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**Neuroethological studies on auditory modulation of  
wind-elicited walking behavior in the cricket**

コオロギの気流誘導性歩行運動の聴覚修飾に関する神経行動学的研究

**Matasaburo Fukutomi**

# 学 位 論 文 の 要 旨

博士の専攻分野の名称 博士（生命科学） 氏 名 福 富 又 三 郎

## 学 位 論 文 題 名

Neuroethological studies on auditory modulation  
of wind-elicited walking behavior in the cricket

(コオロギの気流誘導性歩行運動の聴覚修飾に関する神経行動学的研究)

外敵の接近から素早く逃げる逃避行動は、その行動制御に関わる神経回路が少数のニューロンによって構成されているにも関わらず、同一の忌避刺激に対しても状況に応じて運動パターンを柔軟に変化させる。本研究では、逃避行動の状況依存的な変化が、行動を直接引き起こす「トリガー刺激」と状況を表現する「状況刺激」の異種感覚統合によって生じるという作業仮説の下、その神経機構の解明を目的として、フタホシコオロギ (*Gryllus bimaculatus*) を用いて行動実験及び電気生理学実験を行った。コオロギは腹部末端に尾葉と呼ばれる気流感覚器官をもち、短い気流刺激に対してすばやく遠ざかる逃避行動を示す。また、前肢には鼓膜器官が存在し、同種のオスの求愛歌や捕食者であるコウモリの発する超音波に対して、それぞれ正または負の走性を示す。これは、コオロギの気流感覚系、聴覚系ともに刺激源方位に関する空間情報を認識する機能を有することを意味している。さらに、これら生得的定位行動の神経機構についての詳細な神経行動学的知見が蓄積されているため、コオロギは本研究の目的に適した実験材料である。

第一章では、音刺激（10 kHz トーン音）が気流刺激で誘発される逃避行動を修飾するか、また、音と気流刺激の方向一致性が音刺激による修飾作用に影響するかを検証した。音刺激を気流刺激に対して 800 ミリ秒先行して呈示し、続く 200 ミリ秒間気流刺激を音と同時に側方から与えたところ、逃避行動の移動方向が後方へ偏り、気流刺激に対する反応閾値が上昇した。これらの行動変化は、音刺激を前方から呈示しても気流刺激と同じ側方から呈示しても変化せず、2つの刺激の方向一致性の影響は見られなかった。音刺激による移動方向の変化と反応閾値の上昇は初回の試行から観察され、繰り返して実験しても変化しなかったことから、経験依存的な学習の効果ではないと考えられる。以上の結果から、コオロギは生得的に聴覚系と気流感覚系を統合する機構をもち、その異種感覚間相互作用が行動を変化させることが示唆された。

第二章では、音刺激による気流逃避行動の修飾に対する両刺激の時間的關係性の影響を調べるため、気流刺激に対する音刺激の先行時間と両刺激の重複時間が異なる複数の刺激パターンを

用いた。その結果、音刺激の先行時間を 200 ミリ秒に短縮しても移動方向は後方へ偏ったが、同時に呈示した場合は移動方向の変化は見られなかった。また、先行音を気流刺激前に終了させ、重複呈示しない場合でも、移動方向の変化が観察された。驚くことに音刺激終了から 600 ミリ秒の間隔を開けて気流刺激を呈示しても、移動方向の変化が生じた。これらの結果は、移動方向の変化には音刺激が気流刺激に対して先行することが必要であり、さらに音刺激の情報は終了後少なくとも 600 ミリ秒間は保持されることを示している。一方、反応閾値は、音刺激のタイミングにかかわらず上昇した。したがって、音刺激による移動方向の変化と反応閾値の上昇は、異なる神経メカニズムによって修飾/制御されていると考えられる。

第三章では、音刺激の周波数を変化させ、気流逃避行動の修飾における聴覚による状況依存性を調べた。コオロギ求愛歌の搬送周波数である 5 kHz トーンと、飛行中に回避運動を引き起こす 15 kHz トーンを状況刺激として使用した。これらの音刺激を第一章と同じ時間関係で気流刺激に先行して呈示し、周波数音の違いによる影響を比較した。5 kHz トーンは移動方向を若干後方へ偏らせたものの反応閾値の上昇は見られなかった。一方、15 kHz トーンは移動方向の変化と反応閾値の上昇に加え、移動距離の増大とターン角度のばらつきの上昇をもたらした。すなわち、忌避的な信号である高周波の音ほど気流逃避行動への影響が大きいことがわかった。これらの結果から、コオロギは音の周波数から状況を判断し、続く気流刺激に対する逃避戦略を変化させていると考えられる。

第四章では、音刺激による気流逃避行動の修飾を支配する神経機構を調べるため、細胞内電位記録法を用いて、先行音によって気流応答が変化するニューロンを探索した。本研究では、先行研究においてすでに音・気流刺激の両方に応答することが明らかにされている胸部神経節に存在する Ascending neuron 2 (AN2) に着目した。第三章の実験と同様に、15 kHz トーンを気流刺激に対して 800 ミリ秒先行して呈示したところ、AN2 の気流刺激に対する応答が気流刺激のみの応答よりも減少することが明らかになった。しかし本実験で用いた気流刺激は噴出ノイズを伴うことから、AN2 の応答にはノイズに対する聴覚応答成分が含まれてしまう。そこで尾葉感覚神経束を電気刺激し、AN2 の純粋な機械感覚応答に対する先行音の影響を調べた。その結果、先行音は尾葉電気刺激に対する応答も減弱させた。これらの結果から、行動実験で観察された逃避行動の修飾と同様に、AN2 の気流応答も聴覚入力によって変化することが分かった。AN2 は鼓膜器官の聴覚受容ニューロンから入力を直接受け取っていることから、状況依存的な気流誘導性逃避行動の変化に重要な役割を持っている可能性がある。

本学位論文では、コオロギを材料として、動物が状況依存的に行動を変化させる神経機構を調べるための新規な実験パラダイムを構築した。これを用いて、2 種の感覚刺激の時空間一様性を検証するとともに、状況依存的な行動の変化の発端である可能性をもつニューロンを特定した。本研究成果を足がかりとして、ニューロンの応答性の変化と状況依存的な運動の変化の相関や因果関係を調べることにより、行動の状況依存性を支配する神経機構の全容を解明することが期待される。

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## General Introduction

### *Escape behavior as a model system for understanding sensorimotor processing*

Transformation of the sensory information into the motor output is the most fundamental function in the nervous system. To elucidate the sensori-motor transformation, a lot of system neuroscientists have focused on an escape behavior and thought that the escape behavior is regulated by a relatively-simple neural circuit consisting of a small number of cells which enables the quick response (Card, 2012). For example, the sound-elicited escape behavior in the fish is mediated by the neural circuitry in which the key neuron is identified as Mauthner cell (M-cell). The M-cells, a pair of giant descending neurons, are excited by auditory input from hair cells in the ear contra-lateral to the M-cell's axon and subsequently induce the muscle contraction via activation of the ipsilateral motor neurons, resulting in triggering 'C-start' escape response (Eaton et al., 2001; Korn and Faber, 2005). In the crayfish (*Procambarus clarkia*), two key neurons underlying different types of escape behavior were identified: the medial giant interneurons (MGs) activated by rostral tactile stimulation trigger bending of all abdominal segments, which thrust the animal directly backwards; the lateral giant interneurons (LGs) activated by caudal stimuli elicit bending of only the more rostral abdominal segments, which causes the animal to jump upward and to rotate its tail forwards (Edwards et al., 1999). Several species of insects including fly, locust, cockroach, and cricket have also been well-studied to identify the neural mechanisms underlying sensorimotor processing for the escape behavior (reviewed in Card, 2012). The previous studies provide beautiful neuronal architectures based on the assumption of one-by-one correspondence between the sensory stimulus and the induced reaction. However, it is uncertain whether the escape behavior

really such a stereotyped behavior regulated by a hard-wired neuronal circuit.

### *Context-dependent change of escape behavior*

Many behavioral studies have demonstrated that the performance of the escape behavior largely depends on the surrounding context (reviewed in Domenici, 2010; Domenici et al., 2011a). For example, presence of refuges or burrows affects the escape trajectories in various species of animals (Ellard and Eller, 2009; Hemmi, 2005; Kanou et al., 2016; Zani et al., 2009). If the animals perceive barriers or obstacles, they alter their escape trajectories to avoid both an approaching predator and collision to the obstacles (Eaton and Emberley, 1991; Ingle and Hoff, 1990; Ritzmann et al., 1991). These findings suggest that the animals use the visual cues of environment to modulate their escape route to avoid predators. Furthermore, the startle response in solitary herring fish (*Clupea harengus*) are more varied in the turn angle of escape response than that in the herd of conspecifics (Domenici and Batty, 1997), indicating that the animals flexibly change their escape strategies either taking distance accurately from a predator or exhibiting variable responses unpredictable for the predator, depending on the surrounding context. Taken together, the escape behavior is thought to be neither simple nor stereotyped, and animals flexibly change their escape behavior adapting the context surrounding them. This leads to a question about how the neuronal circuit consisting of a few neurons enables the context-dependent modulation of escape behavior.

### *Multisensory integration is involved in contextual modulation of escape*

Multisensory integration would be an important neuronal process for the context-dependent modulation of the escape behavior because the animals need to combine



multiple sensory cues informing alert signal triggering the escape response itself and additional signals of surrounding contexts, which are often received by different modalities of sensory organ. The multisensory integration often provides animals with accurate and robust perception of their surrounding environment (reviewed in Driver and Noesselt, 2008; Fetsch et al., 2013; Stein and Stanford, 2008). Neuropsychological studies have shown that the spatio-temporal relationship between two different stimuli is crucial for the enhancement of perception by the multisensory integration. For example, directional coincidence of auditory and visual stimuli facilitates the orientation behavior meanwhile the directional mismatch of them obstructs that behavior in the cat (Jiang et al., 2002). These features of the multisensory enhancement in the behavior was supported by the neurophysiological evidences. For example, the multisensory neurons in the superior colliculus (SC) showed the largest response to the visual and auditory stimuli applied from the same position (Meredith and Stein, 1986). In addition, temporal coincidence of these both stimuli also largely enhanced the responses in the SC neurons (Meredith et al., 1987).

I hypothesized that the context-dependency of escape behavior is mediated by multisensory integration of ‘trigger’ stimulus releasing the escape response and ‘contextual’ stimulus that represents the contextual information. Firstly, I built a new behavioral experimental paradigm using two simple stimuli, which should be applicable to neurophysiological experiments. Next, I examined impacts of the spatial- and temporal relationships between the trigger and contextual stimuli on the behavioral modulation. Finally, I explored multisensory neurons combining the trigger and context stimuli which would play a significant role for the contextual modulation of the behavior.

### *Cercal mechanosensory system and auditory system in the cricket*

In this thesis, I used field cricket (*Gryllus bimaculatus*) because it has two well-studied sensory systems: cercal mechanosensory system and auditory system. The sensory organ of the cercal mechanosensory system is ‘cerci’ that is a pair of appendages of the cricket abdomen. Approximately 500–750 mechanosensory filiform hairs are distributed on the cerci and detect an airflow surrounding the cricket as the displacement of the hairs by air-particle (Palka et al., 1977; Miller et al., 2011; Shimozawa and Kanou, 1984). The receptor neurons of these hairs project their axons into the terminal abdominal ganglion (TAG) in a directionally orderly manner (Jacobs and Theunissen, 1996). The sensory afferents from the mechanoreceptors make synaptic contacts with several wind-sensitive interneurons including the giant interneurons (GIs), which arborize specific morphology of the dendrites and project thick and long axons to all proximal ganglia (Hirota et al., 1993). The GIs encode the direction, frequency, and intensity of airflow detected by the cerci and convey these sensory information to the higher centers including the thoracic ganglia and the brain (Miller et al., 1991; Theunissen and Miller, 1991). The cercal mechanosensory system is thought to mediate wind-elicited escape behaviors and provide important signals for the control of escape direction (Camhi, 1980; Boyan et al., 1986; Kanou et al., 1999; Oe and Ogawa, 2013).

The receptor organ of the cricket auditory system is ‘tympanum’ on the frontal legs, which detects changes in a sound pressure. The auditory receptor neurons in the tympanum project their afferents into the prothoracic ganglion in a frequency-dependent orderly manner (Imaizumi and Pollack, 2005). Homologous to the GIs in the cercal system, two identified auditory projection neurons named AN1 and AN2 receive synaptic inputs from the auditory receptor afferents and project their ascending axons to the brain

(Boyan and Williams, 1982; Hennig, 1988; Wohlers and Huber, 1982). The AN1 and AN2 have distinct sensitivity to the sound frequency and provide the auditory information to the brain (Schildberger and Kleindienst, 1989; Stabel et al., 1989). These neurons were shared by the both sexes (Poulet and Hedwig, 2003). Their activities are thought to mediate positive and negative phonotaxis behaviors in both male and female crickets (Hedwig, 2006; Leonard and Hedrick, 2009; Marsat and Pollack, 2012; Pollack, 1982).

I have proposed the following idea. The cercal sensory system could be used for detecting short air puff as ‘trigger’ stimulus eliciting escape behavior, and the auditory system could be used for sensing acoustic ‘contextual’ stimulus indicating two distinct situations in which the conspecifics or the predators are nearby them. In addition, both of these sensory systems provide the directional information of the stimuli, which allow us to test the effects of spatio-temporal relationship on the contextual modulation. Furthermore, the several key neurons for the neural circuits of the primary sensory processing were identified such as GIs and ANs, which would be useful clues to elucidate neural basis of the multisensory integration underlying the contextual modulation of the behavior.

### *Goal of this thesis*

The ultimate goal of my study is to elucidate a neural circuit underlying contextual modulation of escape behavior. In this thesis, I firstly developed the behavioral experimental paradigm with cricket’ escape behavior using air-puff and pure-tone stimuli and examined the impacts of the directional and temporal coincidence of these stimuli on the auditory modulation of the wind-elicited escape behavior (Chapter 1 and 2). In addition, using two different frequency of the acoustic stimuli, the context-dependency

of this behavioral modulation was tested (Chapter 3). Finally, I performed electrophysiological recording to identify a key neuron involved in the context-dependent modulation of escape behavior (Chapter 4).

# Chapter 1

## Effect of spatial coincidence on auditory modulation of behavior

### 1.1 Introduction

Escape reactions are often regarded as a simple behavior, but 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 (*Priplaneta amiricana*) 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 following mechanical stimulus in gobies (*Gobius niger*) (Turesson et al., 2009). These context-dependent modulations of the escape behavior are mediated by multisensory integration between “trigger” stimuli that release the escape response itself and “context” stimuli that represent the contextual information.

Multisensory integration provides a robust perception, which improves sensitivity (McDonald et al., 2000; Stein et al., 1996) and leads to behavioral changes such as shortened reaction time (Rowland et al., 2007). The cross-modal effects on the perception depend on the spatial and temporal relationships of the multiple stimuli. For example, directional coincidence of auditory and visual stimuli facilitates orientation behavior in cats (Jiang et al., 2002). Temporal coincidence of these stimuli enhanced the neural responses in the superior colliculus neurons in cats (Meredith et al., 1987). In humans, a preceding auditory cue improves the directionality of subsequent visual detection

(McDonald et al., 2000). However, what spatio-temporal relationships between multiple stimuli such as trigger and context stimuli in different modalities have effects on the context-dependent modulation of the escape behavior remains unknown. To address this question, I examined the effects of an auditory stimulus on wind-elicited walking behavior in the cricket, 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 aero-detecting organs. One is an auditory organ called tympanal organs on the front legs, which receive changes in air pressure. The other is a mechanosensory organ called cerci at the rear of the abdomen, which sense the 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), while flying crickets exhibit negative phonotaxis in response to ultrasounds emitted by echolocating bats (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 systems, I made a point to use a 10-kHz pure tone as the auditory stimulus of which frequency did not match the carrier frequencies of the calling song (4–5 kHz) and echo-location call (20 kHz or higher). The reason for this is that I 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 this chapter, I tested the following two questions: (1) how the auditory stimulus would modulate the wind-elicited escape behavior, and (2) whether the directional coincidence of the air-puff and tone would affect those cross-modal modulations.

## 1.2 Materials and Methods

### *Animals*

I used 120 laboratory-bred adult male crickets (*Gryllus bimaculatus*) less than 14 days after adult eclosion throughout the experiments. They were reared under 12/12 h light/dark conditions at a constant temperature of 27°C. The body weight was  $0.62 \pm 0.06$  g (average  $\pm$  standard deviation). I removed their antennae to eliminate the influence of mechanosensory inputs from the antennal organ so as to focus on the interaction between the cercal and auditory systems.

