

RESEARCH ARTICLE

Auditory modulation of wind-elicited walking behavior in the cricket *Gryllus bimaculatus*

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ABSTRACT

Animals flexibly change their locomotion triggered by an identical stimulus depending on the environmental context and behavioral state. This indicates that additional sensory inputs in different modality from the stimulus triggering the escape response affect the neuronal circuit governing that behavior. However, how the spatio-temporal relationships between these two stimuli effect a behavioral change remains unknown. We studied this question, using crickets, which respond to a short air-puff by oriented walking activity mediated by the cercal sensory system. In addition, an acoustic stimulus, such as conspecific 'song' received by the tympanal organ, elicits a distinct oriented locomotion termed phonotaxis. In this study, we examined the cross-modal effects on wind-elicited walking when an acoustic stimulus was preceded by an air-puff and tested whether the auditory modulation depends on the coincidence of the direction of both stimuli. A preceding 10 kHz pure tone biased the wind-elicited walking in a backward direction and elevated a threshold of the wind-elicited response, whereas other movement parameters, including turn angle, reaction time, walking speed and distance were unaffected. The auditory modulations, however, did not depend on the coincidence of the stimulus directions. A preceding sound consistently altered the wind-elicited walking direction and response probability throughout the experimental sessions, meaning that the auditory modulation did not result from previous experience or associative learning. These results suggest that the cricket nervous system is able to integrate auditory and air-puff stimuli, and modulate the wind-elicited escape behavior depending on the acoustic context.

KEY WORDS: Escape behavior, Multisensory integration, Directionality, Context dependence, Cricket, Cercal system

INTRODUCTION

Animals perceive environmental external stimuli via many sensory pathways and their resulting behavior is based on an integration of this sensory information. Multisensory signals provide a robust perception of the environment and lead to behavioral changes, such as a shortened reaction time (Rowland et al., 2007) and an improved sensitivity (McDonald et al., 2000; Stein et al., 1996). Even in escape reactions that are often regarded as a simple behavior, the details of the response, such as its directionality, are flexible depending on the environment or the behavioral context (Card, 2012; Domenici, 2010; Domenici et al., 2011a,b; Ydenberg and Dill, 1986). For example, mechanical contact to the antenna of the

cockroaches modulates their escape trajectory in response to an air-puff stimulus (Ritzmann et al., 1991) and postural curvature caused by a bending response to a weak stimulus affects startle responses evoked by a subsequent mechanical stimulus in gobies (Turesson et al., 2009). However, how spatio-temporal relationships between multiple stimuli in different modalities influence escape behaviors remains unknown. To address this question, we examined the effects of an auditory stimulus on wind-elicited walking behavior in crickets, a response that is considered to be an escape behavior (Gras and Hörner, 1992; Oe and Ogawa, 2013; Tauber and Camhi, 1995).

Crickets have two kinds of aero-detecting organ. The first are auditory tympanal organs on the front legs, which receive changes in air pressure. The other are mechanosensory cerci at the rear of the abdomen, which sense air-particle displacement. Both of these sensory systems can detect directional information, such as the location of a sound and the direction of airflow to mediate distinct 'oriented behaviors'. Female crickets exhibit positive phonotaxis in response to the calling songs of a conspecific male (Hedwig, 2006; Huber and Thorson, 1985), whereas the ultrasounds emitted by echolocating bats elicit negative taxis in flying crickets (Brodfehrer and Hoy, 1990; Moiseff et al., 1978; Pollack and Martins, 2007). Oriented escape walking behavior is also elicited by the gust of air generated by an approaching predator (Gras and Hörner, 1992; Tauber and Camhi, 1995). In this behavior, the direction and turn angle of the resulting walk depends on the stimulus direction (Oe and Ogawa, 2013). However, the details of the interaction between the cercal and auditory systems are unknown.

To elucidate the multisensory interaction between the auditory and cercal system, we made a point to use a 10 kHz pure tone as the auditory stimulus, which was outside the carrier frequencies of the calling song (4–5 kHz) and echo-location call (20 kHz or higher). The reason for this was that we needed the neutral auditory cue that solely causes no reaction to the crickets in order to separate the multisensory interaction and the effect of motor activity evoked by the additional (auditory) stimulus. In particular, we focused on the coincidence in arrival direction between the auditory and airflow stimuli. In humans, a preceding auditory cue improves the directionality of subsequent visual detection (McDonald et al., 2000). We tested whether in crickets a preceding auditory stimulus would alter a corresponding walking behavior in response to an air-puff delivered from the same direction as the auditory cue.

MATERIALS AND METHODS

Animals

Laboratory-bred adult male crickets (*Gryllus bimaculatus* De Geer 1773) (0.50–0.80 g body weight) were used throughout the experiments. They were reared under 12 h light:12 h dark conditions at a constant temperature of 27°C. We removed their antennae to eliminate the influence of mechanosensory inputs from the antennal organ so we could focus on the interaction between the cercal and auditory systems.

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Treadmill system

To monitor a cricket's walking activity during the initial response to the air-puff stimulus, we used a spherical-treadmill system (Fig. 1A), as described in a previous study (Oe and Ogawa, 2013). An animal was tethered on top of a Styrofoam ball using a pair of insect pins bent into an L-shape that were stuck to the cricket's tergite with paraffin wax. The cricket's walking was monitored as rotation of the ball at a 200 Hz sampling rate, using two optical mice mounted orthogonally around the ball. TrackTaro software (Chinou Jouhou Shisutemu, Kyoto, Japan) was used to measure the walking trajectory and to calculate parameters such as translational and angular turn velocities, based on the measured ball rotation.

Air-puff stimulation

An air-puff stimulus was provided to the stationary cricket by a short puff of nitrogen gas from a plastic nozzle (15 mm diameter) connected to a PV820 pneumatic picopump (World Precision Instruments, Sarasota, FL, USA).

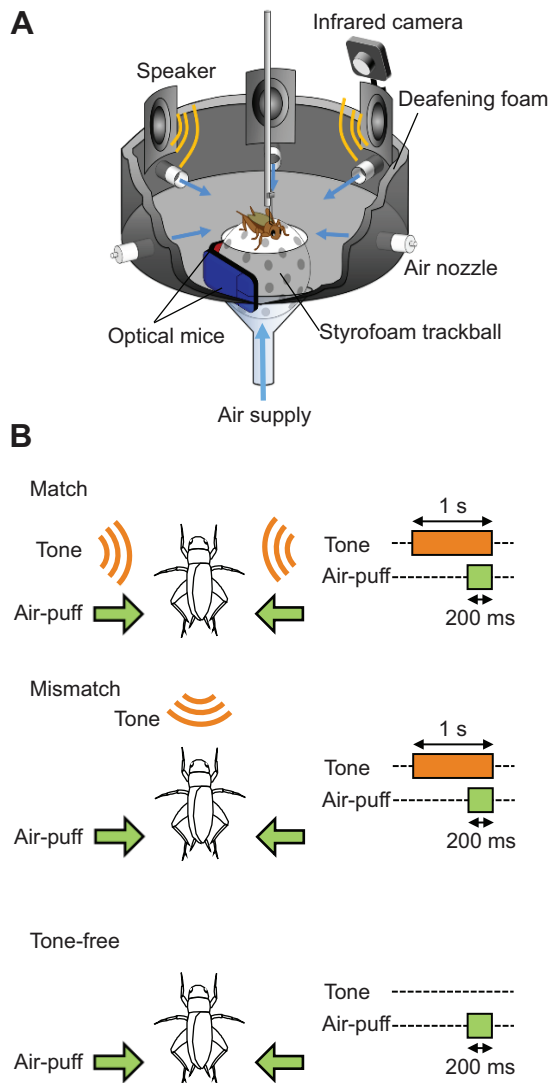


Fig. 1. Experimental apparatus and stimulation protocols. (A) The spherical-treadmill system. The air-puff and acoustic stimulus were delivered from eight nozzles and speakers arranged on the inside wall around the treadmill. (B) Spatial (left) and temporal arrangements (right) of the air-puff and acoustic stimuli for the three different protocols. For the match protocol, both stimuli were delivered from the same direction. In the mismatch protocol, the acoustic stimulus was always given from a speaker located in front of the cricket. In the tone-free protocol, only the air-puff stimulus was given, without the acoustic stimulus.

