

sensory-driven steering information only influences neuronal firing during the correct phase of flight-generated input (Reichert and Rowell, 1985). The neural substrate of context-dependent gating in *Ormia* is unknown but may utilize a similar mechanism. Categorical perception, by contrast, may be influenced by auditory receptor organization. Moths and crickets show behavioral non-discrimination within a broad range of ultrasonic frequencies, consistent with the broad tuning of only a few existing ultrasound receptors and of the neuron controlling evasive steering (Moiseff and Hoy, 1983; Waters and Jones, 1996). Many auditory receptors in *Ormia* (and the tympanic membranes that excite these receptors) are tuned to calling song frequencies but even those are quite broad in their tuning, and the receptors that respond best to ultrasonic frequencies are even more broadly tuned (Miles et al., 1995; Oshinsky and Hoy, 2002). The response ambiguity exhibited by *Ormia* at the category boundary (Fig. 4C) may be an effect of this broad tuning: mid-range frequencies may excite both low- and high-frequency afferents, resulting in a conflicting signal and potential lack of response. This behavior contrasts with that seen in the cricket, where few ambiguous or non-responses occur at category boundary, and where tuning curves of auditory afferents are less broad than in *Ormia* (Imaizumi and Pollack, 1999; Ramsauer and Robert, 2000; Zaretsky and Eibl, 1978). This neural organization could allow a strong sensory signal of a relevant frequency to reliably elicit a given motor response.

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