### *Treadmill system*

To monitor a cricket's walking activity during the initial response to the air-puff stimulus, I used a spherical-treadmill system (Fig. 1-1A), 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). For the behavioral experiments using



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

#### *Acoustic stimulation*

Behavioral 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 delivered by 1.5 inch full-range sealed MM-SPS2 loudspeakers (Sanwa Supply, Okayama, Japan). For the behavioral experiments, eight speakers were located 105 mm from the animal and spaced 45° apart, just above the air-puff nozzles on the inside wall of the arena. The distance from the preparation to each speaker was 130 mm. To eliminate sound reverberation, acoustic absorbent was attached to the inside wall of the arena for the behavioral tests. In addition, the treadmill system was built in the sound insulation box.

#### *Stimulation protocols and 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, I designed three types of stimulation protocols, referred to as the match, mismatch and tone-free protocols (Fig. 1-1B). In all these protocols, a single air-puff stimulus was delivered from a nozzle to the left or right side alternatively. In the match and mismatch protocols, a tone sound of 1-s duration started 800 ms before an air-puff of 200-ms duration. 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, an air-puff of 200-ms duration was delivered without any prior acoustic stimulus, but I 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 had stood still for 1 s or longer.

I 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). 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 because I needed to wait for 1 second of stationary time prior to the stimulation as mentioned above.

### *Behavioral data analyses and statistical methods*

To quantify the walking behavior among the different stimulation protocols, I 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 the 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. 1-5B). 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’. The definitions of walking direction and turn angle were shown in Fig. 1-5B.

Since the reaction time was tentatively measured as a delay from open of the delivery valve in the picopump to 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, I measured the time delays from the valve open 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, 0.90, and 1.11 m/s were  $67.85 \pm 8.37$ ,  $52.86 \pm 4.34$ , and  $35.71 \pm 2.02$  ms (mean  $\pm$  SEM, seven trials for each speed), respectively. Unfortunately, I was unable to detect the movement of the lint caused by the airflow slower than 0.43 m/s. 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, I classified the trials into three types of response (Fig. 1-2). If a cricket started to walk 800 ms before the onset of the air-puff stimulus and the maximum walking speed was  $> 0.01$  m/s, the trial response was categorized as ‘sound-elicited’ (Fig. 1-2A). If a cricket started to walk after the onset of an air-puff stimulus and the maximum walking speed in the initial response

was  $> 0.05$  m/s, the trial response was categorized as ‘wind-elicited’ (Fig. 1-2B). All other trials, including no walking were categorized as ‘no response’ (Fig. 1-2C). The thresholds of 0.01 and 0.05 m/s 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. 1-3).

The auditory response probability was defined as follows:

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

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 follows:

$$\text{Wind response probability} = \frac{N_w}{N_w + N_{no}},$$

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 the cricket during the locomotion responded to the air-puff stimulus or not.

I used R programming software (version 2.15.3, R Development Core Team) for the statistical analysis. To avoid pseudo-replication, the values of the walking activity parameters in all trials throughout the sessions were averaged for each individual. For analysis of the transition during the sessions, angles of walking direction were averaged for each session in each individual. For statistical analysis of the wind-elicited probability, the probabilities were calculated from all trials throughout the sessions or 10 trials in each session for each individual. To assess the significance of the stimulation protocols, I used a one-way factorial ANOVA when comparing the walking direction, turn angle and

auditory response probability among the groups of crickets. If the main effect of the protocols was significant, I then compared the protocol groups using Tukey's HSD post hoc test. I 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. I 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 the nlme package in R software (Pinheiro et al., 2013). I approximated the wind response probability, using the Hill function as follows:

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

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. I compared the threshold velocity of the stimulation protocols.

### 1.3 Results

*Walking reaction was triggered by an air-puff, but not by a preceding sound.*

Prior to the investigation of the auditory effects on wind-elicited walking, I 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), and there were no significant differences in this probability among the three stimulation protocols ( $p = 0.128$ , one-way factorial ANOVA, Fig. 1-4A). This indicates that the walking activities during auditory stimulus before the air-current stimulus were voluntarily initiated, and that preceding auditory cue alone could not elicit the cricket locomotion. I 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, I examined cricket’s walking activity in response to air-puffs delivered at 0.90 m/s to the crickets’ 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. 1-5A). 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 orientations between the protocols, I 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. 1-5B). The angular value of the walking direction in the match and mismatch protocols were greater than 90° ( $113.79 \pm 7.33^\circ$ ,  $112.64 \pm 7.99^\circ$ , respectively), while the walking direction in the tone-free protocol was less than 90° ( $71.29 \pm 9.30^\circ$ ) (Fig. 1-5C). There were significant differences between the match and tone-free results ( $p = 0.004$ , Tukey's HSD test), and between the mismatch and tone-free ( $p = 0.005$ ). However, the walking direction in the match protocol was not significantly different from that in the mismatch protocol ( $p = 0.995$ ). This result means that the alteration of the walking orientation is 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  $19.43 \pm 2.07^\circ$  for match,  $30.88 \pm 8.58^\circ$  for mismatch and  $27.11 \pm 3.42^\circ$  for tone-free, respectively (Fig. 1-5C). 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.

To analyze the details of auditory effect on the walking orientation, I compared frequency distributions of walking direction between the three stimulation protocols (Fig. 1-6). The results for the match and mismatch protocols revealed similar distributions, in which the walking directions were mainly distributed on the backward ( $> 90^\circ$ ) and their peaks were  $135\text{--}150^\circ$ . In contrast, the walking directions in the tone-free protocol were distributed around  $90^\circ$  and its peak was  $30\text{--}45^\circ$ . The forward walks ( $< 90^\circ$ ) 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 of wind-elicited responses), whereas the backward walks ( $> 90^\circ$ ) 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. 1-6 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, I delivered five different velocities of air-puff stimulus (0.26, 0.43, 0.61, 0.90, and 1.11 m/s) 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 ( $p < 0.001$  for the velocity, and  $p < 0.001$  for the protocol, in a two-way factorial ANOVA) (Fig. 1-7). This indicated that the preceding auditory stimulus affected the dependency of the response probability on the stimulus velocity. Next, I estimated the response threshold based on Hill curves fitted to plots of pooled data of the response probability in each stimulation protocol (Fig. 1-7). 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 for the match, 0.80 m/s for the



mismatch, and 0.59 m/s 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, I compared various walking activity parameters of the responses to the different velocity air-puffs (0.43–1.11 m/s) delivered from the lateral side of the tested crickets, among the three stimulation protocols (Fig. 1-8). The effects of the stimulation protocols and the air-puff velocities on these parameters were tested using a two-way factorial ANOVA (Table. 1-1). The walking directions are independent of the stimulus velocity ( $p = 0.787$ ), while the auditory alterations in the walking direction were significant ( $p < 0.001$ ) (Fig. 1-8A). 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 (Fig. 1-8C). 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) (Fig. 1-8B, D, E). There was also no statistical 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 velocity (0.43–1.11 m/s), 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, I considered the influence of experience on the auditory effects. When the experimental session was extended, the response probability of walking triggered by the auditory stimulus alone remained low (Fig. 1-4B). Both the stimulation protocol type, the 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, I compared the walking direction and the response probability, both of which were altered by the preceding sound, between the four experimental sessions (Fig. 1-9). Regardless of the air-current velocity, the walking direction did not depend on the order of sessions (Fig. 1-9A). In contrast, there were significant effects of the stimulation protocols at the 0.43 – 0.90 m/s velocities (Table 1-2, two-way repeated measures ANOVA), meaning that the walking direction was altered by the preceding auditory stimulus from the first session. As shown in figure 1-7, 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. I 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 0.61 m/s), 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), the response probability gradually declined as the session progressed (Fig. 1-9B). The crickets possibly habituated to the faster air-puff stimuli. In all cases, however, there was no statistical significance of the interaction between the stimulation protocol and the experimental session (Table 1-2, 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.

## 1.4 Discussion

*Cricket has an intrinsic mechanism for auditory modulation of wind-elicited escape*

I revealed that the cricket hearing a sound beforehand altered their wind-elicited behavior, in which the backward walking was facilitated, and the response threshold was elevated. These changes did not result from the associative learning between the preceding tone and air-puff, which means that the cricket has an intrinsic neural mechanism to integrate the auditory and cercal mechanosensory inputs to modulate the escape behavior.

Prior to the experiments, I had hypothesized that a preceding auditory stimulus would more largely or differently modulate escape response triggered by an air-puff delivered from the same direction as the auditory cue. However, neither the modulation of walking direction nor the elevation of the response threshold correlated with coincidence of the two stimulus directions. Responding to the acoustic or air-puff stimuli, the crickets exhibited distinct locomotion depending on the stimulus orientation. The turn angular velocity of female crickets during phonotaxis towards the calling song depends on the location of the sound source (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). Numerous studies have illustrated the neural mechanisms underlying the processing of directional information in the cricket auditory and cercal mechanosensory 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 in this chapter showed no evidence that the directional coincidence affected the auditory modulation, it is still possible that the cricket nervous system is capable of integrating the directional information of different modal stimuli.

### *Cross-modal effect on directionality of locomotion*

Directionality 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, 2011b; Humphries and Driver, 1970). As shown by the results for the tone-free protocol in Fig. 1-6, crickets walked in various directions distributed on the opposite side to a lateral sir-puff stimulus. In addition, the animal's 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 startle stimulus in goldfish (*Carassius auratus*) (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 wall, alters the direction of the escape response in order to avoid collision (Eaton and Emberley, 1991; Ritzmann et al., 1991). My results showed that a preceding auditory stimulus biased walking direction in the wind-elicited walking behavior (Fig. 1-5). Although the ethological relevance for this modulation remains unclear, the change in the directionality could have any impact on the successful escape in a certain specific situation.

Despite abundant studies on the context-dependent change in directionality for

escape behavior, what and how neural mechanism regulates the directional modulation has been unknown. My findings could provide a useful platform to explore the underlying neural mechanism because the modulation of the escape direction in the crickets was caused by combination of two simple sensory stimuli, for which the auditory system and cercal mechanosensory system were studied well (Hedwig, 2006; Jacobs et al., 2008).

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. 1-6 indicates that not only the frequency of forward walking was reduced but backward walking was more frequently induced. That is, the angular distribution of the walking directions could be drastically altered. Furthermore, a preceding auditory input did not alter the turn angle (Fig. 1-5C), which means that the preceding auditory stimulus increased the frequency of backwards stepping rather than turning. The previous study has shown 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 backward 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 mechanism underlying the context-dependent change in directional control of escape.

### *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 Shimozawa, 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 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). For crickets, high-frequency (> 10 kHz) sounds may signal that there are insectivorous bats nearby because a flying cricket displays avoidance steering behavior in response to such sounds (Moiseff et al., 1978; Popov and Shuvalov, 1977). My result of increasing response threshold indicates that the cricket on the ground may reduce unnecessary responses to air-puff in order not to be found from the bats. Further experiments using a higher-frequency sound would illuminate the behavioral function of the auditory effects on the threshold of the air-current-evoked response (see Chapter 3).

### *Potential effects of ablation of antennae*

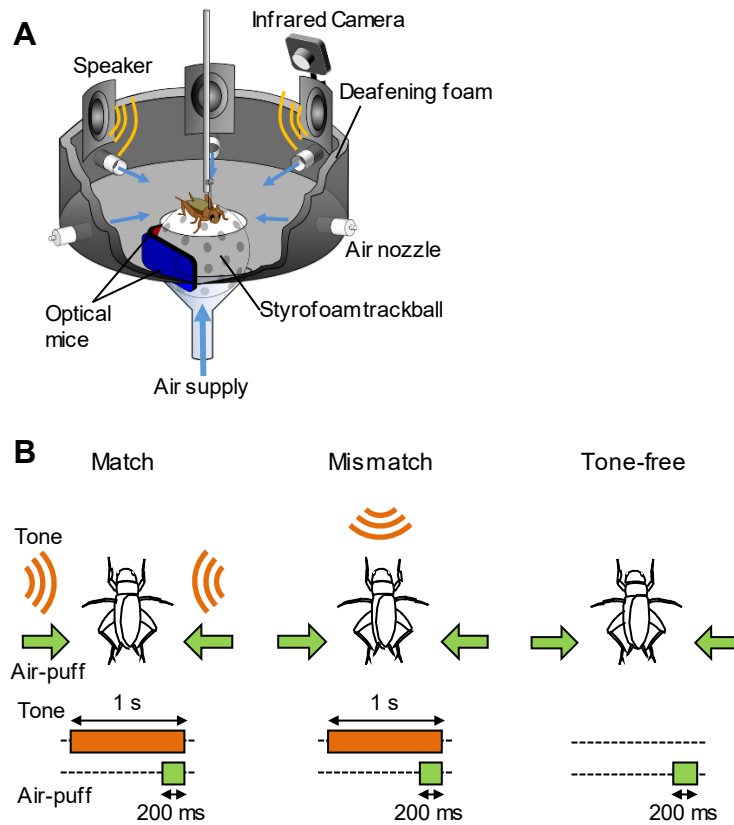
In order to focus on the cross-modal interaction between the cercal and auditory system,

I removed the antennae in this study. There is no reports on the contribution of antennae to the wind-elicited walking behavior in the crickets. However, the previous study shows that the removal of antennae has little effects on the cockroach's wind-elicited escape behavior, which is mediated by the cercal system as well as crickets (Stierle et al., 1994). In addition, the cricket with both intact antennae indicates walking direction in response to the lateral air-puff in the distribution as same as the cricket without antennae for the tone-free protocol, which means little effect of removal antennae on the walking direction (Oe and Ogawa, 2013). However, it remains unknown if the removal of antennae affects the escape responses to other stimulus angles. Possibly, the antennae might play any roles in wind-elicited escape response to the frontal stimuli. The contribution of the antennae on the wind-elicited escape behavior should be examined in the future studies.



## 1.5 Figures

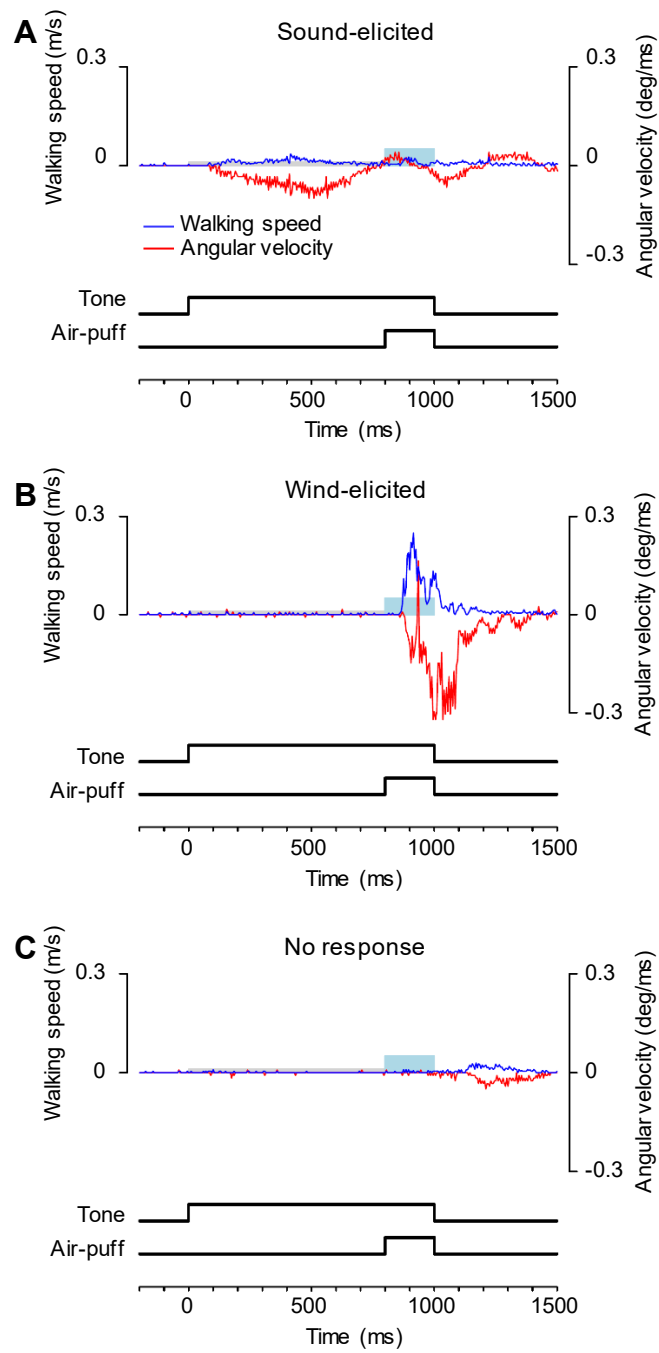
(Figure 1-1)



**Figure 1-1. Experimental apparatus and stimulation protocols for behavioral tests.**

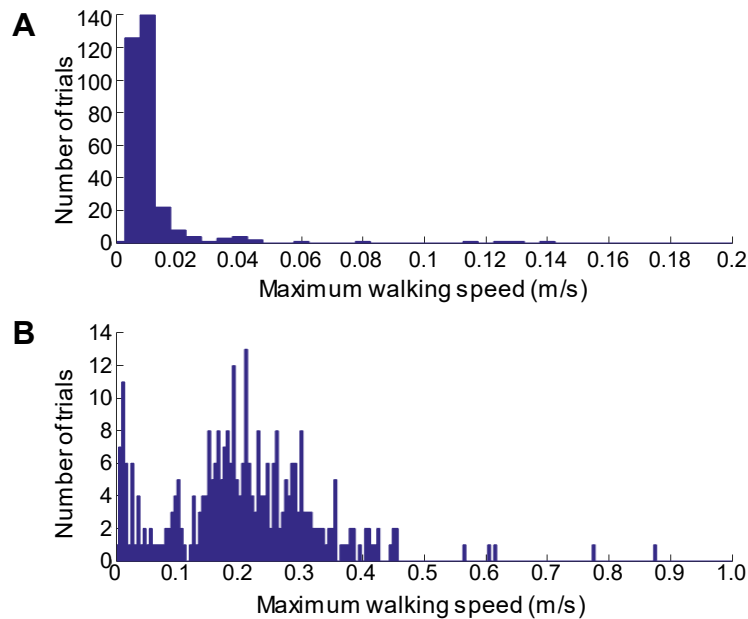
(A) The spherical-treadmill system. The air-puff and acoustic stimulus were delivered from eight nozzles and speakers arranged on the inside wall around the treadmill. (B) Spatial (upper diagram) and temporal arrangements (lower diagram) of the air-puff and acoustic stimuli for the three different protocols in the spatial relationship tests. 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.