Eight air-puff nozzles were arranged on the inside wall of the arena, on the same horizontal plane as the animal (Fig. 1A), but only two nozzles positioned at right and left sides of the cricket were used for this study. The nozzle ends were arranged at 45 deg angles and at a distance of 105 mm from the animal. The velocity of the air-puffs was controlled by adjusting the delivery pressure of the picopump. To measure the response threshold, we used air-puffs of different velocities, 0.26, 0.43, 0.61, 0.90 and 1.11 m s⁻¹ measured at the center of the arena with a thermal anemometer (405-V1, Testo, Yokohama, Japan).

Acoustic stimulation

All of the experiments were conducted in a sound-proof chamber, with a 150-mm-thick wooden wall. The acoustic stimuli were 10 kHz pure tones, synthesized using RpvdsEx software (Tacker Davis Technologies, Alachua, FL, USA) and transduced and attenuated using a RM1 processor (TDT). The sounds were calibrated at an average of 70 dB SPL and were delivered by 1.5 inch (3.81 cm) full-range sealed loudspeakers (MM-SPS2, Sanwa Supply, Okayama, Japan). Eight speakers were located 105 mm from the animal and spaced 45 deg apart, just above the air-puff nozzles (Fig. 1A), but three speakers positioned at anterior and lateral (left and right) sides of the cricket were used for this study. To avoid sound reverberation, deafening foam was attached to the inside wall of the arena.

Experimental procedure

To test any relationships between the directional coincidence of the acoustic and air-puff stimuli, and cross-modal effects on the walking activity parameters in wind-elicited walking, we designed three types of stimulation protocol, referred to as the match, mismatch and tone-free protocols (Fig. 1B). In all these protocols, a single air-puff stimulus was delivered from a nozzle to the left or right side alternately. In the match and mismatch protocols, a tone sound of 1 s duration started 800 ms before an air-puff for 200 ms. In the match protocol, the direction of the acoustic stimulus consistently corresponded to that of the air-puff stimulus. In the mismatch protocol, the acoustic stimulus was presented from a speaker located in front of the animal, regardless of the direction of the air-puff. In the tone-free protocol, a 200 ms air-puff was delivered without any prior acoustic stimulus, but we monitored the cricket's walking activity during the 800 ms silent time prior to the air-puff. A sequence of stimulation in all protocol types was started only after the cricket remained still for 1 s or longer.

We divided the crickets into 15 groups for three different protocols (match, mismatch and tone-free) using five different velocities of the air current (0.26, 0.43, 0.61, 0.90 and 1.11 m s⁻¹). As each experimental group consisted of 8 individuals, 120 crickets were used for the experiments in total. For each individual cricket, four sessions of the experiments, each of which comprised 10 trials, were performed using the same protocol and air-current velocity. The inter-trial interval was >1 min and the inter-session interval was >10 min.

Behavioral analysis

To quantify the analysis of walking behavior among the different stimulation protocols, we focused on 'wind-elicited' initial responses, and measured some walking activity parameters including walking direction, turn angle, reaction time, maximum walking speed and walking distance. Definition and calculation of these parameters were the same as those in our previous study (Oe and Ogawa, 2013). The *x*- and *y*-axes were defined as the lateral and antero-posterior axes of the cricket at the start position, respectively (Fig. 2B). The translational velocity on the *x*-*y* plane was defined as 'walking speed'. An initial, continuous walking trot followed by a stationary moment was defined as the 'initial response'.

Since the reaction time was measured as a delay from opening of the delivery valve in the picopump to the start of the initial response, this value contained not only actual reaction time of the animal but also the travel time of air currents from the nozzle to the center of the arena. To estimate the actual reaction time, we measured the time delays from the valve opening to movement of the micro lint placed at the center of arena, based on a movie (640×480 pixels, 200 Hz) monitored with a CH130EX high-speed video camera (Shodensha, Osaka, Japan). The travel times of air currents of 0.61,

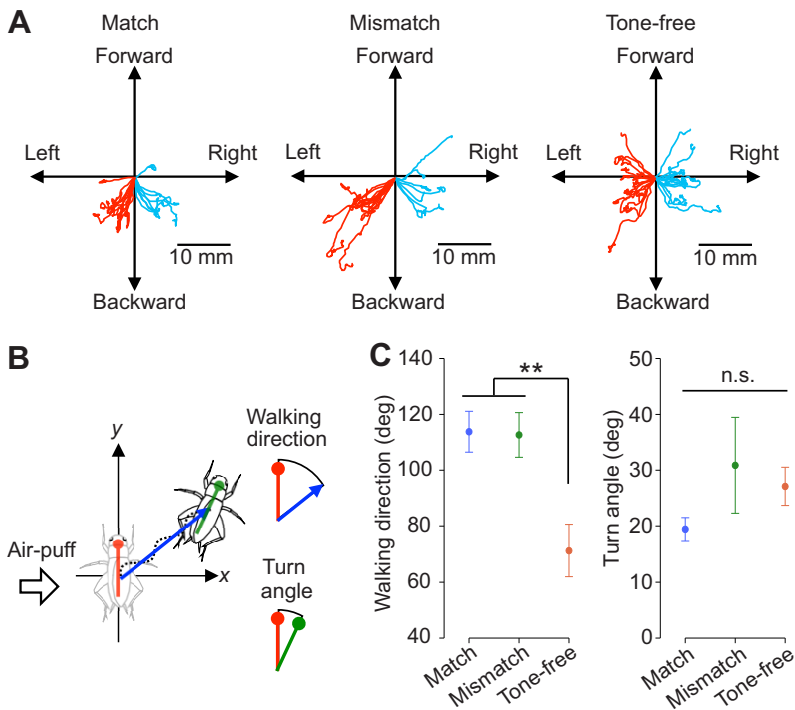


Fig. 2. Auditory effects on directionality in wind-elicited walking. (A) Typical walking trajectories of an individual cricket in response to air-puffs (0.90 m s^{-1}) from lateral sides in the three different protocols. Red and light blue traces show the walking trajectories elicited by the stimulus from the right and left sides, respectively. (B) Definition of walking direction and turn angle in the initial response of wind-elicited walking behavior. The left diagram shows the crickets before (gray drawing) and after (black drawing) the initial response to an air-puff stimulus on the virtual plane, in which the x- and y-axes were defined as lateral and antero-posterior axes of the cricket at the start position. The walking direction was measured as the angle between the body axis at the start point (red line) and the line connecting the start and finish points of the initial response (blue arrow). The turn angle was measured as the angle made by the body axes at the start (red line) and finish points (green line). Both walking direction and turn angles were relative to forward direction, which was set as 0 deg. If the air-puff was delivered from the left side of the cricket, these parameters were arranged clockwise as plus, and counterclockwise as minus. In the responses to the stimulus from the right side, they were arranged clockwise as minus and counterclockwise as plus. (C) Pooled data of walking direction (left) and turn angle (right) in the three stimulation protocols. The data recorded in all responses categorized as ‘wind-elicited’ to the left- and right-side stimuli were combined. Each plot shows the mean values of the data obtained from 40 trials for each individual and error bars indicate \pm s.e.m. ($N=8$ animals for each protocol). ** $P < 0.01$ (Tukey’s HSD test).