(Figure 1-2)



**Figure 1-2. 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'.

(Figure 1-3)



**Figure 1-3. 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, I 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

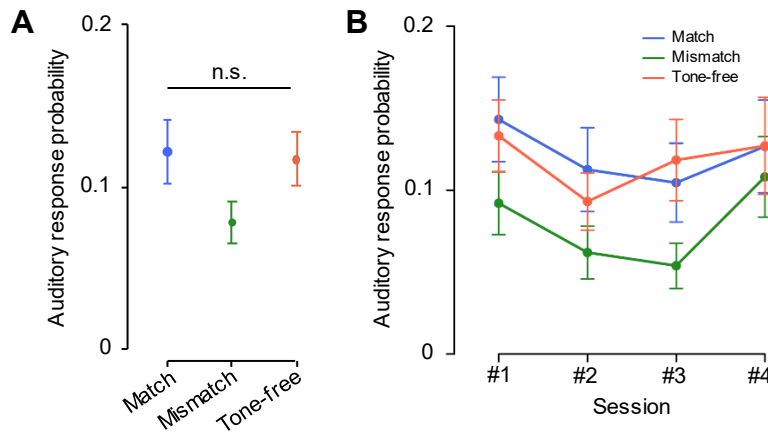
observed: Larger group with high speed will represent the air-current-evoked responses,

and smaller group with low speed will represent the voluntary walking and artificial

noise. Thereby, to identify the air-current-evoked responses, I set the threshold to 0.05

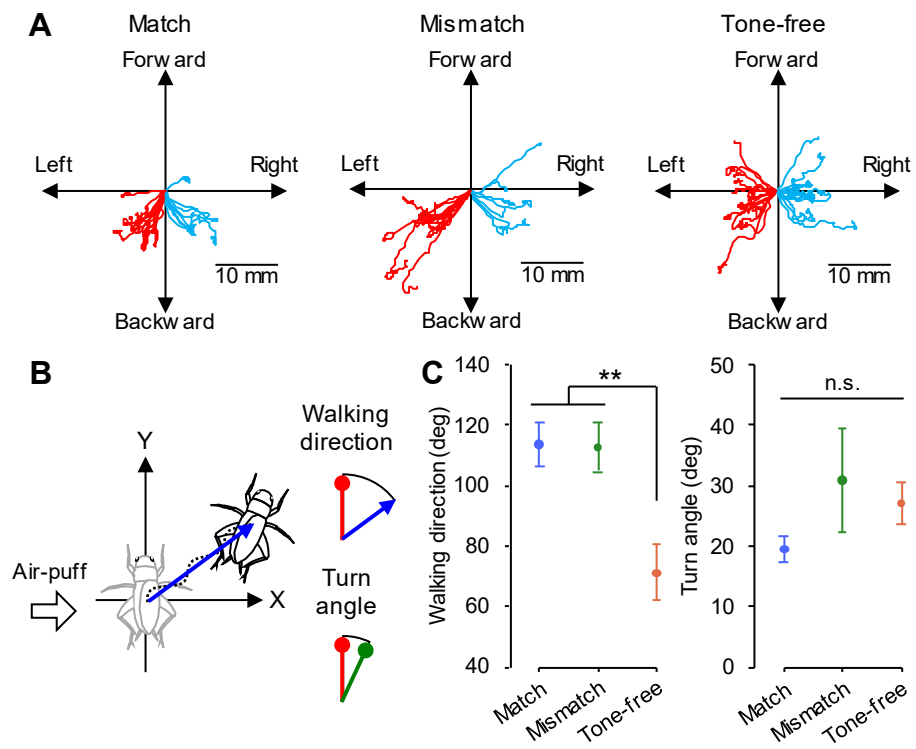
m/s that can divide these two groups.

(Figure 1-4)



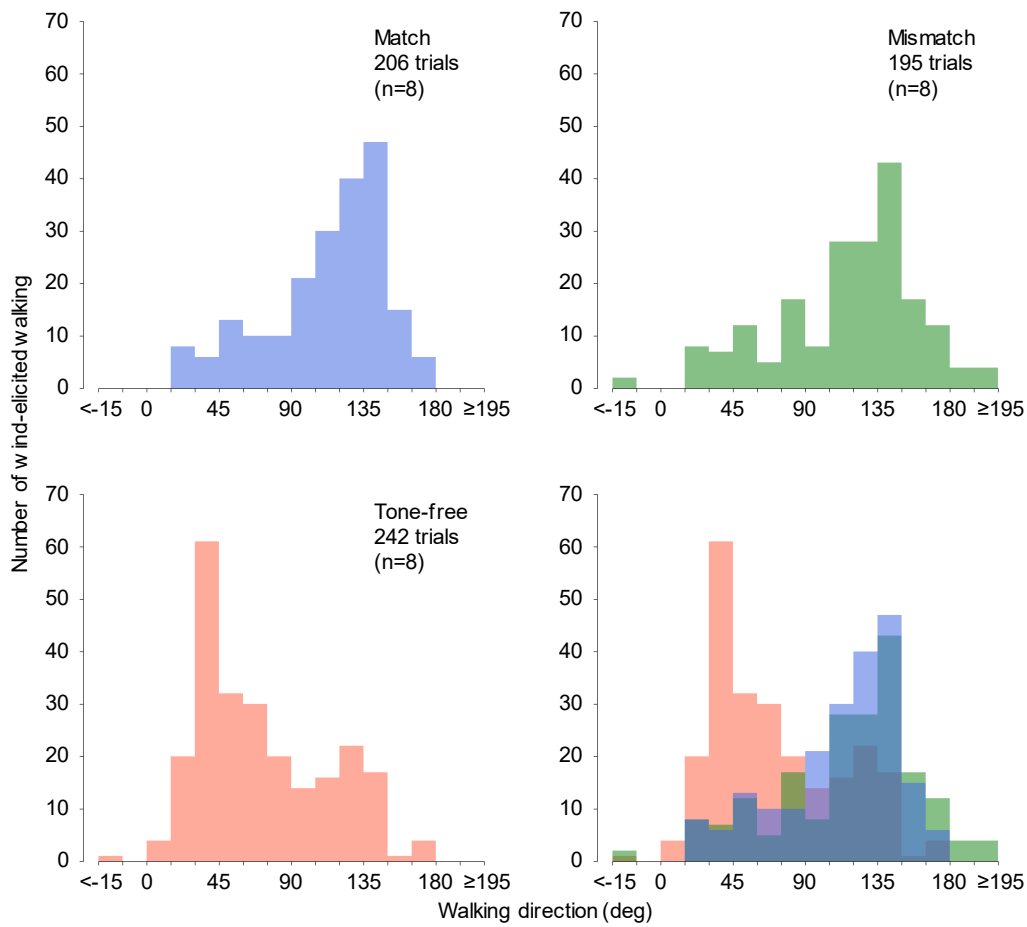
**Figure 1-4. A preceding acoustic stimulus rarely triggered walking in crickets on its own.** (A) Probability of a walking response for the period of 800 ms before the onset of an air-puff in the three stimulation protocols. The data in (A) and (B) contain the results of responses to the air-puffs at 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).

(Figure 1-5)



**Figure 1-5. 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) 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 arranged for forward as 0°. 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 average of the mean values of the data obtained from 40 trials for each individual, and error bars indicate  $\pm$  SEM ( $n = 8$  animals for each protocol). \*\*  $p < 0.01$  (Tukey’s HSD test).

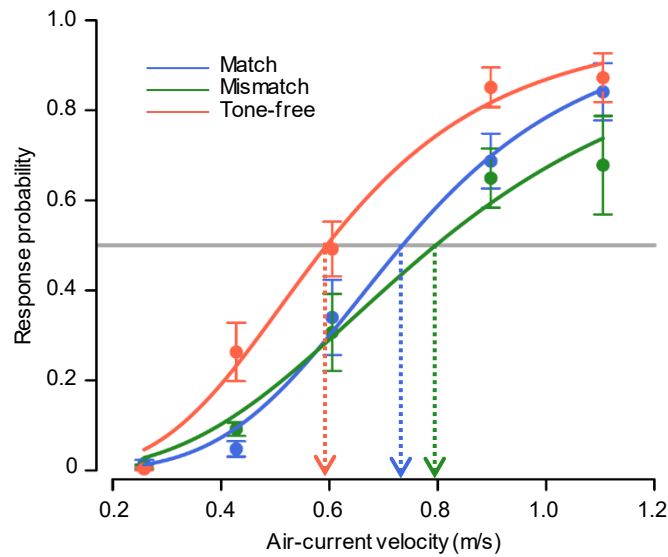
(Figure 1-6)



**Figure 1-6. Frequency distributions of walking directions.** Histograms show the number of trials in which the crickets walked in that direction in responses to three stimulation protocols. Total numbers of the ‘wind-elicited’ responses were 206, 195, and 242 trials for the match (blue), mismatch (green), and tone-free (red) protocols, respectively. The data were obtained from the same experiments shown in Fig. 1-5C (N = 8 animals for each protocol, air-current velocity = 0.90 m/s). The bottom-right shows the overlaid histogram of the frequency distributions in three stimulation protocols.

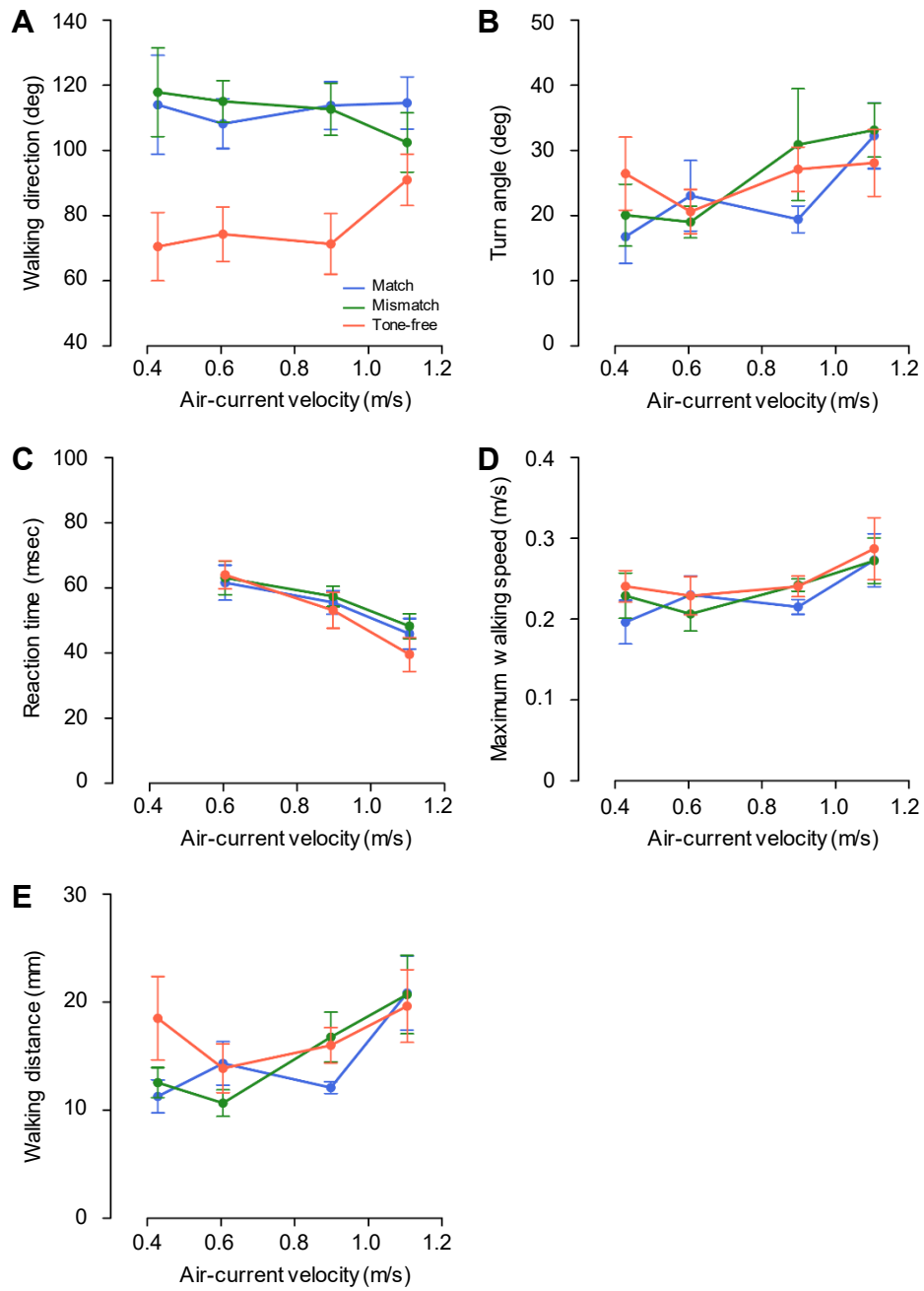


(Figure 1-7)



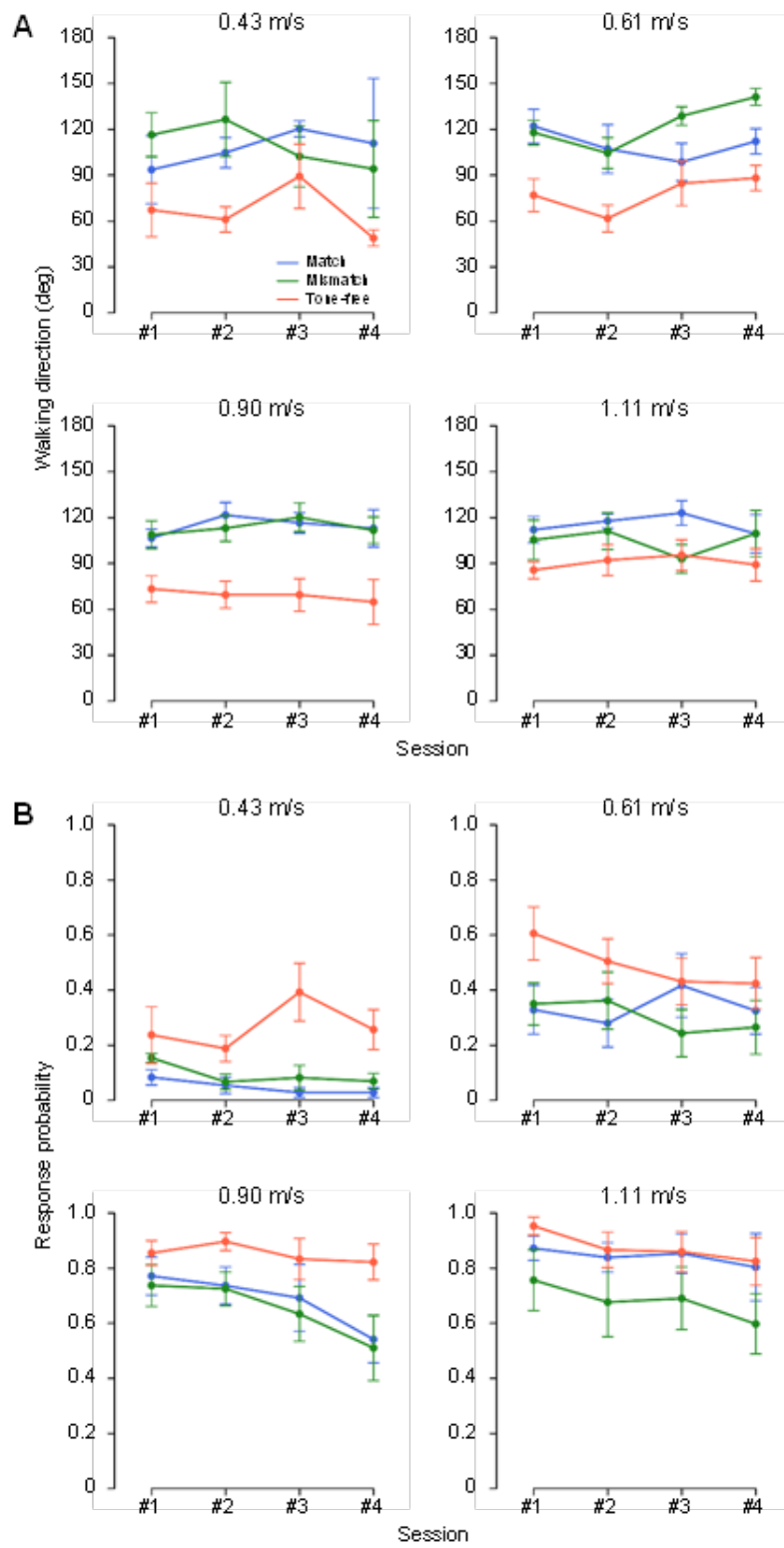
**Figure 1-7. Auditory effect on response threshold.** The probabilities of a walking response to the air-puff stimuli in the different protocols were plotted against the stimulus velocity. The colors of plots and lines represent the stimulation protocols: blue = match, green = mismatch, and red = tone-free. Each plot represents the average of the response probabilities in 40 trials for each individual, and error bars indicate  $\pm$  SEM ( $n = 8$  animals for each protocol and for each air-current velocity). Solid color lines represent curves fitted with the Hill function (see Material and Methods), for which the parameters were as follows:  $h = 4.19$ , and  $v_{1/2} = 0.735$  (match);  $h = 3.16$ , and  $v_{1/2} = 0.796$  (mismatch);  $h = 3.64$ , and  $v_{1/2} = 0.595$  (tone-free). 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.