0.90 and 1.11 m s^{-1} were 67.85 ± 8.37 , 52.86 ± 4.34 and $35.71 \pm 2.02 \text{ ms}$ (mean \pm s.e.m., seven trials for each speed), respectively. Unfortunately, we were unable to detect the movement of the lint caused by airflows slower than 0.43 m s^{-1} . Thereby, the putative reaction time was defined as the difference calculated by subtracting mean value of the travel time for each airflow speed from the reaction time measured with a treadmill.

According to the walking speed and timing of the initial response, we classified the trials into three types of response. If a cricket started to walk 800 ms before the onset of the air-puff stimulus and the maximum walking speed was $>0.01 \text{ m s}^{-1}$, the trial response was categorized as ‘sound-elicited’ (Fig. S1). If a cricket started to walk after the onset of an air-puff stimulus and the maximum walking speed in the initial response was $>0.05 \text{ m s}^{-1}$, the trial response was categorized as ‘wind-elicited’. All other trials, including no walking were categorized as ‘no response’. The thresholds of 0.01 and 0.05 m s^{-1} for these classifications were determined based on the frequency distribution of the maximum walking velocity before and after the air-puff in the tone-free protocol (Fig. S2). The auditory response probability was defined as:

$$\text{Auditory response probability} = \frac{N_s}{N_s + N_w + N_{no}}, \quad (1)$$

where N_s , N_w and N_{no} are the number of trials categorized as a ‘sound-elicited’ response, a ‘wind-elicited’ response and a ‘no response’, respectively.

The wind response probability was defined as:

$$\text{Wind response probability} = \frac{N_w}{N_w + N_{no}}, \quad (2)$$

which indicates that the trials categorized as ‘sound-elicited’ were eliminated from the calculation of the wind response probability. The reason is that it was impossible to determine correctly whether a cricket responded to the air-puff stimulus or not during the locomotion.

Data analysis and statistical methods

To avoid pseudo-replication, the averaged values of the walking activity parameters in all trials throughout the sessions in each individual were used for statistical analysis. For analysis of the transition during the sessions, angles of the walking direction were averaged for each session in each

individual. For statistical analysis of the wind response probability, the probabilities were calculated from all trials throughout the sessions or 10 trials in each session for each individual. That is, only a single value was used for each individual for all statistics in this study.

We used R programming software (v2.15.3, R Development Core Team) for the statistical analysis of walking activity parameters. To assess the significance of the stimulation protocols, we used a one-way factorial ANOVA when comparing the walking direction and auditory response probability for the different groups of crickets. If the main effect of the protocols was significant, we then compared the protocol groups using Tukey’s *post hoc* test. We used a two-way factorial ANOVA to assess the significance of the stimulation protocols and the air-current velocity, for the response probability and for the various walking activity parameters. We used a two-way repeated-measures ANOVA to assess the significance of the stimulation protocols and session progress for the walking direction, wind response probability and auditory response probability.

Estimation of the threshold velocity of the air-puff was performed using R software. We approximated the wind response probability, using the Hill function as:

$$\text{Response probability} = \frac{v^h}{v^h + v_{1/2}^h}, \quad (3)$$

where v is the air-current velocity, h is the Hill coefficient and $v_{1/2}$ is the threshold velocity of the air-puff that provides a 50% chance of eliciting walking behavior. We compared the threshold velocity of the stimulation protocols.

RESULTS

Walking reaction is triggered by an air-puff but not by a preceding sound

Prior to the investigation of the auditory effects on wind-elicited walking, we checked whether a 10 kHz pure tone auditory stimulus triggered walking when delivered 800 ms before the air-puff stimulation. The auditory response probabilities were relatively low in all stimulation protocols ($12.16 \pm 1.96\%$, $7.77 \pm 1.31\%$ and $11.69 \pm 1.66\%$ for the match, mismatch and tone-free protocols, respectively), and there were no significant differences in this

probability among the three stimulation protocols ($P=0.128$, one-way factorial ANOVA, Fig. S3A). This indicates that the walking activities during auditory stimulus before the air-current stimulus were voluntarily initiated, and that a preceding auditory cue alone could not elicit the cricket locomotion. We then focused on walking triggered by the air-puff stimulus, categorized as a ‘wind-elicited response’, and compared the various parameters among the three stimulation protocols.

Auditory effect on walking orientation in wind-elicited behavior

First, we examined cricket walking activity in response to air-puffs delivered at 0.91 m s^{-1} to their lateral sides in the three different protocols. The recorded trajectories of the initial responses (Oe and Ogawa, 2013) on virtual planes measured with the spherical treadmill indicated characteristic reciprocal locomotion in all types of stimulation protocols; that is, air-puffs delivered from the left side elicited walking to the right side and vice versa (Fig. 2A). When the air-puff stimulus was delivered without the preceding sound in the tone-free protocol, crickets walked in a diametrically opposite direction to the air-puff, and its trajectory was distributed around the lateral axis. In contrast, when the tone sound preceded the air-puff stimulus in the match and mismatch protocols, the crickets walked backwards more often. To compare the walking orientation between the protocols, we measured two walking activity parameters; the walking direction and the turn angle, based on the trajectory data combined from the initial responses to stimuli from the left and right sides (Fig. 2B). The angular value of the walking direction in the match and mismatch protocols was greater than 90 deg ($113.79 \pm 7.33 \text{ deg}$, $112.64 \pm 7.99 \text{ deg}$, respectively), while the walking direction in the tone-free protocol was less than 90 deg

($71.29 \pm 9.30 \text{ deg}$) (Fig. 2C). There were significant differences between the match and tone-free results ($P=0.0042$, Tukey’s HSD test), and between the mismatch and tone-free ($P=0.0052$). However, the walking direction in the match protocols was not significantly different from that in the mismatch protocol ($P=0.9947$). This result means that the alteration of the walking orientation was independent of any coincidence in stimulus direction between the sound and the air-puff. In contrast, the turn angles of the initial walking responses were $19.43 \pm 2.07 \text{ deg}$ for match, $30.88 \pm 8.58 \text{ deg}$ for mismatch and $27.11 \pm 3.42 \text{ deg}$ for tone-free, respectively (Fig. 2C). There was no significant difference in the turn angle between the stimulation protocols ($P=0.339$, one-way factorial ANOVA). These results indicated that the preceding tone sound changed the walking orientation, but did not alter the turning motion in the initial response to the air-puff. Our previous study reported that the walking direction and turn angle in the initial response to an air-puff depends on the stimulus angle but that these relationships followed different rules (Oe and Ogawa, 2013). Crickets exposed to the auditory stimulus would probably exhibit side- or backwards stepping without a large turn.