(Figure 1-8)



**Figure 1-8. Air-current velocity dependency of auditory effects on the walking activity parameters.** The colors of plots and lines represent the stimulation protocols: blue = match, green = mismatch and red = tone-free. 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, and error bars indicate  $\pm$  SEM (n = 8 animals for each protocol and for each air-current velocity). The reason for no plot of the reaction times in responses to the airflow of 0.43 m/s is because the travel time of that stimulus was undetectable.

(Figure 1-9)



**Figure 1-9. 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). The colors of plots and lines represent the stimulation protocols: blue = match, green = mismatch and red = tone-free. 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  $\pm$  SEM (n = 8 animals for each protocol). The data from session #1 to session #4 were measured continuously from each individual.

## 1.6 Tables

**Table 1-1. Summary of two-way factorial ANOVAs testing the significance of stimulation protocols and air-current velocity.** Significance of the main effects was represented in bold.

Parameter	Main effect	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Walking direction	Protocol	2	26619	13309	20.439	<b>&lt; 0.001</b>
	Velocity	1	48	48	0.073	0.787
	Procotol × Velocity	2	2212	1106	1.699	0.189
Turn angle	Protocol	2	117	58.4	0.326	0.723
	Velocity	1	1454	1454.1	8.11	<b>0.005</b>
	Procotol × Velocity	2	327	163.5	0.912	0.406
Reaction time	Protocol	2	194	97	0.591	0.556
	Velocity	1	3912	3912	23.885	<b>&lt; 0.001</b>
	Procotol × Velocity	2	225	113	0.688	0.506
Maximum walking speed	Protocol	2	0.0055	0.00275	0.604	0.549
	Velocity	1	0.0358	0.03575	7.868	<b>0.006</b>
	Procotol × Velocity	2	0.0005	0.00023	0.051	0.95
Walking distance	Protocol	2	85	42.4	0.824	0.442
	Velocity	1	513	513.4	9.981	<b>0.002</b>
	Procotol × Velocity	2	137	68.7	1.336	0.268

**Table 1-2. Summary of two-way repeated measures ANOVAs testing the significance of stimulation protocols and the order of sessions.** Significance of the main effects was represented in bold.

Parameter	Velocity	Main effect	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Walking direction	0.43 m/s	Protocol	2	24554	12277	5.98	<b>0.012</b>
		Session	1	199	199.1	0.173	0.68
		Protocol × Session	2	1656	828	0.719	0.495
	0.61 m/s	Protocol	2	30430	15215	10.143	<b>0.001</b>
		Session	1	1118	1117.6	1.909	0.172
		Protocol × Session	2	2295	1147.7	1.961	0.15
	0.90 m/s	Protocol	2	42574	21287	10.28	<b>&lt; 0.001</b>
		Session	1	2	2.4	0.009	0.926
		Protocol × Session	2	436	218.2	0.786	0.46
	1.11 m/s	Protocol	2	9990	4995	2.382	0.119
		Session	1	2	1.8	0.004	0.949
		Protocol × Session	2	98	48.9	0.113	0.894
Response probability	0.43 m/s	Protocol	2	0.868	0.434	8.853	<b>0.002</b>
		Session	1	0.0038	0.003821	0.238	0.627
		Protocol × Session	2	0.0616	0.03078	1.918	0.155
	0.61 m/s	Protocol	2	0.634	0.3172	1.652	0.216
		Session	1	0.0997	0.9967	3.907	0.052
		Protocol × Session	2	0.1155	0.05774	2.264	0.112
	0.90 m/s	Protocol	2	0.734	0.367	3.482	<b>0.049</b>
		Session	1	0.3707	0.3707	11.632	<b>0.001</b>
		Protocol × Session	2	0.0936	0.468	1.468	0.237
	1.11 m/s	Protocol	2	0.7	0.3501	1.778	0.193
		Session	1	0.1458	0.14581	8.15	<b>0.006</b>
		Protocol × Session	2	0.016	0.00798	0.446	0.642

## Chapter 2

# Effect of temporal relationship on auditory modulation of behavior and descending neural signal

### 2.1 Introduction

The nervous system integrates sensory information of multi-modal stimuli to generate the robust perception and adaptive action selection in both vertebrates and invertebrates (e.g. Ohyama et al., 2015; Stein et al., 1989). As well as the directional coincidence of multiple stimuli examined in Chapter 1, the temporal relationship between them is another crucial factor for the multisensory integration. For example, the temporal coincidence of auditory and visual stimuli enhances the multisensory response in the superior colliculus neurons of cats, which mediates attentive and orientation behavior (Meredith et al., 1987). In humans, a preceding auditory cue improves the directionality of subsequent visual detection (McDonald et al., 2000). In addition to the results in the mammalian CNS, a preceding visual input increases response probability of sound-evoked escape in larval zebrafish (*Danio rerio*) (Mu et al., 2012).

In the previous chapter, I demonstrated that a preceding auditory input biased walking direction backward and increased response threshold in the wind-elicited escape behavior in crickets, and that the directional coincidence of the tone and air-puff stimuli was not essential for these modulations. This finding allows us to speculate that it would not matter for the crickets whether the acoustic and airflow stimuli originate from the same source or not, but the presence of sound would be important. The next question should be what temporal relationship between these two stimuli is required for the auditory modulation of wind-elicited escape. Especially, I focused on the following two



points in the temporal relationship: (1) the precursor time of the tone sound against the air puff, and (2) the temporal overlap of these two stimuli. Furthermore, I recorded extracellularly descending activities from the cephalic ganglia which would mediate the directional control of cricket's escape behavior like the superior colliculus neurons that mediates walking orientation in cats (Meredith et al., 1987; Oe and Ogawa, 2013). To test the effects of the temporal relationships on the behavioral and neural responses, I used several types of combined stimulation of different temporal patterns.

## 2-2 Materials and Methods

Treadmills, and stimulators were the same as in Chapter 1.

### *Animals*

I used 79 laboratory-bred adult male crickets (*Gryllus bimaculatus*) less than 14 days after adult eclosion throughout the experiments. They were reared under 12/12 h light/dark conditions at a constant temperature of 27°C. The body weight for 66 crickets used in the behavioral experiments was  $0.60 \pm 0.06$  g (average  $\pm$  standard deviation). The body weight for 13 crickets used in the extracellular recording was not measured. I removed their antennae to eliminate the influence of mechanosensory inputs from the antennal organ so as to focus on the interaction between the cercal and auditory systems.

### *Extracellular recording*

The cricket was held dorsal side up using insect pins on a silicon platform and its tergite was removed to expose the ventral nerve cord (VNC) after anesthetized with ice. Extracellular recordings of descending spikes were made using a glass suction electrode placed on distal cut of a right VNC between the subesophageal ganglion (SEG) and prothoracic ganglion (PTG). The recorded signals were digitized at 20 kHz through a Powerlab 4/30 A-D converter (ADInstruments) and filtered with low cut filter with 150 Hz.

### *Stimulation protocols for behavioral experiments*

To test the cross-modal effects of temporal relationships between the acoustic and air-puff stimuli on wind-elicited walking behavior, I designed six types of stimulation

protocol, referred to as the long-delayed, short-delayed, simultaneous, short-traced, long-traced, and tone-free (Fig. 2-1). For five protocols except for tone-free, the direction of acoustic stimulus (10-kHz pure tone, 70 dB) consistently corresponded to that of air-puff stimulus in similar to the match protocol used in Chapter 1 (Fig. 1-1B). In the long-delayed protocol, a tone sound of 1000-ms duration started 800 ms before an air-puff for 200 ms, which is the same stimulus as used in the match protocol. In the short-delayed protocol, a tone sound of 400-ms duration started 200 ms before an air-puff for 200 ms. In the simultaneous protocol, a tone sound of 200-ms duration and an air-puff for 200 ms were applied simultaneously. In the short-traced protocol, a tone sound of 800-ms duration started 800 ms before an air-puff for 200 ms. In the long-traced protocol a tone sound of 200-ms duration started 800 ms before an air-puff for 200 ms. In all protocols for this test, a single air-puff stimulus was delivered from the left and right nozzles alternatively with >1-min interval. I divided the crickets into six groups for different protocols (long-delayed, short-delayed, simultaneous, short-trace, long-trace, and tone-free). As each experimental group consisted of 11 individuals, 66 crickets were used for the experiments in total. For each individual cricket, five sessions using different air-current velocities (0.26, 0.43, 0.61, 0.90 and 1.11 m/s), each of which comprised 10 trials, were performed using the same protocol. The inter-trial interval was >1 min, and the inter-session interval was >10 mins.

#### *Stimulation protocols for extracellular recording*

The tone-free, long-delayed and simultaneous protocols were used for the electrophysiological experiment. For air-puff stimulation, the air currents at a velocity of 0.90 m/s were used throughout the electrophysiological recording. The directions of air-

puff and tone were applied from the nozzle contralateral to the recording side (-90°). The descending signals were recorded in 13 individuals and 10 trials were performed for each protocol in each individual.

### *Behavioral analysis*

Categorization of the behavioral responses and definition of the locomotory parameters were the same as described in Chapter 1.

For the statistical analysis, R programming software (version 2.15.3, R Development Core Team) was used. Considering individual differences and sample size effects due to air-current velocity, I used linear mixed-effects models (LME) in which air-current velocity nested within cricket ID were considered as random effects, to test the significance of the several temporal relationships of air-puff and acoustic stimuli on walking direction and on turn angle (Fig. 2-3 and 2-5). To assess the cross-modal effects of temporal relationships of air-puff and acoustic stimuli on the relationship between response probability and air-current velocity, I used generalized linear mixed-effect model (GLMM) with a bimodal distribution (Fig. 2-4 and 2-6). In this analysis, I compared the three models, referred as model A, B, and C. Model A had a fixed effect of air-current velocity only. Model B had fixed effects of air-current velocity, stimulation protocol, and the interaction. Model C had fixed effects of air-current velocity, acoustic stimulus regardless of timing, and the interaction. I employed the best model of the three candidates by comparing the AIC.

### *Analysis of descending neural activities*

I performed spike extraction using LabChart software (Ver.7, ADInstruments). The

threshold for extracting the spikes was set to five times of standard deviation of voltages during spontaneous activities before stimulation of the ‘tone-free’ protocol for each individual. Based on the peri-stimulus-time histogram of the spike count with 10-ms bins, I analyzed peak value of firing rate, time of the peak firing-rate and decay time using add-on software (Spike Histogram Extension for LabChart 7, ADInstruments). Based on the exponentially-decaying curve approximated to the decay phase of the response, the decay time was determined as a duration from the peak to half of the firing rate.

For statistical analysis of descending signals, I used Wilcoxon signed-rank test. The p-values were corrected by the Holm method.

## 2.3 Results

*Auditory effect on the walking direction requires preceding but not simultaneous input.*

To test the impact of precursor time of the auditory stimuli on the cross-modal effects, I designed four stimulation protocols which differed from each other in the time lag between the onsets of the tone sound and the air puff, referred to as long-delay, short-delay, simultaneous, and tone-free protocols (Fig. 2-1). The long-delay protocol was the same temporal arrangement of the two stimuli as used for the match and mismatch protocols in Chapter 1. The auditory stimulus 200 ms prior to air-puff in the short delay protocol biased walking direction backward in similar as the long-delay protocol (Fig. 2-3A). However, the crickets stimulated by the simultaneous protocol walked laterally to the opposite direction to the air puff, which was similar to the results in the tone-free protocol. The mean angular values of the walking direction response to air-puff at different velocities (0.43-1.11 m/s) in the long- and short-delay protocols were larger than 90° ( $102.28^\circ \pm 2.99^\circ$  for long-delay,  $109.26^\circ \pm 2.46^\circ$  for short-delay), while those in the simultaneous and tone-free protocols were smaller than 90° ( $81.71^\circ \pm 2.82^\circ$  for simultaneous  $79.72^\circ \pm 3.19^\circ$  for tone-free). LME revealed statistical significant differences between the long-delay and tone-free protocols ( $p = 0.010$ ), between the short-delay and tone-free protocols ( $p = 0.003$ ), between the long-delay and simultaneous protocols ( $p = 0.042$ ), and between the short-delay and simultaneous protocols ( $p = 0.017$ ). In contrast, there was no significant difference between the long-delay and short-delay protocols ( $p = 0.701$ ) and between the simultaneous and tone-free protocols ( $p = 0.564$ ) (Fig. 2-3B). The mean angular values of turn angle in the four protocols were  $23.71^\circ \pm 1.43^\circ$  for the long-delay protocol,  $35.82^\circ \pm 2.19^\circ$  for the short-delay protocol,  $25.79^\circ \pm 1.51^\circ$  for the simultaneous protocol, and  $24.65^\circ \pm 1.59^\circ$  for the tone-free protocol (Fig. 2-

3C). There was no significant difference in the turn angle between any pairs of the stimulation protocols ( $p = 0.186$  for the long-delay and short-delay protocols,  $p = 0.731$  for the long-delay and simultaneous protocols,  $p = 0.635$  for the long-delay and tone-free protocols,  $p = 0.324$  for the short-delay and simultaneous protocols,  $p = 0.384$  for the short-delay and tone-free protocols, and  $p = 0.899$  for the simultaneous and tone-free protocols, LME) (Fig. 2-3C). These results demonstrated that not simultaneous but preceding auditory input altered the walking direction and that auditory stimulus had no impact on the turn angle regardless of its precursor time.

To examine the effect of the precursor time on the auditory modulation of the response threshold, I analyzed air-current-velocity-dependent curve of the response probability in these four stimulation protocols (Fig. 2-4). The response thresholds calculated from the Hill-function curves fitted to the data plots were 0.67 m/s for the long-delay, 0.75 m/s for the short delay, 0.73 m/s for the simultaneous, and 0.60 m/s for the tone-free protocols, respectively. To test statistical effects of the temporal coincidence on response probability of wind-elicited walking behavior, I adopted model selections using a generalized linear mixed-effect model (GLMM) with a bimodal distribution (see also Materials and Methods). In this analysis, I compared three models, which were model A containing a fixed effect of air-current velocity only, model B containing fixed effects of air-current velocity, protocols, and their interaction, and model C containing fixed effects of air-current velocity, presence or absence of the auditory stimulus (regardless of precursor time), and their interaction. AIC values were 342.9 for model A, 347.8 for model B, and 342.0 for model C, meaning that the model C is the most interpretive model. This result suggests that auditory inputs elevated the response threshold regardless of the precursor time to the air-puff stimuli. The fact that the auditory effects on the walking

direction and response threshold followed different rules of the temporal relationship, also imply that the auditory modulation in the wind-elicited behavior should be involved in multiple stages of neural circuits for cross-modal interaction.

*Auditory effects did NOT require temporal coincidence of acoustic and air-puff stimuli.*

Next, I focused on temporal coincidence (overlap) between the acoustic and air-puff stimuli and the duration of tone preceding to air-puff. Here, I designed four different stimulation protocols which differed from each other in the time lag between the offsets of the tone sound and the air puff, referred to as the long-delay, short-trace, long-trace, and tone-free protocols (Fig. 2-1). The preceding auditory stimulus without temporal coincidence with the air puffs in the short- and long-trace protocols also biased the wind-elicited walking backward (Fig. 2-5A). The mean angular values of the walking direction in the short- and long-trace protocols were larger than  $90^\circ$  ( $99.54^\circ \pm 3.63^\circ$  for short-trace,  $105.57^\circ \pm 2.90^\circ$  for long-trace) (Fig. 2-5B). LME revealed significant differences between the long-delay and tone-free protocols ( $p = 0.015$ ), between the short-trace and tone-free protocols ( $p = 0.046$ ), and between the long-trace and tone-free protocols ( $p = 0.009$ ). In contrast, there was no significant difference between the long-delay and short-trace protocols ( $p = 0.648$ ), between the long-delay and long-trace protocols ( $p = 0.848$ ), and between the short- and long-trace protocols ( $p = 0.517$ ) (Fig. 2-5B). The mean angular values of turn angle in the short- and long-trace protocols were  $21.44^\circ \pm 1.82^\circ$  and  $30.45^\circ \pm 2.12^\circ$ , respectively. There was no significant difference in the turn angle between any pairs of the stimulation protocols ( $p = 0.875$  for the long-delay and short-trace protocols,  $p = 0.250$  for the long-delay and long-trace protocols,  $p = 0.642$  for the long-delay and tone-free protocols,  $p = 0.323$  for the short- and long-trace protocols,  $p = 0.761$  for the



short- and tone-free protocols, and  $p = 0.483$  for the long-trace and tone-free protocols, LME) (Fig. 2-5C). These results indicated that even without temporal coincidence the preceding auditory inputs altered walking direction.

I also tested the effects of the temporal coincidence on the modulation of the response threshold. The response thresholds based on the Hill-function curve were 0.84 m/s for short-trace and 0.69 m/s for long-trace protocols, respectively (Fig. 2-6). Using GLMM with a bimodal distribution, I compared three models A, B, and C mentioned above. The model C containing fixed effects of air-current velocity, presence or absence of the auditory stimulus (regardless of the temporal coincidence), and their interaction was selected as the most interpretive model (AIC values, 364.1 for model A, 364.4 for model B, and 361.9 for model C). This result also indicated that auditory input elevated the response threshold regardless of the temporal coincidence between the acoustic and air-puff stimuli and the duration of tone preceding to air-puff.

*Preceding auditory inputs decreased descending activity evoked by air-puff stimulus.*

To test the contribution of cephalic ganglia to the cross-modal interaction, I compared descending activities among three different stimulation protocols referred as to tone-free, delay, and simultaneous (N = 13 animals). In all stimulation protocols, the descending neurons transiently responded to an air-puff stimulus from contralateral side to the recording site (Fig. 2-7A). The bursting response in the delay protocol was slightly lower than those in tone-free and simultaneous protocol. In addition to the wind-evoked burst, the descending neurons also responded to 10-kHz pure tone, but this auditory response was very transient, and the descending activity quickly returned to spontaneous level before the air-puff stimulation (Fig. 2-7A). Focusing on the transient responses to air-puff

stimulus in different stimulation protocols, the time course of the averaged firing rate was similar to each other (Fig. 2-7B). The response to the delay stimulation, however, displayed lower peak of the firing rate than tone-free and simultaneous stimulation protocols. There were significant differences between the delay and tone-free protocols ( $p = 0.029$ , Wilcoxon signed-rank test corrected by Holm's method) and between the delay and simultaneous protocols, ( $p = 0.020$ ). In contrast, there was no significant difference between the tone-free and simultaneous types ( $p = 1.000$ ) (Fig. 2-7C). On the other parameters such as time of the peak firing rate and decay time, there was no significant difference among the three stimulation protocols (Fig. 2-7D, E). These results indicated that the auditory input reduced the descending activity evoked by the following air-puff stimulus.

## 2.4 Discussion

### *Auditory effects depend on temporal relationship between multisensory inputs*

The auditory effect on the walking direction depends on precursor time of acoustic stimulus. However, the fact that repeated perceptions of the paired stimuli of tone and air-puff had no impacts on wind- or auditory-evoked behavior demonstrated that this auditory modulation did not result from associative learning (shown in Chapter 1). Temporal relationships between conditioning stimulus (CS) and unconditioned stimulus (US) were crucial rules for classical conditioning inducing the associative learning (Mazur, 2006). In a classical eye-blink conditioning in mammals, for example, the sound stimulus as CS preceding air puff as US was required for the successful learning, but simultaneous exposure of CS and US is not effective in that conditioning. That is because the animals need some time to recognize the CS and to associate the CS with the following US. Probably, this reason accounts for my results that auditory modulation of the walking direction required precursor time. If so, the auditory cue of 200–800 ms duration is sufficient for the cricket to perceive the auditory context to modulate the wind-elicited behavior. In contrast, the elevation of the response threshold was induced also by the simultaneous stimulation of the tone and air puff. The auditory modulation of the response threshold may result from simpler interaction between the auditory and cercal sensory systems.

### *Working memory of acoustic signal may be involved in the behavioral modulations*

Interestingly, the auditory inputs even in the trace stimulation protocols biased the walking direction backward. Since the auditory inputs are terminated 600 ms before the onset of the air puff in the long-trace protocol, the auditory context needs to be memorized

for at least 600 ms for the cross-modal modulation. The recent study on the eye-blink conditioning in rabbits reported that rostral medial prefrontal cortex (rmPFC) neurons counts CS-US time intervals with dominant firing peaks at three precise time dependent on the duration of CS-US time interval (Caro-Martin et al., 2015). In the cricket, the coincidence detector neuron identified as LN3 within the brain recognize the interval of acoustic pulse of male's calling song (Schöneich et al., 2015). It is possible that the crickets have some neural mechanism to memorize the contextual information. Further investigation to clarify how long the time interval between auditory cue and air puff can modulate walking direction will reveal the capability of the working memory for the cross-modal modulation in the cricket.

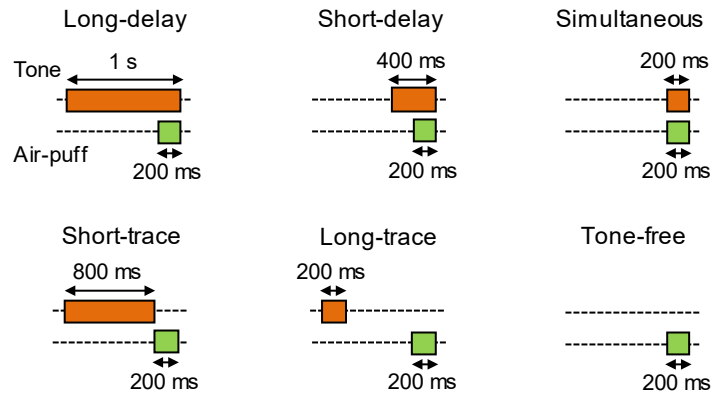
*Modulation of walking direction should be mediated by neural circuits via brain.*

Auditory modulations of walking direction and response threshold showed the different temporal-pattern dependency, which suggests that the modulations were mediated by different neural circuits respectively as mentioned above. In addition, I also showed the firing rate of descending neuronal was reduced only by the preceding tone but not modulated by the simultaneous one. That is, the auditory modulations of descending activity and of walking direction seem to follow the similar rule in the temporal relationship between the auditory and cercal sensory inputs. As mentioned in Discussion of Chapter 1, the descending activity is crucial for the control of the voluntary movement such as directional control in the locomotion. For the cricket's wind-elicited escape behavior, the descending neurons have a significant role for directional control of walking, because hemi-severance of the VNC containing descending axons between the subesophageal and the thoracic ganglia abolished stimulus-angle-dependent control (Oe

and Ogawa, 2013). Taken together, the auditory modulation of walking direction should be mediated by neural circuits via brain.

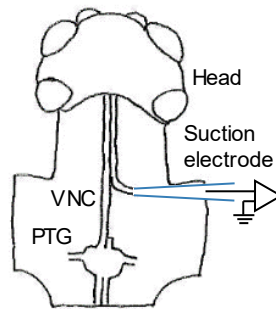
## 2.5 Figures

(Figure 2-1)



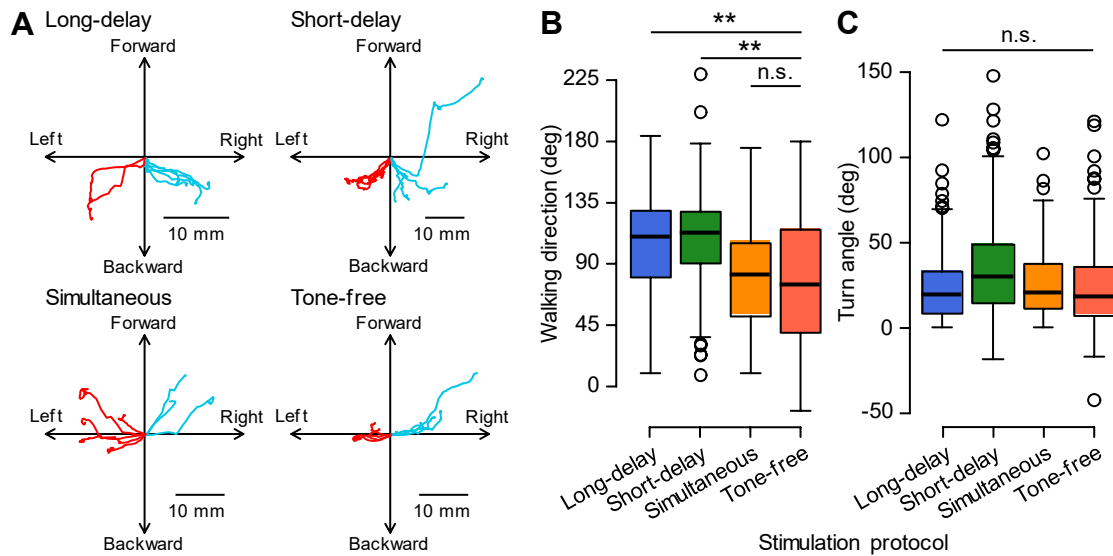
**Figure 2-1. Stimulation protocols for the temporal relationship test.** Temporal arrangements of the air-puff and acoustic stimuli for the six different protocols in the temporal relationship tests. Both stimuli were delivered from the same direction in the same way as the match protocol.

(Figure 2-2)



**Figure 2-2. Diagram of extracellular recording.** Descending signals from the right ventral nerve cord were recorded with the suction electrode. VNC: ventral nerve cord. PTG: Prothoracic ganglion.

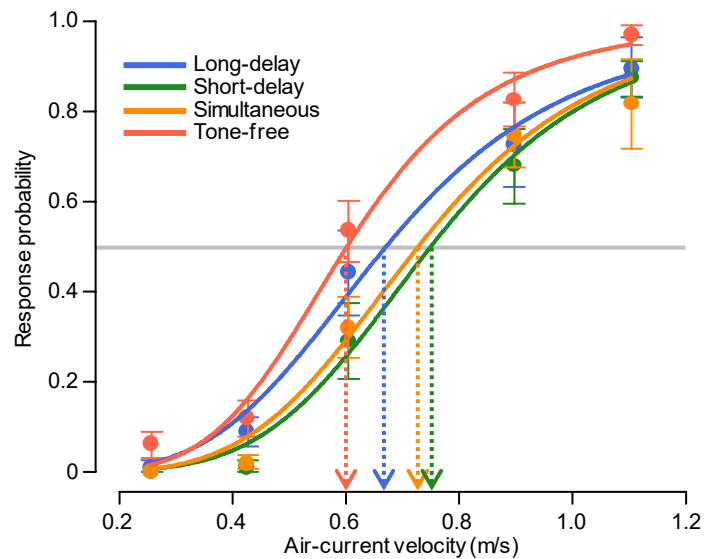
(Figure 2-3)



**Figure 2-3. Effects of precursor time of acoustic stimulus on the directional modulation.** (A) Typical walking trajectories of an individual cricket in response to air-puffs (0.90 m/s) from lateral sides in the four different protocols. Red and light blue traces show the walking trajectories elicited by the stimulus from the right and left sides, respectively. Pooled data of walking direction (B) and turn angle (C) in the four different protocols, for each of which the air-current stimuli were applied at five different velocities. The box plot whiskers indicate the  $1.5 \times$  interquartile range of the lower and upper quartiles; box limits indicate the lower, median, and upper quartile from bottom to top; the circles indicate outlier data. \*\*  $p < 0.01$  (linear mixed-effects models).

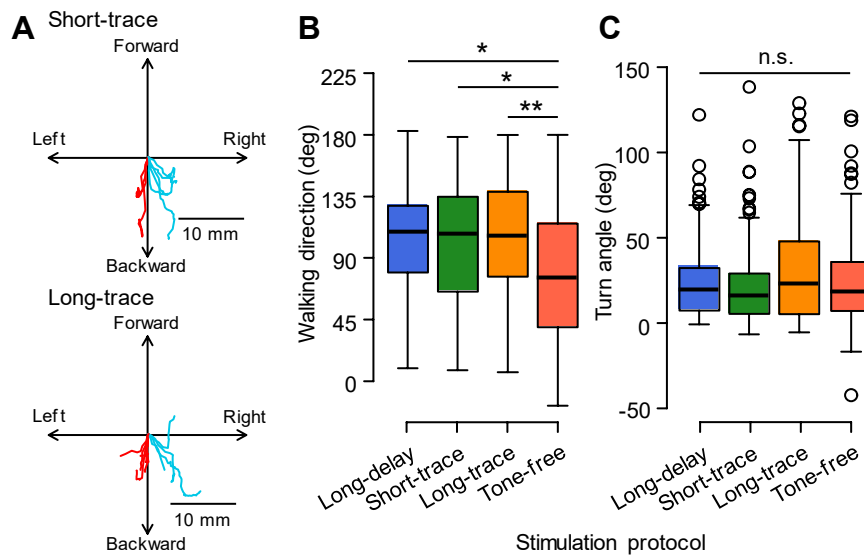


(Figure 2-4)



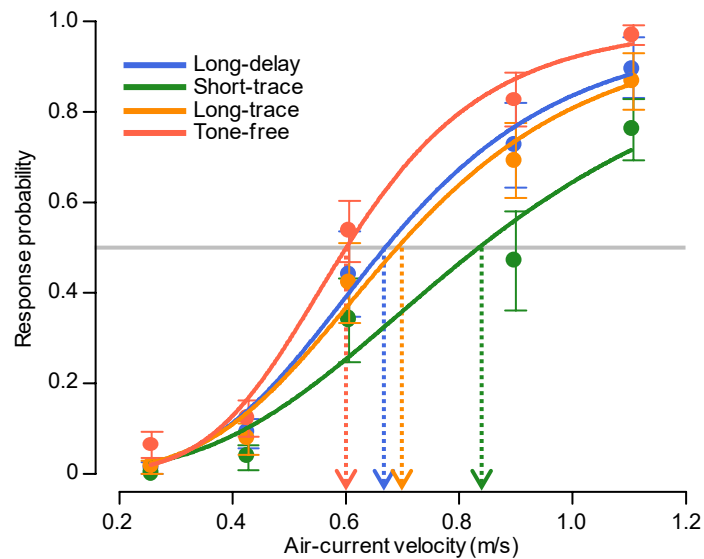
**Figure 2-4. Effects of precursor time of acoustic stimulus on the elevation of response threshold.** The probabilities of walking response to the air-puff stimuli in the different protocols against the stimulus velocity. Each plot represents the mean response probabilities of 11 animals for each protocol and error bars indicate  $\pm$  SEM. Solid color lines represent curves fitted with the Hill function (see the Material and methods), for which the parameters were follows: long-delayed,  $h = 4.09$  and  $v_{1/2} = 0.672$ ; short-delayed,  $h = 4.81$  and  $v_{1/2} = 0.752$ ; simultaneous,  $h = 4.59$  and  $v_{1/2} = 0.730$ ; tone-free,  $h = 4.78$  and  $v_{1/2} = 0.604$ . 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  $v_{1/2}$ .