To analyze the details of an auditory effect on the walking orientation, we compared frequency distributions of walking direction between the three stimulation protocols (Fig. 3). The results for the match and mismatch protocols revealed similar distributions, in which the walking directions were mainly distributed on the backward ($>90 \text{ deg}$) and their peaks were $135\text{--}150 \text{ deg}$. In contrast, the walking directions in the tone-free protocol were distributed around 90 deg and its peak was $30\text{--}45 \text{ deg}$. The forward walks ($<90 \text{ deg}$) in the tone-free protocol were more than those in the match and mismatch protocols (match=47/206, mismatch=51/195 and tone-free=168/242; N of forward walks/ N

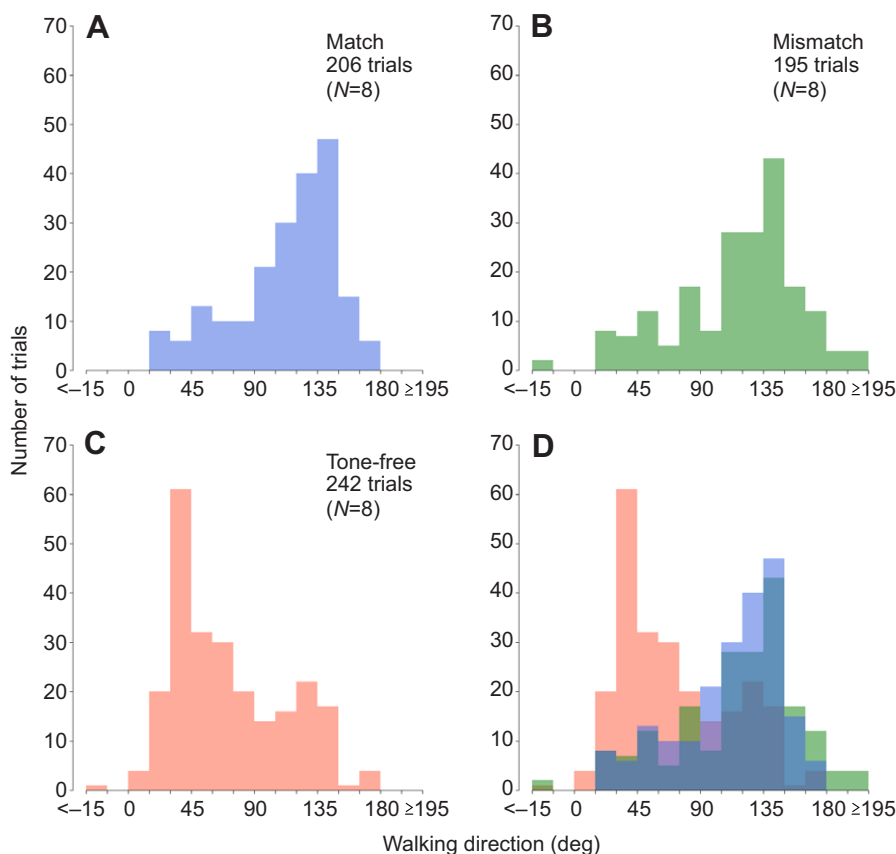


Fig. 3. Frequency distributions of walking direction. Histograms show the number of trials in which the crickets walked in that direction in response to three stimulation protocols. Total numbers of the ‘wind-elicited’ responses were 206, 195 and 242 trials for the match (A, blue), mismatch (B, green) and tone-free (C, red) protocols, respectively. The data were obtained from the same experiments shown in Fig. 2C ($N=8$ animals for each protocol, air-current velocity= 0.90 m s^{-1}). (D) Overlaid histogram of the frequency distributions in three stimulation protocols.

of wind-elicited responses), whereas the backward walks (>90 deg) in the tone-free protocol were fewer than those in the match and mismatch protocols (match=159/206, mismatch=144/195 and tone-free=74/242; *N* of backward walks/*N* of wind-elicited responses). This result demonstrates that the preceding auditory stimulus did not simply reduce the probability of forward walking but also increased the probability of backward walking.

Auditory effect on the response threshold

The results shown in Fig. 3 also revealed that total numbers of the wind-elicited responses in match and mismatch protocols were smaller than that in the tone-free protocol, suggesting that the preceding auditory stimulus reduced the wind response probability. To examine the auditory effects on the threshold of wind-elicited walking, we delivered five different velocities of air-puff stimulus (0.26, 0.43, 0.61, 0.91 and 1.11 m s⁻¹) for each stimulation protocol and calculated the wind response probability from the number of trials in which a walking response was observed for the air puff from the lateral side of the cricket. The response probability was significantly affected not only by the air-current velocity, but also by the stimulation protocol ($P<0.0001$ for the velocity and $P=0.0005$ for the protocol, in a two-way factorial ANOVA). This indicated that the preceding auditory stimulus affected the dependency of the response probability on the stimulus velocity. Next, we estimated the response threshold based on Hill curves fitted to plots of pooled data of the response probability in each stimulation protocol (Fig. 4). The air-current velocity that induced the walking response in 50% of the trials was defined as the response threshold and these were 0.74 m s⁻¹ for the match, 0.80 m s⁻¹ for the mismatch and 0.59 m s⁻¹ for the tone-free protocols, respectively. This indicates that a preceding auditory

stimulus can increase the threshold of wind-elicited walking behavior. In contrast, the difference in the threshold between the match and mismatch protocols was smaller. There is no evidence that the effect of the preceding auditory stimulus on the response threshold is correlated to coincidence with the stimulus directions.

Air-current velocity dependency of auditory effects

Next, we compared various walking activity parameters of the responses to the different velocity air-puffs (0.43–1.11 m s⁻¹) delivered from the lateral side of the tested crickets, among the three stimulation protocols (Fig. 5). The effects of the stimulation protocols and the air-puff velocities on these parameters were tested using a two-way factorial ANOVA (Table 1). The walking directions were independent of the stimulus velocity ($P=0.787$), while the auditory alterations in the walking direction were significant ($P<0.001$ for match versus tone-free and $P<0.001$ for mismatch versus tone-free, Tukey's HSD test). The reaction time decreased with increasing stimulus velocity ($P<0.001$). But, there was no difference in this value between the protocols ($P=0.557$), suggesting that the reaction time could be unaffected by the preceding auditory stimulus. Other walking activity parameters including the turn angle, maximum walking speed and walking distance increased depending on the air-current velocity ($P=0.005$ for turn angle, $P=0.006$ for maximum walking speed and $P=0.002$ for walking distance), but did not differ between the stimulation protocols ($P=0.723$ for turn angle, $P=0.549$ for maximum walking speed and $P=0.442$ for walking distance). There was also no significance of the interaction between the stimulus velocity and stimulation protocol for any of the parameters ($P=0.189$ for walking direction, $P=0.406$ for turn angle, $P=0.861$ for reaction time, $P=0.950$ for maximum walking speed and $P=0.268$ for walking distance). These results demonstrate that, for a range of air-current velocities (0.43–1.11 m s⁻¹), a preceding auditory stimulus consistently alters the walking direction but has no effect on the other walking activity parameters.

Auditory effects did not result from associative learning

Here, we considered the influence of experience on the auditory effects. Even if the experimental session was extended, the response probability of walking triggered by the auditory stimulus alone remained low (Fig. S3B). The stimulation protocol type, experimental session number and their interaction had no effect on the auditory response probability ($P=0.145$, 0.942 and 0.694 for protocols, sessions and interaction, respectively, two-way repeated measures ANOVA). This result indicates that crickets could not learn the association between the auditory cue and the air-puff stimulus.