(Figure 2-5)



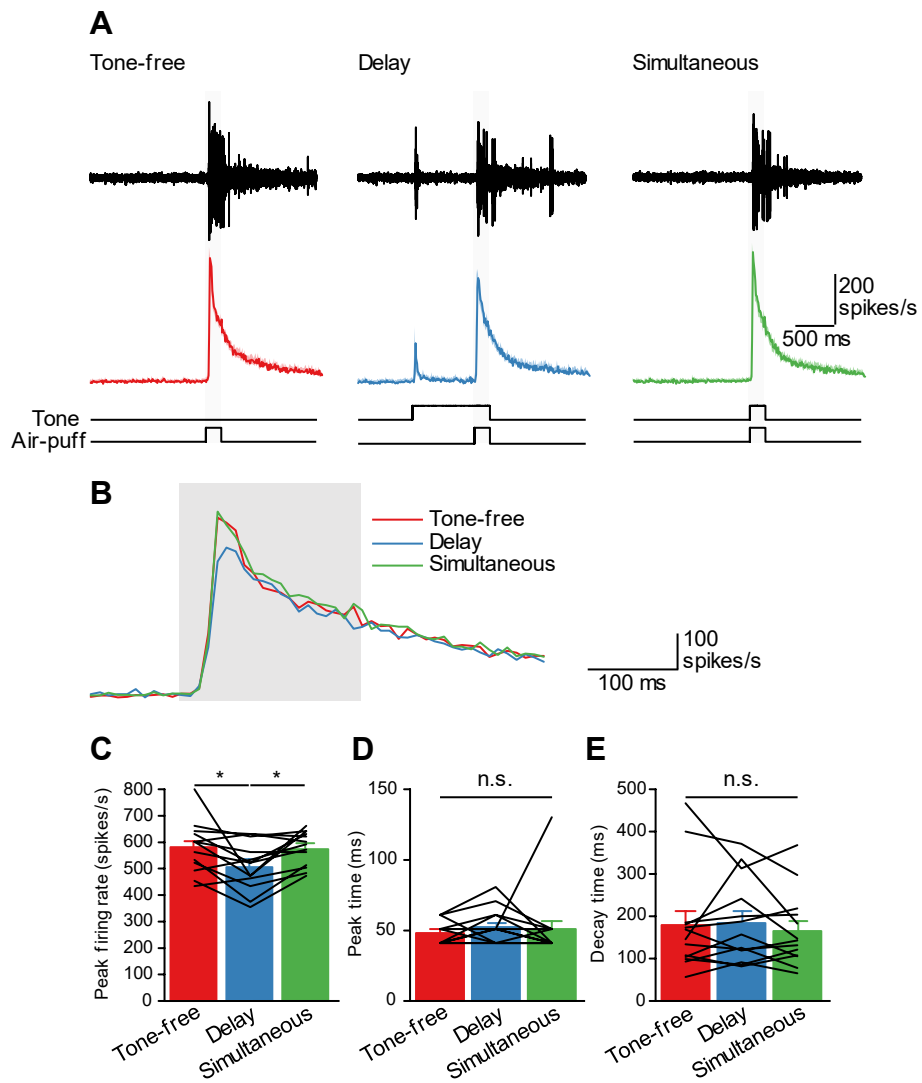
**Figure 2-5. Effects of temporal overlap of acoustic and air-puff stimuli on the directional modulation.** (A) Typical walking trajectories of an individual cricket in response to air-puffs (0.90 m/s) from lateral sides in the four different protocols. Red and light blue traces show the walking trajectories elicited by the stimulus from the right and left sides, respectively. Pooled data of walking direction (B) and turn angle (C) in the four different protocols, for each of which the air-current stimuli were applied at five different velocities. The box plot whiskers indicate the  $1.5 \times$  interquartile range of the lower and upper quartiles; box limits indicate the lower, median, and upper quartile from bottom to top; the circles indicate outlier data. \*  $p < 0.05$ , \*\*  $p < 0.01$  (linear mixed-effects models).

(Figure 2-6)



**Figure 2-6. Effects of temporal overlap of acoustic and air-puff stimuli on the elevation of response threshold.** The probabilities of walking response to the air-puff stimuli in the different protocols against the stimulus velocity. Each plot represents the mean response probabilities of 11 animals for each protocol and error bars indicate  $\pm$  SEM. Solid color lines represent curves fitted with the Hill function (see the Material and methods), for which the parameters were follows: long-delay,  $h = 4.09$  and  $v_{1/2} = 0.672$ ; short-trace,  $h = 3.31$  and  $v_{1/2} = 0.840$ ; long-trace,  $h = 3.90$  and  $v_{1/2} = 0.694$ ; tone-free,  $h = 4.78$  and  $v_{1/2} = 0.604$ . 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  $v_{1/2}$ .

(Figure 2-7)



**Figure 2-7. Effects of preceding auditory input on descending neuronal activities.**

(A) Typical responses of descending neuronal activities recorded from the same preparation (upper traces) and time-course changes of the firing rates of descending spikes (lower colored traces) in three different protocols including tone-free, delayed, and simultaneous. For lower traces, solid lines indicate mean of firing rate and colored shadows indicate  $\pm$  SEM. Light gray rectangles show the durations of air-puff stimulus.

(B) Enlarged traces showing time course of the firing rate in (A) for three stimulation protocols (red, tone-free; blue, delayed; green, simultaneous).

(C-E) Pooled data of the peak value of the firing rate (C), time of the peak firing rate (D), and decay time (E) in three different protocols. Each column represents the average of mean values of the data obtained from 10 recordings in each sample and error bars indicate  $\pm$  SEM (N = 13 samples for each protocol). \*  $p < 0.05$  (Wilcoxon rank-signed test).

## Chapter 3

# Acoustic context with sound carrier frequency alters auditory modulation of behavior

### 3.1 Introduction

Audition is one of the essential sensory modalities used to sense environments and direct appropriate behaviors. Similar to vertebrates, insects detect various auditory cues to serve in adaptive decision making. For mating-related communication in several insect species, male insects use acoustic signals (i.e. calling songs) to attract conspecific female insects (Coen et al., 2016; Nakano et al., 2014; ter Hofstede et al., 2015). Meanwhile, most nocturnal flying insects detect the ultrasonic echolocation calls of bats and exhibit avoidance behaviors in response (Hoy et al., 1989; Pollack, 2015; ter Hofstede and Ratcliffe, 2016). These acoustic signals are considered trigger stimuli which induce stereotyped behaviors (Hildebrandt, 2014; Hildebrandt et al., 2015). In vertebrates, the acoustic signals which trigger no response alone can provide ‘acoustic context’ used for behavioral modulation when adapting to environmental conditions (Roy et al., 2009; Maren et al., 2013). However, in insects, it remains unclear whether audible but non-triggering stimuli can modulate the behavior elicited by other sensory inputs.

The cricket has been used as a prominent model animal for neuroethological studies of acoustic behavior (Horsh et al., 2017). Carrier frequency of sound is a major factor of acoustic context for crickets. During flight, crickets exhibit two distinct behaviors depending on the sound frequency: first, a positive steering induced by a conspecific male cricket’s calling song, of which the carrier frequency is ~5 kHz; second, an avoidance reflex in response to a high-frequency sound imitating a foraging bat's

echolocation call ( $> 20$  kHz); these behaviors are known as positive and negative phonotaxis, respectively (Moiseff et al., 1978; Popov and Shuvalov, 1977; Ulagaraj and Walker, 1973; Wyttenbach et al., 1996). Two ascending auditory neurons, identified as AN1 and AN2, within the prothoracic ganglion project their axons to the brain (Boyan and Williams, 1982; Hennigm 1988; Schildberger, 1984). AN1 and AN2 are sensitive to different frequency ranges: AN1 is tuned to a low frequency corresponding to the calling song, while AN2 responds mainly to a higher frequency spanning the echolocation calls (Boyan and Williams, 1982; Hennigm 1988; Schildberger, 1984). These neurons are involved in positive and negative phonotaxes, respectively. For positive phonotaxis, AN1 conveys information about the temporal pattern of a conspecific's calling song to a neural circuit within the brain (Kostarakos and Hedwig, 2015; Schildberger, 1984; Schöneich et al., 2015). Firing activity of AN2 encodes the information of high-frequency sound and triggers inflection of the cricket's abdomen during flight, one of the negative phonotaxis behaviors (Pollack, 2015; ter Hofstede and Ratcliffe, 2016). These evidences demonstrate that the crickets can distinguish acoustic contexts by hearing the difference in sound frequency. On the ground, however, female crickets approach the sound source of the calling song but exhibit no behavioral response to the high-frequency sound like the bat echolocation call, which should be heard by the crickets (Hedwig, 2006; ter Hofstede et al., 2009). In addition, firing activities of AN1 or AN2 evoked by pure tone sounds elicit no specific behavior in the standing crickets (Nolen and Hoy, 1984; Schildberger, 1984). On the other hand, the cricket exhibits an escape response to a short air-puff detected by cerci, which is considered as a defensive behavior against lunging predators such as spider (Oe and Ogawa, 2013; Dangles et al., 2006). In the previous chapters, I explored the auditory impact on the wind-elicited walking behavior and revealed that a preceding

acoustic stimulus of a 10-kHz pure tone that evoked no response alone modulated moving direction and response threshold of wind-elicited walking. This fact means that a cross-modal interaction between auditory and cercal sensory systems caused behavioral changes in escape strategy, suggesting that crickets perceived the acoustic signals and interpreted them as acoustic contexts. However, it was unclear whether the crickets used sound-frequency information representing distinct contexts for the modulation of the wind-elicited escape behavior.

To address this issue, I adopted the frequency-dependent acoustic context to test the behavioral framework of auditory modulation of wind-elicited walking behavior in the cricket. I applied two different frequencies of sound (a 5- or 15-kHz pure tone), which were initiated preceding an air puff and terminated simultaneously, and measured wind-elicited escape walking using a spherical treadmill system.



### 3.2 Materials and Methods

Treadmill system, and air-puff stimulation were the same as used in Chapters 1 and 2.

#### *Animals*

I used 27 laboratory-bred adult male crickets (*Gryllus bimaculatus*) less than 14 days after adult eclosion throughout the experiments. They were reared under 12/12 h light/dark conditions at a constant temperature of 27°C. The body weight was  $0.68 \pm 0.06$  g (average  $\pm$  standard deviation). I removed their antennae to eliminate the influence of mechanosensory inputs from the antennal organ so as to focus on the interaction between the cercal and auditory systems.

#### *Acoustic stimulation*

The tone sounds were synthesized and delivered by the same system as used in Chapters 1 and 2. For the acoustic stimuli, 5- or 15-kHz pure tone were used. The sound pressure for each frequency sound was calibrated at 70 dB SPL at the center of the treadmill ball with a sound-level meter (TYPE 6224, ACO CO., LTD.). These auditory stimuli also elicited little walking responses as well as the 10-kHz pure tone used in the previous chapters (Fig. 3-1)

#### *Stimulation protocol*

To test dependency of sound carrier frequency of the auditory effects on wind-elicited walking, I used three types of stimulation referred to as tone-free, 5-kHz tone, and 15-kHz tone (Fig. 3-2). For the cross-modal stimulation, 5- or 15-kHz tone sound of 1-s duration was initiated 800 ms before an air-puff for 200 ms. For the uni-modal stimulation

referred to as tone-free, a 200-ms air-puff was delivered without any prior acoustic stimulus, but the cricket's walking activities were monitored during the 800-ms silent time prior to the air-puff. In all types of stimulation in this chapter, a sequence of stimuli was started only if the cricket remained at rest for 1 s or longer. The acoustic and air-current stimuli were always delivered from the same direction like Chapter 2 (inset in Fig. 3-2).

Each individual cricket was randomly exposed to three types of stimulation for 20 trials each, leading to 60 trials in total. The inter-trial interval was >1 minute. Each cricket received all stimuli from left or right relative to the cricket's anteroposterior body-axis, for 14 crickets stimulated from the left side and for 13 crickets from the right side. For the data analysis, I combined the results acquired from both groups, therefore, the sample size were 27 crickets.

#### *Data analysis*

The method of data acquisition and the definition of response probability were the same as used in Chapter 1. I measured the following locomotor parameters in the 'initial response' of the wind-elicited walking: walking distance, reaction time, maximum walking speed, walking direction, and turn magnitude. The walking direction was defined for forward as  $0^\circ$  so that the direction opposite to stimulus angle was  $90^\circ$  and backward direction was  $180^\circ$  (see insets in Fig. 3-4A). Instead of turn angle used in Chapter 1 and 2, the turn magnitude was measured as an absolute value to compare the magnitude of turning movement. In Fig. 3-5D and E, I used absolute walking direction defined as follows:  $0^\circ$  means the cricket moved in the forward direction and  $180^\circ$  means the cricket moved in the backward direction. For statistical analyses of the scalar parameters such as

reaction time, maximum walking speed, walking distance, and turn magnitude, I calculated average values in trials categorized into ‘wind-elicited response’ for each individual to avoid pseudo-replication. For analysis of the walking direction that was a circular parameter, I calculated mean of angle and circular variance for each individual using a package in R programming for circular statistics (Agostinelli and Lund, 2013). Prior to the statistical test of significance of the stimulation types, I checked the distribution of all dataset for mean and S.D. values for each individual, using Kolmogorov-Smirnov test. To assess the significance of the stimulation types for response probability (Fig. 3-3A), maximum walking speed (Fig. 3-3C), reaction time (Fig. 3-3D), walking direction (Fig. 3-4B, C), and turn magnitude (Fig. 3-5B,C), of which the data were distributed in Gaussian, I used one-way repeated-measures analysis of variance (ANOVA). If the main effect of stimulation types was significant, I used a paired t-test followed by Holm’s correction as a post hoc test. To assess the significance of the stimulation types for walking distance (Fig. 3-3B) and auditory response probability (Fig. 3-1), of which the data were not distributed in Gaussian, I used Friedman’s test and Wilcoxon paired-sample test instead of ANOVA and t-test. To compare the variance in distributions of the walking direction among the three types of stimulation, I used Wallraff’s test using the R programming package of circular statistics (Agostinelli and Lund, 2013; Wallraff, 1979). To compare the variance in distributions of the turn magnitude among the stimulation types, I used an F test. To assess the significance of sound frequency for the relationships between the walking direction and the turn magnitude, I used a multiple regression analysis for their plots in absolute values considering an interaction effect of the walking direction and the stimulation type. For analysis of the correlation between the walking direction and the variance of turn

magnitude, I calculated standard deviations of the turn magnitude for ranges of every 30° (i.e. 0°–30°, 30°–60°, 60°–90°, 90°–120°, 120°–150°, and 150°–180°) of the walking direction. Further, I tested the significance of the stimulation type for that relationship, using multiple regression analysis.

### 3.3 Results

#### *Frequency dependency of the auditory modulation of locomotor activities in wind-elicited escape walking.*

Firstly, I examined the carrier-frequency dependence on the cross-modal impacts of the sound on the escape response to air-puff stimulus from the lateral side (Fig. 3-2). I observed significant effects of a sound on the wind-elicited response probability and walking distance, and these effects depended on the sound frequency (Fig. 3-3A, B). The 15-kHz tone reduced response probability, but 5-kHz tone did not affect it ( $p = 0.007$  comparing tone-free and 15-kHz tone protocols,  $p = 0.759$  comparing tone-free and 5-kHz tone protocols,  $p = 0.007$  comparing 5-kHz tone and 15-kHz tone protocols, paired t-tests followed by Holm's correction). In addition, a 15-kHz tone increased walking distance in the initial response whereas 5-kHz tone did not change it ( $p = 0.001$  comparing tone-free and 15-kHz tone protocols,  $p = 0.470$  comparing tone-free and 5-kHz tone,  $p = 0.0982$  comparing 5-kHz tone and 15-kHz tone protocols, Wilcoxon paired-sample test followed by Holm's correction). In contrast, there was no significant difference among the stimulation types in maximum walking speed ( $p = 0.045$ , one-way repeated-measures ANOVA,  $p = 0.095$  for tone-free vs 15-kHz tone,  $p = 0.448$  for tone-free vs 5-kHz tone,  $p = 0.203$  for 5-kHz tone vs 15-kHz tone protocols, Holm's corrected paired t-tests) (Fig. 3-3C) and reaction time ( $p = 0.957$ , one-way repeated-measures ANOVA) (Fig. 3-3D). Taken together, high-frequency sound decreased the responsiveness to the air puff but extended the escaping distance.

#### *Frequency dependency of the auditory modulation of walking direction.*

Typical walking trajectories in response to lateral stimulus showed that a cricket walked

backward more frequently after 15-kHz tone stimulation than after other frequency (Fig. 3-2). Frequency distributions of the walking direction revealed that the 15-kHz stimulation was different at its peak from the tone-free and 5-kHz stimulation (Fig. 3-4A). Comparing the walking direction for individuals among the three types of stimulation, both frequencies of tones facilitated walking in the backward direction ( $p < 0.001$ , one-way repeated-measures ANOVA,  $p = 0.024$  for tone-free vs 5-kHz tone,  $p = 0.003$  for tone-free vs 15-kHz tone, Holm's corrected paired t-test) (Fig. 3b). In addition, the 15-kHz tone facilitated backward movement to a larger degree than the 5-kHz tone ( $p = 0.024$  for 5-kHz tone vs 15-kHz tone). This suggests that the higher-frequency tone has a larger impact on walking direction. To determine whether these auditory modulations on walking direction were derived from the inhibition of forward walking, I compared the dispersion of the frequency distributions and the individual changes of circular variance of walking direction among the three stimulation types. I observed any significant differences in neither frequency distribution of the population ( $p = 1$  for all pairs, Wallraff's test followed by Holm's correction) nor circular variance for each individual ( $p = 0.605$ , one-way repeated-measures ANOVA) (Fig. 3-4A, C). These results demonstrated that the tones did not simply inhibit forward walking but also induced walking in the backward direction more frequently.

#### *Frequency dependency of the auditory modulation of turn magnitude.*

In the wind-elicited walking behavior, the walking direction linearly correlates with the stimulus angle, meaning that the crickets always move in the direction opposite to the air puff (Oe and Ogawa, 2013). In contrast, the turn angle depends on the stimulus angle according to a sin function because backward motion can consist of one of two movement

strategies, one of which is turnaround followed by straight walking, and the other is backward stepping without turn (Bidaye et al., 2014; Sen et al., 2017). Here, to examine which strategy of walking in the backward direction was enhanced by tones, I analyzed the cross-modal effects on the absolute value of turn angle (termed ‘turn magnitude’, see Methods) and their frequency dependency.

The frequency distributions of the turn magnitudes were similar to each other at their peaks among the three stimulation types (Fig. 3-5). There was no significant difference in mean turn magnitude among the three stimulation types ( $p = 0.007$ , one-way repeated-measures ANOVA,  $p = 0.644$  for tone-free vs 5-kHz tone,  $p = 0.057$  for tone-free vs 15-kHz tone,  $p = 0.057$  for 5-kHz tone vs 15-kHz tone, Holm’s corrected paired t-test) (Fig. 3-5B). Focusing on the variance of the distributions, however, the turn magnitude after 15-kHz tone stimulation showed broader distribution than those after tone-free or 5-kHz tone stimulation ( $p < 0.001$  for tone-free vs 15-kHz tone,  $p < 0.001$  for 5-kHz tone vs 15-kHz tone,  $p = 0.090$  for tone-free vs 5-kHz tone, F test followed by Holm’s correction). This indicates that the 15-kHz tone induced larger turns more frequently. For each individual, the turn magnitude fluctuated more after the 15-kHz tone stimulation than after tone-free or 5-kHz tone stimulation ( $p < 0.001$ , one-way repeated-measures ANOVA,  $p = 0.001$  for tone-free vs 15-kHz tone,  $p = 0.006$  for 5-kHz tone vs 15-kHz tone,  $p = 0.319$  for tone-free vs 5-kHz tone, Holm’s corrected paired t-test) (Fig. 3-5C). Taken together, it is suggested that the 15-kHz tone causes large turns, resulting in broader distribution of the turn magnitude.

The 15-kHz tone facilitated walking in the backward direction (Fig. 3-4B) and increased the variance of the turn magnitude. It is uncertain if these effects of the 15-kHz tone were independent from each other. To answer this question, I examined the

relationships between the walking direction and the turn magnitude. In all types of stimulation, the turn magnitude was positively dependent on the walking direction, but there was no significant cross-modal effect on that dependency ( $p = 0.257$  for tone-free vs 5-kHz tone,  $p = 0.097$  for tone-free vs 15-kHz tone, multiple regression analysis) (Fig. 3-5D). This means that the more backward the crickets moved, the more largely they turned regardless of sound. The Pearson's correlation coefficients for these relationships, however, were low because more walking in the backward direction was accompanied by a more varied turn magnitude. In addition, the scatter plots shown in Fig. 3-5D indicate that large turns were more common after the 15-kHz tone stimulation than after tone-free or 5-kHz tone stimulation. Further, I examined the relationships between the walking direction and the standard deviation of turn magnitude (Fig. 3-5E, see also Methods). The standard deviation of the turn magnitude was strongly correlated with the walking direction for all types of stimulation (Fig. 3-5E). Interestingly, the slope of regression line for the 15-kHz tone was significantly larger than that of tone-free ( $p = 0.003$ , multiple regression analysis), whereas there was no significant difference between the 5-kHz tone and tone-free ( $p = 0.081$ ) (Fig. 4e). Therefore, the high frequency tone facilitated the walking backward, and additionally caused fluctuations in turning movement.



### 3.4 Discussion

*Crickets perceive sound-frequency as information to discriminate between distinct acoustic contexts*

In this chapter, I showed that the sound modulates wind-elicited walking behavior in the response probability, walking distance, walking direction, and variance of turn magnitude. These cross-modal effects depended on the sound carrier frequency of the stimuli. Before the experiments, I had predicted that the 5- and 15-kHz tones would have different impacts on the wind-elicited walking because these frequencies of sounds directly trigger different behaviors in the flying cricket, which are positive and negative phonotaxis (Moiseff et al., 1978; Popov and Shuvalov, 1977). Based on these facts, I hypothesized that crickets can recognize different contexts based on sound frequency. Reduction of the response probability, elongation of the walking distance, and enhancement of the turn-magnitude variability were exerted by 15-kHz but not 5-kHz tones. Although the 5-kHz tone facilitated walking in the backward direction similarly to the 15-kHz tone, this effect of the 5-kHz tone was significantly smaller than that of the 15-kHz tone. These results reveal that crickets alter their wind-elicited escape strategies depending on the acoustic context.

Even innate behaviors of insects are not only simple reflex responses or stimulus-triggered hardwired behaviors but are adaptively modulated depending on the environmental contexts and physiological states, which are mediated by cross-modal interaction between the different sensory systems. Crickets perceiving a ‘shelter’ alter their wind-elicited escape behaviors so that the crickets turn toward the shelter (Kanou et al., 2014). The fruit fly (*Drosophila melanogaster*) reduces CO<sub>2</sub> avoidance behavior in the context of appetitive odor (Lewis et al., 2015). In the previous chapters, I have

demonstrated that crickets alter their wind-elicited walking behavior in moving direction and response threshold after hearing an acoustic stimulus. This suggests that insects' avoidance behaviors can be flexibly modulated by various sensory inputs including visual, olfactory, and auditory signals. Furthermore, other innate behaviors also depend on the sensory parameters representing a context. For example, the male fruit fly modulates the intensity of the calling song to attract the conspecific female depending on the visually estimated distance to the female fruit fly (Coen et al., 2016). My present results demonstrating the sound-frequency dependence of the auditory modulation support the idea that the insect auditory system, which had been considered as simply triggering the specific behaviors (Hildebrandt, 2014; Hildebrandt et al., 2015), is also used for perception of the acoustic context instructing decision making.

The crickets in nature are surrounded by a variety of acoustic signals with complex temporal patterns, some of which will be produced by the various species of predator including bats, rodents, birds and toads (Hedrick and Dill, 1993; Hoy et al., 1989; Rodríguez-Muñoz et al., 2011; Wöhr and Shwartz, 2013). In addition to the echo of bats, rodents communicate each other with ultrasonic squeaks. I have showed that just simple high-frequency tone altered escape behavior of the crickets. This suggests that this auditory modulation of escape behavior would not require the recognition of the temporal patterns of acoustic signals like the conspecific calling song (Hedwig, 2006). However, it is still unknown what effects the different temporal patterns has on the auditory modulation of wind-elicited walking behavior. The question should be addressed by the future studies.

*Impacts of high-frequency sound on response probability, walking direction, and moving*

*distance*

The 15-kHz tone facilitated walking in the backward direction and reduced response probability of wind-elicited walking, which corresponds to my results in the previous chapter. In addition to these effects, the walking distance was elongated by the 15-kHz sound. In general, a longer escape distance results in a higher survival rate from attacking predators (Nair et al., 2017). In contrast, the decrease in the response probability of walking means that the crickets less frequently respond to the air-puff stimuli, which is also one of their escape strategies (Baba and Shimozawa, 1997; Eilam, 2005). In the context of the presence of echolocating bats, which are a predator of the crickets, an inadvertent motion in response to weak air-flows may result in being caught by predators. Indeed, gleaner bats (*Antrozous p. pallidus*) could potentially detect movement of the cricket on the ground by passive hearing and locate to capture it (Fuzessery et al., 1993), while the standing cricket exhibits no response to the ultrasound emitted by bats (ter Hofstede et al., 2009). The elevation of the response threshold would be, therefore, effective to avoid the detection by bat's echolocation call. In addition to possible ethological advantages, the elongation of escape distance and the decrease of response probability may be not contradictory, because two neural mechanisms at different levels are possibly involved in these modulation, one of which is for decision-making of go or no-go and the other of which is for regulating the walking distance (Domenici, 2010). Auditory inputs of the preceding tone will affect these two neural mechanisms independently. The ethological meaning of the facilitation of walking backward with a 15-kHz tone remains unknown. The walking in the backward direction that is an unanticipated movement contrary to the bat's expectation may be also one of the effective escape strategies.

### *Cross-modal effects on angular variability in escape turning*

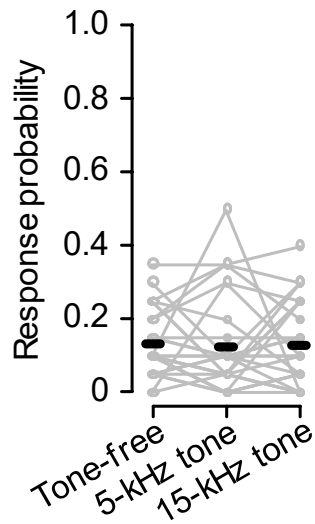
Another effect specific to the 15-kHz tone was increase in variance of the turn magnitude. The turn magnitude measured in the experiments for this chapter indicates the orientation to which the cricket faced at the termination of initial response. Although the walking direction was altered by the sound, its variance was unaffected, meaning that the auditory inputs made no change in the directional variability of the initial responses. In the context of the presence of predators, therefore, crickets may utilize their second responses to further move in various directions. For the behavioral analysis in the present study, I focused on the initial response but paid no attention to the second or later responses because few second responses were observed in this study (data were not shown). It is possible that repetitive air-puffs may uncover the directional variability of second or later responses (Ogawa and Oka, 2015).

Variability in the escape behavior is thought to be not only noise but also an adaptive trait, which leads to unpredictability for protection from predators (Brembs, 2011; Domenici et al., 2008; Humphries and Driver, 1970). Some previous studies on fish demonstrate that the escape direction was varied depending on the context (Domenici, 2010). For example, acute changes in environmental conditions, such as hypoxia and cooling, facilitate the movement of fish toward the noxious stimulus instead of away from it, resulting in greater variability of escape direction (Lefrançois et al., 2005; Preuss and Faber, 2003). In addition, herring schools exhibit escape responses away from the stimulus more frequently than a solitary fish (Domenici and Batty, 1997). These facts suggest that animals alter the variability in the escape behaviors depending on the surrounding contexts. Despite the significance of these findings, the neural mechanism

producing the behavioral variability remains unknown. An insect's escape behavior being regulated by a small neural circuit consisting of a few accessible, identified neurons is an appealing behavioral model (Card, 2012). The cross-modal effect on the behavioral variability in the cricket escape behavior will be useful to understand the neural mechanisms that regulate the behavioral variability.

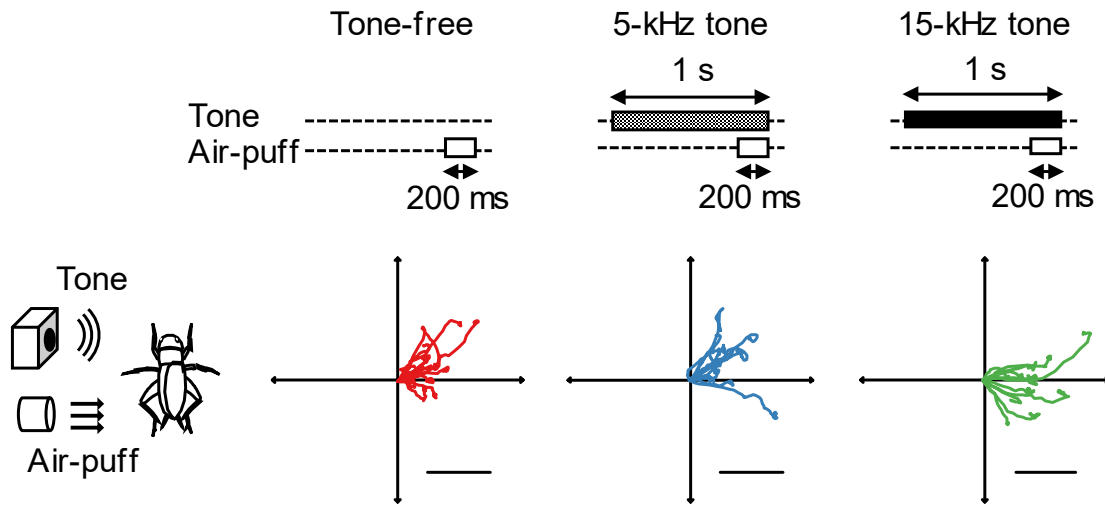
### 3.5 Figures

(Figure 3-1)



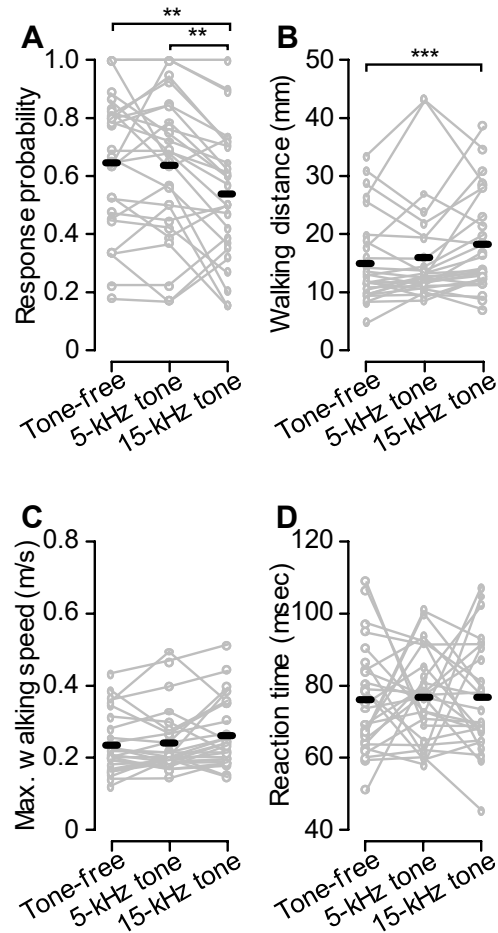
**Figure 3-1. Probability of walking initiation for the period of 800 ms before the onset of an air-puff.** There was no significant difference in the response probabilities among the three types of stimulation ( $p = 0.3679$ , Friedman's test), meaning that an auditory stimulus of 5- or 15-kHz frequency triggered no walking in crickets on its own.

(Figure 3-2)



**Figure 3-2. Experimental designs and typical walking trajectories of initial responses to air puffs combined with tone sounds of different frequencies.** Upper diagrams show the temporal arrangements of auditory and air-puff stimuli. Three types of stimuli were randomly applied to an individual cricket. Left diagrams show the spatial arrangements of the stimulation. Color traces show typical walking trajectories in response to different types of stimuli in an individual. Scale bars indicate 10 mm on the virtual plane.