To further test the influence of experience on the auditory effects, we compared the walking direction and response probability, both of which were altered by the preceding sound, between the four experimental sessions (Fig. 6). Regardless of the air-current velocity, the walking direction did not depend on the order of sessions (Fig. 6A). In contrast, there were significant effects of the stimulation protocols at the 0.43–0.90 m s⁻¹ velocities (Table 2, two-way repeated-measures ANOVA), meaning that the walking direction was altered by the preceding auditory stimulus from the first session. As shown in Fig. 4, the auditory impact on the response probability depended on the air-current velocity, because the faster the air-puff, the greater the likelihood of a walking response. We then focused on differences in the transition of the response probability throughout the sessions, among the stimulation protocols. When the air-puff stimulations were slower (0.43 and

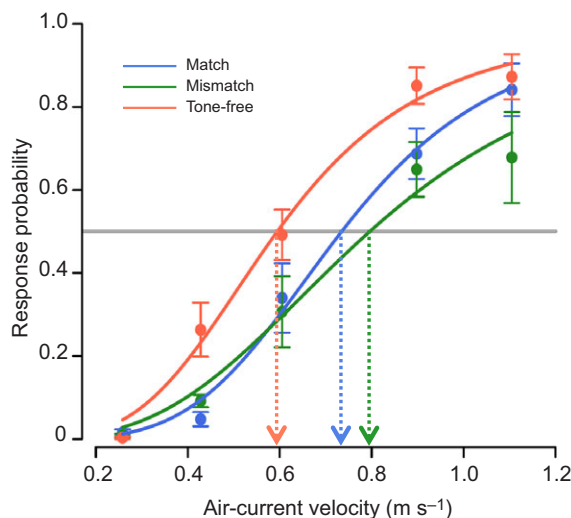


Fig. 4. Auditory effect on response threshold. The probabilities of a walking response to the air-puff stimuli in the different protocols plotted against the stimulus velocity. Each plot represents the average of the mean response probabilities in 40 trials for each individual and error bars indicate \pm s.e.m. ($N=8$ animals for each protocol and for each air-current velocity). Solid color lines represent curves fitted with the Hill function (see the Materials and methods), for which the parameters were as follows: match, $h=4.19$ and $v_{1/2}=0.735$; mismatch, $h=3.16$ and $v_{1/2}=0.796$; tone-free, $h=3.64$ and $v_{1/2}=0.595$. Dotted color lines indicate the velocities at which air-puffs triggered the walking response, with the 50% probability shown by the gray line. These velocities correspond to values of $v_{1/2}$. The value of $v_{1/2}$ in the tone-free protocol was smaller than in the match and mismatch protocols.

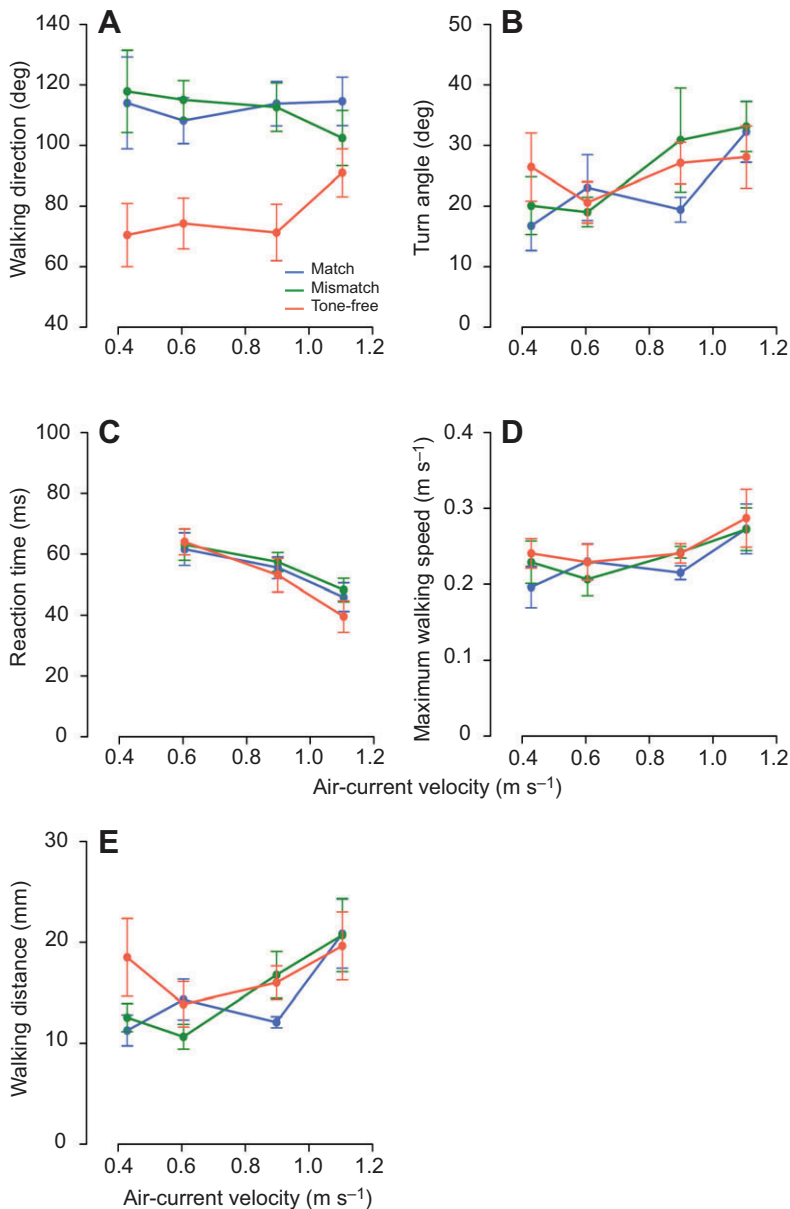


Fig. 5. Air-current velocity dependency of auditory effects on walking activity parameters. Each plot represents the average of mean values of data, including the walking direction (A), turn angle (B), reaction time (C), maximum walking speed (D) and walking distance (E), obtained from 40 trials for each individual. Error bars indicate \pm s.e.m. ($N=8$ animals for each protocol and for each air-current velocity). There is no plot of the reaction times in response to the airflow of 0.40 m s^{-1} because the travel time of that stimulus was undetectable.

0.61 m s^{-1}), the response probability was not correlated with the order of the sessions; however, when the air-puffs were faster (0.90 and 1.11 m s^{-1}), the response probability gradually declined as the session progressed (Fig. 6B). The crickets possibly habituated to the faster air-puff stimuli. In all cases, however, there was no significance of the interaction between the stimulation protocol and the experimental session (Table 2, two-way repeated measures

ANOVA), meaning that the auditory effect on the response probability also did not vary as sessions progressed. This suggests that the effect of the preceding auditory stimulus is not a result of associative learning.

DISCUSSION

Cross-modal effect on directionality of locomotion

Directionality of locomotion is one of the most important aspects in escape behavior by which animals maximize their chances of survival. An attempt to escape in the wrong direction could result in predation. The escape direction, however, is plastic rather than stereotypical, such as a habitual movement in the exact opposite direction to the predator. An individual animal, even in the same environmental situation, shows variability in the direction of its escape movement in order to confound the predator's prediction of the prey's likely displacement (Domenici et al., 2008; Humphries and Driver, 1970). As shown by the results for the tone-free protocol in Fig. 3, crickets walked in various directions distributed on the opposite side to a lateral air-puff stimulus. In addition, the animals'

Table 1. *P*-values from two-way factorial ANOVAs testing the significance of stimulation protocols and air-current velocity

Parameter	Main effect		
	Protocol	Velocity	Interaction
Walking direction	<0.001	0.787	0.189
Turn angle	0.723	0.005	0.406
Reaction time	0.557	<0.001	0.506
Maximum walking speed	0.549	0.006	0.950
Walking distance	0.442	0.002	0.268

Significance of the main effects is represented in bold.

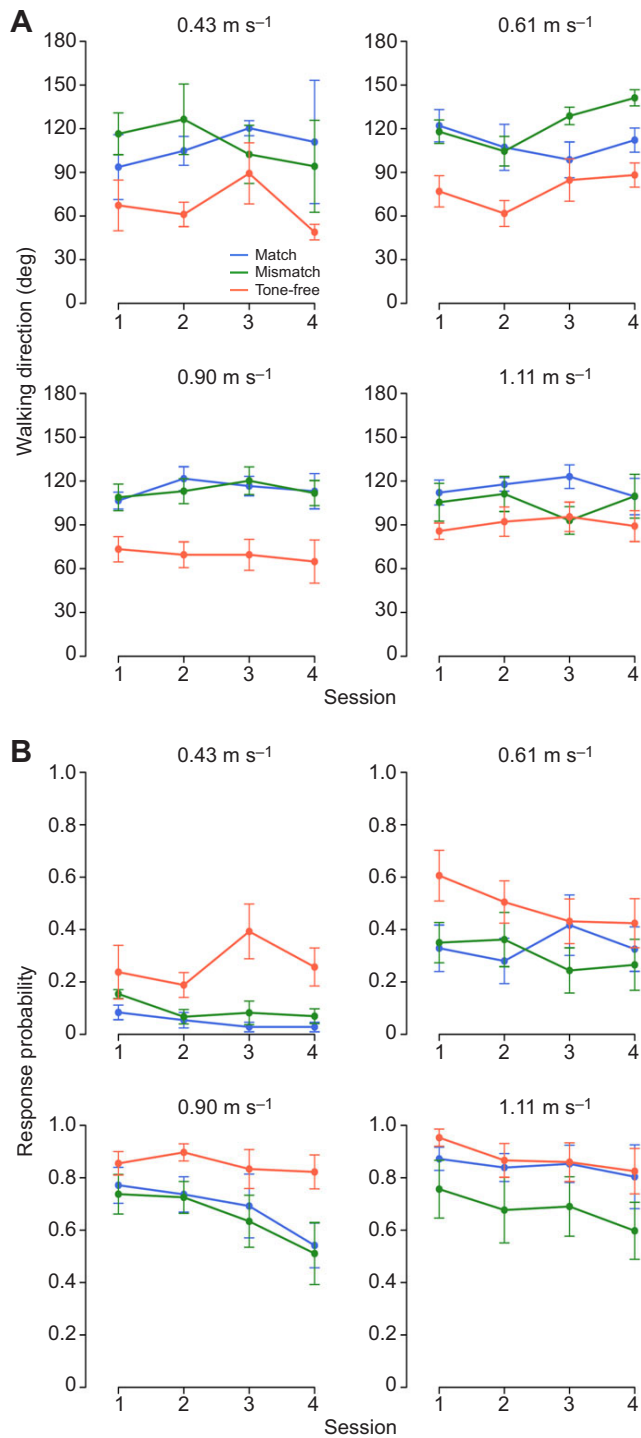


Fig. 6. Changes in auditory effects throughout the experimental sessions. Relationships between the order of sessions and the walking direction (A) or response probability (B) at different velocities of air-puff stimuli (0.43, 0.61, 0.90 and 1.11 m s⁻¹). Each plot of the walking direction represents the average of mean values of the data obtained from 10 trials for each individual, and the plot of the response probability represents the mean probability in 10 trials for each individual. Error bars indicate \pm s.e.m. ($N=8$ animals for each protocol). The data from session 1 to session 4 were measured continuously from each individual.

environmental context and/or behavioral state greatly affect the directionality of the escape locomotion. For example, acute cooling of the surrounding water increases the rate of motion towards the

Table 2. *P*-values from two-way repeated measures ANOVAs testing the significance of stimulation protocols and the order of sessions

Parameter	Velocity (m s ⁻¹)	Main effect		
		Protocol	Session	Interaction
Walking direction	0.43	0.012	0.680	0.495
	0.61	0.001	0.172	0.150
	0.90	0.001	0.926	0.460
	1.11	0.119	0.949	0.894
Response probability	0.43	0.002	0.627	0.155
	0.61	0.216	0.052	0.112
	0.90	0.049	0.001	0.237
	1.11	0.193	0.006	0.642

Significance of the main effects is represented in bold.

startle stimulus in goldfish (Preuss and Faber, 2003), while schooling herring exhibit more frequent escape responses away from the stimulus than do solitary fish (Domenici and Batty, 1997). A preceding weak stimulus causing postural bending enhances the locomotor performance of anti-predator responses (Turesson et al., 2009). Presence of an obstacle, such as a wall, alters the direction of the escape response in order to avoid collision (Eaton and Emberley, 1991; Ritzmann et al., 1991).

One possible mechanism underlying the backward walking biased by the preceding auditory stimulus is a selective inhibition of forward walking. However, the frequency distribution of the walking directions shown in Fig. 3 indicates that not only was the frequency of forward walking reduced but backward walking was also more frequently induced. That is, the angular distribution of the walking directions could be drastically altered. This means that a preceding auditory stimulus modulates the direction of wind-elicited walking and suggests an environmental context-dependent change of the directionality of an escape behavior. Acoustic signals, such as the songs of conspecific males and the echolocation calls of foraging bats, provide significant information to crickets (Moiseff et al., 1978; Wytenbach et al., 1996). Crickets exhibit different behaviors depending on the sound frequency when responding to the same temporal pattern of acoustic stimuli: e.g. a positive phonotaxis to sounds in the range 4–5 kHz but a negative one to sounds over 30 kHz (Moiseff et al., 1978; Wytenbach et al., 1996). As mentioned above, we purposely used a 10 kHz pure tone, which can be heard by the cricket but causes no specific behavior alone, because the stimulus frequency did not match to the carrier frequencies of the cricket calling song and the bat echo-location call. Nevertheless, the 10 kHz pure tone can bias the walking response triggered by an air-puff stimulus to a backwards direction. The ethological relevance of the cross-modal effect on the directionality of the escape response remains unclear. Why would a cricket alter the direction of its escape behavior depending on the auditory context? However, a behaviorally relevant sound may alter the wind-elicited walking behavior more distinctly than the pure tone used in our experiments.

Elevation of the response threshold

Air-puffs detected by the cercal system of crickets and other insects elicit at least 14 distinct reactions including evasion, flight, offensive reactions, scanning and freezing, and the response depends on the behavioral state and environmental context of the individual concerned (Baba and Shimozaawa, 1997; Casas and Dangles, 2010). This implies that cercal-mediated behavior can be modulated by additional sensory information from other modalities. Elevation of the threshold of escape walking means

that a cricket becomes less responsive to the air currents. As a result, the freezing strategy, which is a defensive response employed by various animal species, would be observed more frequently. Escape reactions such as walking, running and jumping can increase the possibility of successful predator evasion, but also provide the predator with clues to capture the prey. If employed before the predator detects the prey, a ‘no-response’ strategy may be the most effective one (Eilam, 2005). As mentioned above, a flying cricket displays negative taxis to high-frequency (>10 kHz) sounds (Moiseff et al., 1978; Wyttenbach et al., 1996). In contrast, crickets on the ground do not exhibit any specific anti-predator behavior in response to the echolocation calls of gleaning bats (ter Hofstede et al., 2009). This study demonstrates that a preceding auditory stimulus (10 kHz pure tone) can increase the threshold of wind-elicited walking. Further experiments using a high-frequency sound, such as an echolocation call, will illuminate the behavioral function of the auditory effects on the threshold of the air-current-evoked response.

Neural basis of cross-modal modulation in escape behavior

Prior to the experiments, we had hypothesized that a preceding auditory stimulus would greatly or differently modulate the walking response triggered by an air-puff delivered from the same direction as the auditory cue. However, neither the modulation of the walking orientation nor the elevation of the response threshold correlated with the coincidence of the two stimulus directions. The crickets exhibited distinct locomotion depending on the direction of the acoustic or air-puff stimuli. The turn angular velocity of female crickets during phonotaxis towards the calling song depends on the location of the sound (Schildberger et al., 1989), while both the walking direction and turn angle in the initial response to an air-puff depends on the stimulus angle (Oe and Ogawa, 2013). This indicates the possibility of an interaction between the auditory and cercal sensory pathways at various levels, including lower-level processing within the thoracic or abdominal ganglia. Multisensory integration at higher levels within the brain is also possible and would suggest that crickets have the ability to recognize the direction of both auditory and cercal stimuli. Numerous studies have illustrated the neural mechanisms underlying the processing of directional information in the cricket auditory and cercal sensory systems (reviewed by Hedwig, 2006; Jacobs et al., 2008). However, it remains unknown whether crickets can perceive the coincidence of the directions of different modal stimuli. Although the present results failed to demonstrate that crickets are able to recognize directional coincidence of stimuli, it remains possible that they can integrate the directional information of different modal stimuli such as cercal/visual or auditory/visual cues at various processing levels.

Other results show that the effect of the auditory stimulus on the walking direction and the threshold of the wind-elicited response were not the results of previous experience (Fig. 6), suggesting that the cricket nervous system has the innate ability to integrate auditory and cercal sensory information. The information provided by the air currents, such as velocity, direction and frequency, was extracted and processed by the neural circuitry within the terminal abdominal ganglion, where the mechanosensory afferents project and make synaptic contact with the ascending projection neurons and local interneurons (Jacobs and Theunissen, 2000; Ogawa et al., 2008). Several pairs of ascending neurons, identified as giant interneurons (GIs), extend their axons to the thoracic and

cephalic ganglia (Hirota et al., 1993). Based on the behavioral effects of the air-puff and selective cell ablations, some types of GIs are probably involved in initiation and modulation of wind-elicited walking behavior (Gras and Kohstall, 1998; Oe and Ogawa, 2013). However, the GIs are sensitive to the direction and dynamics of air particle displacement, rather than the high-frequency vibration used as the auditory cue in this study (Miller et al., 1991; Theunissen and Miller, 1991; Theunissen et al., 1996).

The primary auditory center is located within the prothoracic ganglion, into which the receptor afferents from the tympanal organ project (Eibl and Huber, 1979), and processed auditory signals are also carried by the auditory ascending interneurons. The ascending neurons 1 (AN1) and 2 (AN2) are tuned to different frequency sounds: AN1 is sensitive to 4–5 kHz sound while AN2 is broadly tuned to higher frequencies (>10 kHz) (Hennig, 1988; Poulet, 2005; Schildberger, 1984b; Wohlers and Huber, 1982). The 10 kHz chirp sound at 70 dB, which is similar in frequency and sound intensity to the tone pulse used in this study, activates AN2 (Schildberger, 1984b) and can elicit avoidance behavior during flight (Hoy et al., 1989; Nolen and Hoy, 1984). A preceding auditory stimulus of a 10 kHz tone may be detected by AN2 as an alert signal warning of the presence of flying predators. The excitation of AN2 causes no reaction in crickets standing on the ground (Hoy et al., 1989; Nolen and Hoy, 1984), but may modulate wind-elicited escape walking.

Direct or indirect interactions between the cercal and auditory ascending neurons were previously unknown. It has recently been reported that corollary discharges during singing inhibit the GI's responses to air-puff stimuli (Schöneich and Hedwig, 2015). However, some multimodal interneurons that respond to both auditory and air-puff stimuli have been identified in the cephalic and prothoracic ganglia (Gras et al., 1990; Schildberger, 1984a). It is possible that the cercal and auditory signals are combined in the cephalic and thoracic ganglia. The auditory pathway possibly has influence on the motoneurons or central pattern generator circuits for wind-elicited walking, directly or indirectly mediated by the descending neurons. Interestingly, a preceding auditory stimulus increased the threshold of the wind-elicited escape response, but did not affect its reaction time (Fig. 5). In general, greater intensity sensory stimuli induce a higher probability of reaction and shorter reaction times (Diederich and Colonius, 2004; Vaughan et al., 1966). Reaction time directly reflects the duration of the physiological processes of signal transduction and the transmission of sensory information (Vaughan et al., 1966). A lack of effect on reaction time, therefore, suggests that the auditory modulation of the response threshold does not result simply from desensitization or suppression during earlier sensory processes, but involves a sensory integration process or delayed decision making to give more time to select the best escape strategy at a higher center, such as the brain.

Another interesting finding is that a preceding auditory input biased the backward walking orientation but did not alter the turn angle (Fig. 2C). This means that the preceding auditory stimulus increased the frequency of backwards stepping rather than turning. In a previous study, we showed that walking direction and turn angle of wind-elicited walking behavior might be regulated by different neural circuits (Oe and Ogawa, 2013). Backward walking should require different descending command signals from those for forward walking. Descending neurons specifically

triggering backward walking were recently identified in *Drosophila* (Bidaye et al., 2014). It is likely that a preceding auditory signal could further activate the backward-specific descending neurons and thus, bias wind-elicited walking in a backwards direction. Further investigations of how multi-modal interneurons respond to auditory and air-puff stimuli, and of how synaptic connections are made between the descending projection neurons within the brain and thoracic ganglia will allow us to better understand the neural basis underlying the multisensory regulation of animal behavior.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.F., M.S. and H.O. designed the experiments and interpreted the data. M.F. performed the experiments and analyzed all data. M.F. and H.O. wrote the manuscript.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.128751/-/DC1>

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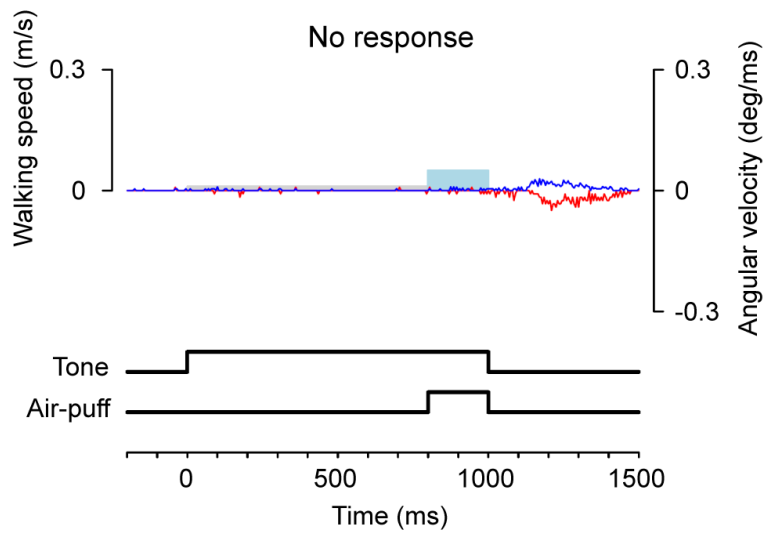
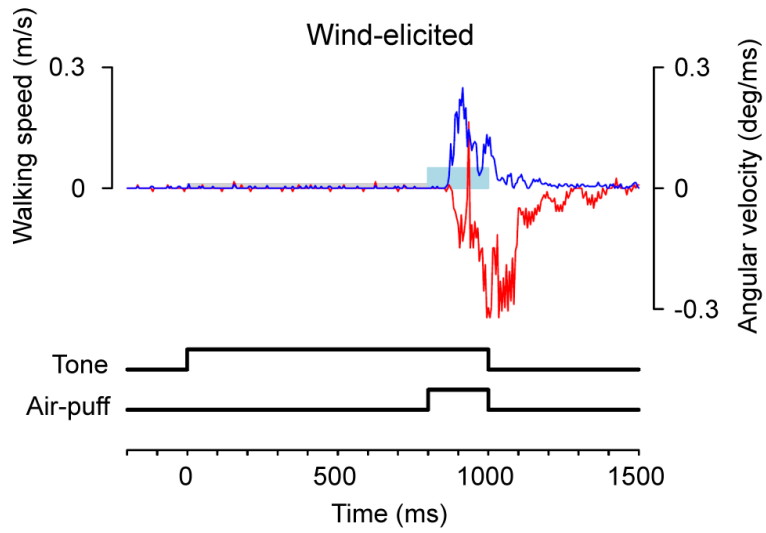
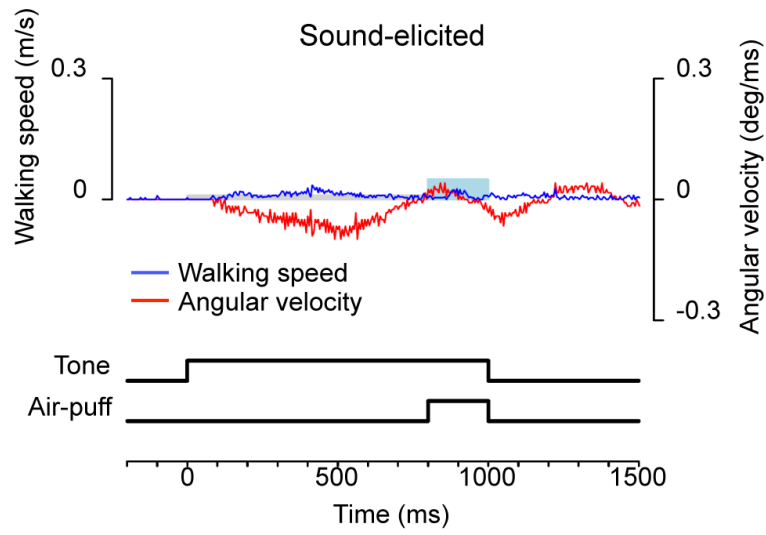


Fig. S1. Classification of behaviors in response to multimodal stimulation. The typical courses of walking speed (red traces) and turn angular velocity (blue traces) during the pure tone for 1 s and air-puff for 200 ms (lower black traces). Based on their time course and magnitude, a cricket's behavior was classified into three responses termed 'wind-elicited', 'sound-elicited' and 'no response'. If a cricket started to walk 800 ms before the air-puff stimulus and the maximum walking speed was >0.01 m/s (indicated by the gray-shaded area), then its response was classified as a 'sound-elicited' response. If a cricket started to walk after the onset of the air-puff stimulus and the maximum walking speed in the initial response was >0.05 m/s (indicated by the blue-shaded area), then its response was classified as a 'wind-elicited' response. Non-responses, including no walking, were classified as a 'no response'.

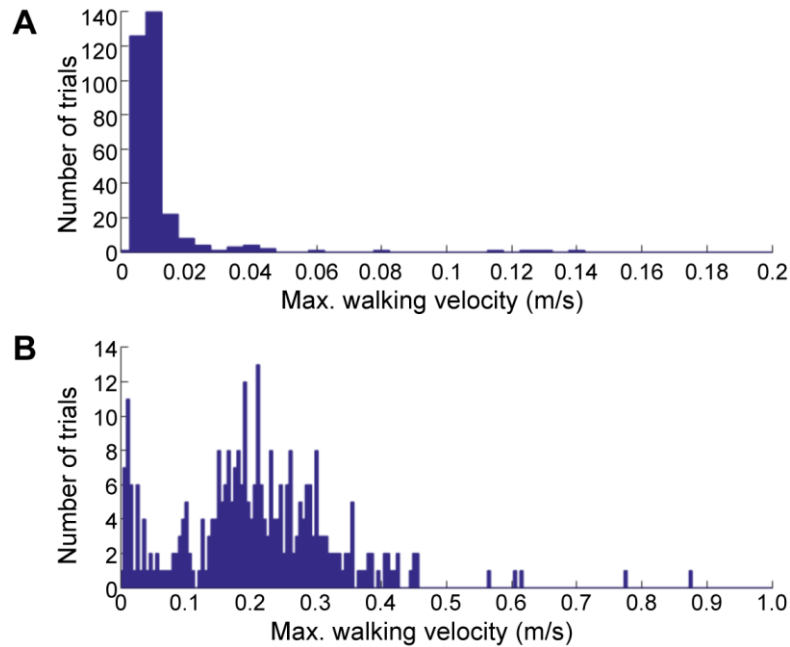


Fig. S2. Frequency distribution of the maximum walking velocities in control

(tone-free) group. (A) Distribution of the walking speed data recorded in the period of 800 ms before the air-puff stimulus of 0.90 m/s. The walking at the speed of >0.01 m/s are very rare, and most of these movements will result from voluntary walking activity. Even if the cricket stood still on the treadmill, the optical sensor detected vibration of the Styrofoam ball lifted by airflow. To separate this vibration noise and the slow rotation caused by voluntary walking, we set the threshold to 0.01 m/s. (B) Distribution of the walking speed after the air-puff stimulus (0.90 m/s). Distinct two groups were observed: Larger group with high speed will represent the air-current-evoked responses, and smaller group with low speed will represent the voluntary walking and artificial noise. Thereby, to identify the air-current-evoked responses, we set the threshold to 0.05 m/s that can divide these two groups.

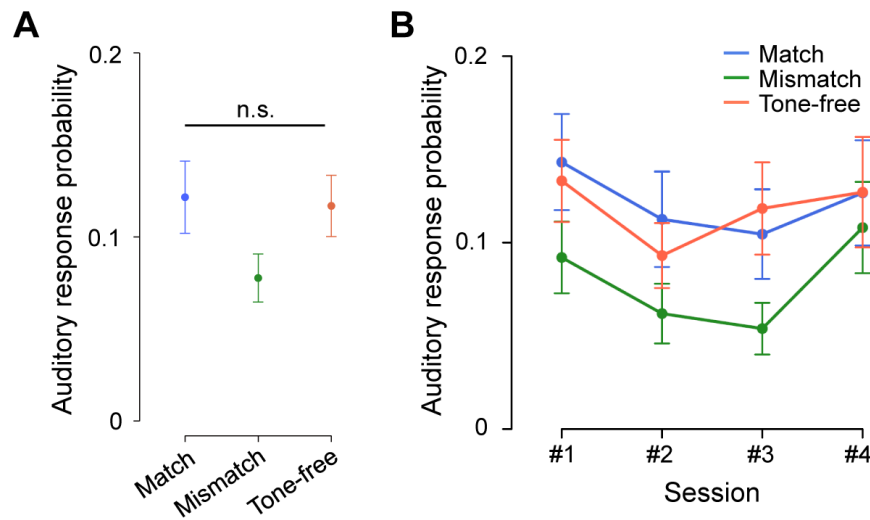


Fig. S3. A preceding acoustic stimulus rarely triggered walking in crickets on its own.

(A) Probability of a walking response for the period of 800 ms before the onset of an air-puff in the three stimulation protocols. The data in (A) and (B) contain the results of responses to the air-puffs at five different velocities (0.26 – 1.11 m/s) in the two-directions test. Each plot in (A) represents the mean response probability in 40 trials for each individual, and the error bars indicate \pm SEM ($n = 40$ animals for each protocol). There were no significant differences in the response probabilities among the stimulation protocols ($p = 0.128$, one-way factorial ANOVA). (B) Relationships between the order of sessions and the auditory response probability. Each plot in (B) represents the average probability in 10 trials for each individual, and the error bars indicate \pm SEM ($n = 40$ animals for each protocol).