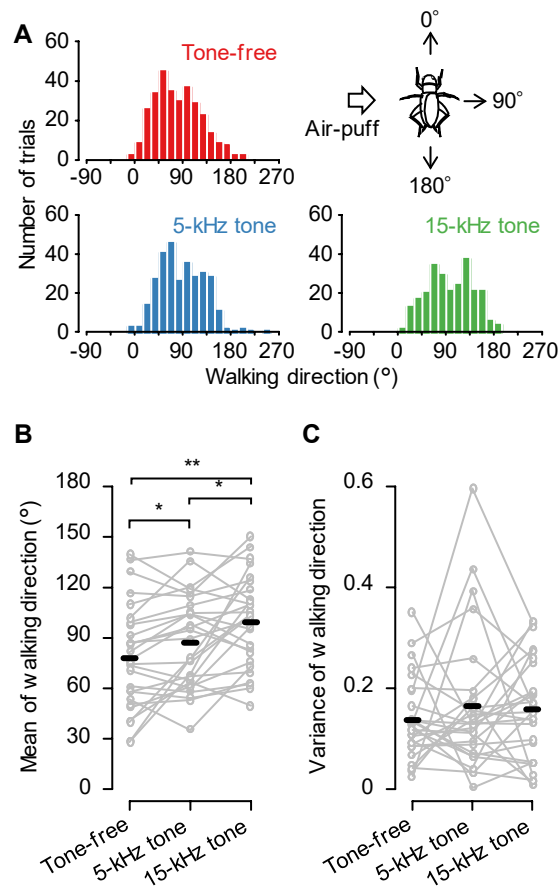
(Figure 3-3)



**Figure 3-3. Auditory effects of different frequencies of tones on the wind-elicited escape.** Four different locomotion parameters including response probability (A), mean walking distance (B), maximum walking speed (C), and reaction time (D) were compared among the three types of stimulation. Each black bar indicates average value across the population for each type of stimulation, and grey circles denote individuals (N = 27 individuals). \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$  (paired t-test or Wilcoxon paired-sample test followed by Holm's correction).

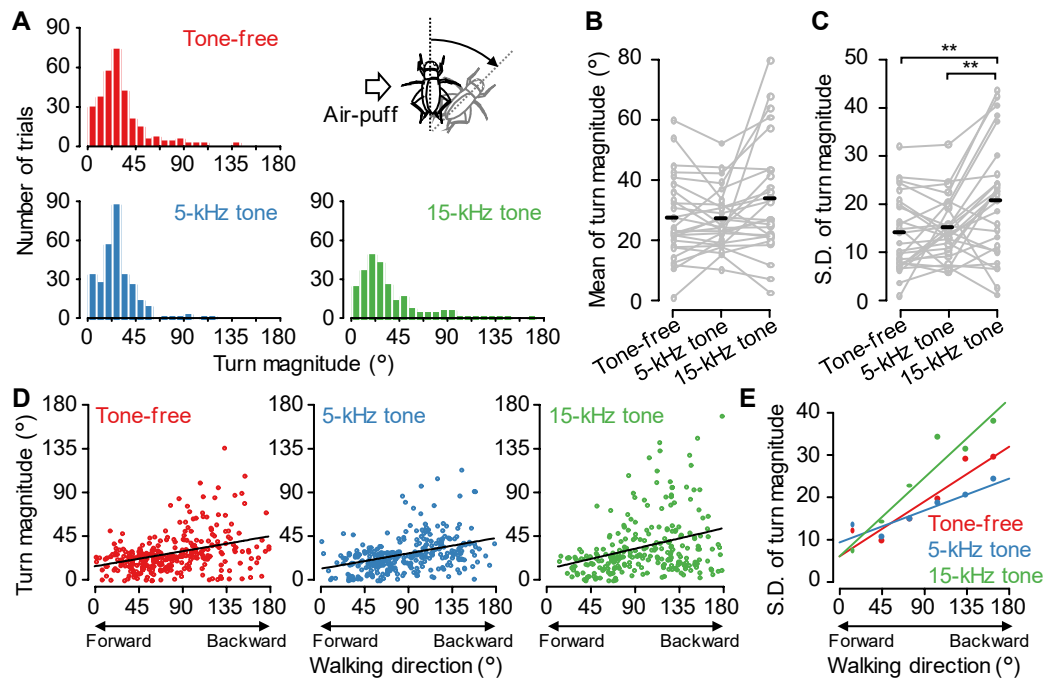


(Figure 3-4)



**Figure 3-4. Auditory effects of different frequencies of tones on directionality in wind-elicited escape.** (A) Histograms of walking direction for three types of stimulation in 15-degree bins ( $n = 299$  trials for tone-free,  $n = 299$  trials for 5-kHz tone, and  $n = 250$  trials for 15-kHz tone). Upper right inset indicates a definition of the walking direction. Direction of walking opposite to the stimulus angle was defined as  $90^\circ$  and walking forward and backward resulted in  $0^\circ$  and  $180^\circ$ , respectively. (B, C) Mean angle and circular variance of walking direction in response to three types of stimulation. ( $N = 27$  individuals)  $** p < 0.01$ ,  $* p < 0.05$  (paired t-test followed by Holm's correction).

(Figure 3-5)



**Figure 3-5. Auditory effects of different frequencies of tones on turn movement in wind-elicited escape.** (A) Histograms of turn magnitude for three types of stimulation in 7.5-degree bins. Upper right inset indicates a definition of turn magnitude. The turn magnitude was measured as an absolute angular value of turn regardless of the rotational direction. (B, C) Mean and standard deviation of turn magnitude among the three types of stimulation. (D) Correlations between walking direction and turn magnitude for the tone-free ( $r = 0.373$ ,  $p < 0.001$ ), 5-kHz tone ( $r = 0.379$ ,  $p < 0.001$ ), and 15-kHz tone ( $r = 0.327$ ,  $p < 0.001$ ) stimulations. Unlike the aligned walking direction shown in figure 3, the walking direction was measured as an absolute value in which forward and backward walks resulted in  $0^\circ$  and  $180^\circ$ , respectively.  $** p < 0.01$ , (paired t-test followed by Holm's correction). (E) Correlations between walking direction and standard deviation of turn magnitude calculated in 6 bins equally-spaced per  $30^\circ$  ( $0^\circ$ – $30^\circ$ ,  $30^\circ$ – $60^\circ$ ,  $60^\circ$ – $90^\circ$ ,  $90^\circ$ –

120°, 120°–150°, and 150°–180°). The standard deviation was positively correlated with walking direction in all types of stimulation (for tone-free,  $r = 0.944$ ,  $p = 0.005$ ; for 5-kHz tone,  $r = 0.928$ ,  $p = 0.008$ ; for 15-kHz tone,  $r = 0.958$ ,  $p = 0.003$ ).

## Chapter 4

### Preceding sound alters AN2 response to air puff

#### 4.1 Introduction

Even if animals receive an identical stimulus, animals do not always exhibit the same behavioral response to those stimuli because the behavioral performance strongly depends on the surrounding contexts (e.g. Domenici, 2010). What and how neuronal circuit mediates the context-dependent change of behavior remains unclear.

Based on the assumption that the context-dependent behavioral change arises from multisensory integration between “trigger” and “context” stimuli, I examined the cross-modal effects on cricket’s wind-elicited walking behavior in the previous chapters. My findings have indicated that the preceding high-frequency sound modulates the wind-elicited walking in several motor parameters: facilitating the backward walking, decreasing the response probability, elongating the walking distance, and increasing the variability of turn magnitude (see also Chapter 3). These findings have also suggested that multisensory neurons responding to both airflow and acoustic stimuli should be involved in this auditory modulation of wind-elicited walking behavior. In addition, the Chapter 2 result that descending activity was modulated by combined stimulation of airflow and sound suggested that the context-dependent modulation should be mediated by the neural circuit within the brain. However, it remains unclear which neurons firstly integrate auditory and mechanosensory inputs involved in the context-dependent change.

The candidate neuron for multisensory integration for the contextual modulation is an identified auditory ascending neuron called AN2. The cell body and dendrites of AN2 locates within the prothoracic ganglion and project the ascending axon to the brain

(Fig. 4-1). The AN2 receive mono-synaptic inputs from the auditory receptor neurons of the tympanum organs located in the tibia of the frontal legs (Hennig, 1988; Wohlers and Huber, 1982). The AN2 responds to high-frequency sound (including 15-kHz sound) and mediate avoiding steering behavior during flight, which is considered to be one of the escape responses to echolocation calls by bats as a predator (Hennig, 1988; Nolen and Hoy, 1984; Popov and Shuvalov, 1977; ter Hofstede et al., 2015). Recently, Someya and Ogawa (2018) reported that the AN2 also responds to airflow stimuli which is detected by the cercal organs and that the simultaneous inputs of the 15-kHz tone and airflow facilitate burst firings in AN2. These findings indicate that the AN2 can convey not only auditory information of high-frequency sound to trigger the avoidance flight steering but also cercal-mediated mechanosensory signals to elicit the walking escape behavior. In other words, it is possible that the auditory and cercal sensory inputs could be integrated by AN2. If the sensitivity of AN2 to the air-puff is altered by its auditory response to the preceding sound, the mecahnosensory signals conveyed by AN2 to the brain is modulated, resulting in contextual modulation of wind-elicited walking behavior.

In this chapter, I intracellularly recorded the membrane potential responses of AN2 to examine whether its wind-elicited activities are modulated by the preceding high-frequency sound, using the stimulation protocols used in Chapter 3.

## 4.2 Materials and Methods

### *Animals and preparation*

Adult male crickets of which developmental stage and rearing condition were the same as in the previous chapters were used for all electrophysiological experiments. For the intracellular recording, the crickets were anesthetized by cooling on crushed ice before the surgery. The cricket was positioned dorsal side-up on a silicon platform (Fig. 4-2) after removal of the antennae, the wings, and the middle- and hind legs. The frontal legs were positioned orthogonally to the anterior-posterior axis with wax. To expose the brain, part of the head cuticle was carefully removed. The brain was stabilized with a stainless-steel platform and a silver wire ring. In the electrical stimulation experiments (Fig. 4-1C), after an incision along the dorsal midline of the abdomen, the gut, internal reproductive organ, and surrounding fat were removed.

### *Electrophysiology*

A glass microelectrode for intracellular recording were made of borosilicate glass capillaries (1B100F-4, World Precision Instruments; outer diameter, 1.0 mm; inner diameter, 0.58 mm;) using a laser puller (P2000, Sutter Instruments). The electrode (75–170 M $\Omega$ ) filled with 8% Lucifer Yellow CH lithium salt (Sigma-Aldrich) in 200-mM LiCl was inserted into the axon of AN2 within the brain (Fig. 4-2). The recorded membrane potential was amplified with a single-electrode voltage-clamp amplifier (CEZ-3100, Nihon Kohden). To confirm the recording from AN2, the fluorescent dye (Lucifer Yellow CH) was loaded iontophoretically in to the cell for 3 min or longer through a glass microelectrode at hyperpolarizing current of -3.0 nA after the recording. After the electrophysiological experiments, the brain was dissected, fixed in 4% paraformaldehyde

in phosphate buffer (pH = 7.2) at 4°C for 3–12 hours, dehydrated for over 5 min each in a series of 50, 70, 90, 100, and 100% ethanol, and cleared in methyl salicylate. After clearing, about 100 optical section images (3  $\mu\text{m}$  of Z-steps for each) of whole brain were acquired using a multi-photon microscope (FV1000MPV, OLYMPUS), and reconstructed with Flour View Software (OLYMPUS) (Fig. 4-1A). In some samples, the morphology of AN2 within the prothoracic ganglion was also observed through the same processes (Fig. 4-1B). In the electrical stimulation experiment, the ascending signals from TAG were extracellularly recorded using a pair of hook electrode positioned under the abdominal ventral nerve cord (VNC) between the 4th and terminal abdominal ganglia. The signals were amplified and filtered by high-pass filter with 150 Hz with a dual-channel bioelectric amplifier (MEG-2100, Nihon Kohden). Electrophysiological signals were digitalized at 20 kHz with an analog-to-digital converter (PowerLab 4/26, ADInstruments), and analyzed with a LabChart version 7 software (ADInstruments).

#### *Airflow, acoustic, and electrical stimulation*

The air-puff stimulation was generated by the same system as used in the previous chapters. The velocity and duration of air-puff stimulation was fixed at 0.90 m/s and 200 ms. For acoustic stimulation, the 15-kHz pure tone (70 dB SPL) were synthesized by the same system as used in the previous chapters, but delivered from a loudspeaker (MM-SPL6BK, Sanwa Supply) different from that used in the behavioral experiments. For the electrical stimulation, a single or train of pulses (electric current: 0.3 mA; pulse duration: 100  $\mu\text{s}$ ; frequency: 500 Hz) was applied using a pair of hook electrodes positioned under the cercal nerve cord ipsilateral to the recorded side.

### *Stimulation protocols*

For the air-puff stimulation combined with sound stimulus, two types of stimulation protocols that were Tone-free and 15-kHz tone protocols used in the Chapter 3, were used. In addition to the normal bimodal stimulation, where the both stimuli were delivered from the right side of the cricket, I turned the air-puff nozzle upward to discriminate the acoustic artifacts of the air-puff belching from a nozzle (unimodal stimulation). In this condition, the cricket receive little mechanosensory stimulus but sound artifact accompanying the belching the air puff.

For the electrical stimulation combined with sound stimulus, three types of stimulation protocols were used, which were referred as Tone-free, Delay and Trace protocols. In the Tone-free protocol, only electrical stimulation was applied. In the Delay protocol, a 15-kHz pure tone of 1-s duration was started 800 ms before an onset of the stimulation pulses. In the Trace protocol, a 15-kHz pure tone of 200-ms duration was started 800 ms before an onset of stimulation pulses, whose temporal structure was the same as that of the Long-trace protocol used for the behavioral experiments in Chapter 2. In addition, I used five different number-of-pulses (1, 2, 5, 10 and 20 pulses). In total, fifteen patterns of stimulation were applied to each individual (Fig. 4-5).

### *Data analysis*

The AN2's responses to air-puff stimuli combined with sound were recorded from eleven cells in different crickets. From six neurons of these eleven samples, the responses to sound artifacts delivered from under the nozzle turned upward were also recorded. The responses to the electrical stimulation to the cercal nerve were recorded from five neurons different from samples described above.



For the processing and statistical analysis of the electrophysiological data, I used custom algorithms with R programming software (version 3.5.1, R Development Core Team).

As the first process for the spike detection in the membrane potential changes, raw traces of the intracellular recordings were subtracted by the traces filtered by a median filter with a 50-ms window to remove subthreshold graded potential and fluctuation due to the unstable recording. Next, I measured the time points when the subtracted membrane potential firstly exceeded the threshold, which was determined manually (7.5–10.0 mV in this study) so that all individual spikes were detected. The firing rate were measured for 10-ms time bins.

Putative mechanosensory-evoked response ( $r_{mechano}$ ) was defined as

$$r_{mechano} = r_{normal} - r_{nozzle-up} ,$$

where  $r_{normal}$  and  $r_{nozzle-up}$  indicate the mean firing-rate during the air-puff stimulus delivered from a nozzle toward (normal) and upward (nozzle-up) against the cricket (see also Fig. 4-4A).

In the electrical stimulation experiments, the evoked spikes were counted for 200 ms after the first stimulation pulse for each trial. The spike latency was measured as the time delay of first spike to the first stimulation pulse. If the spike latency was longer than 50 ms, that data were discarded in the following analysis because almost all spike were evoked within 50 ms after the onset of the electrical stimulation.

For the spike extraction from the extracellularly-recorded ascending activities, the detection threshold was set to five times of standard deviation of voltage fluctuation during spontaneous activity. The following procedure of the measurement of spike time

and of firing rate were the same as used for intracellularly-recorded AN2's responses.

Relative response magnitude of the ascending activities was defined as

$$Relative\ response = \frac{r_{evoked}}{r_{spontaneous}},$$

where  $r_{evoked}$  indicates the mean firing-rate for 200 ms after the first stimulation pulse and  $r_{spontaneous}$  indicates the mean firing-rate for 1,000 ms before the onset of the acoustic stimulation in the Delay and Trace protocols. In the Tone-free protocol, the mean firing-rate for  $r_{spontaneous}$  was measured for the same duration (1,000 ms) 1,800 ms before the first stimulation pulse.

For statistical analysis, the data obtained from the same samples were averaged for each sample to avoid pseudo-replication. To assess the significant difference in mean firing-rate of AN2's responses to the air-puff stimulus between Tone-free and 15-kHz tone protocols, a paired t-test was used. To assess the significant difference in number of evoked-spikes, spike latency, and their interaction of AN2's responses to the electrical stimulation between the stimulation protocols, a two-way repeated-measures ANOVA was used. To assess the significant difference in the relative response magnitude of the ascending activities evoked by the electrical stimulation between the stimulation protocols, a two-way repeated-measures ANOVA was also used. A  $p$ -value less than 0.05 was considered to be statistically significant.

### 4.3 Results

#### *Auditory modulation of AN2 response to an air-puff stimulus*

To test the effects of the preceding sound on the wind-evoked response of AN2, I compared firing responses of AN2 to an air-puff stimulus from the ipsilateral side to the recording site in Tone-free and 15-kHz tone protocols (N = 11 neurons) (Fig. 4-3A). In both stimulation protocols, the AN2 transiently increased in its firing rate and sustained during the airflow (Fig. 4-3 B), meanwhile the tonic firing was evoked by sound stimulus in the 15-kHz tone protocol (fig. 4-3A2). The wind-evoked response in the 15-kHz tone protocol was smaller in the peak of the firing rate than that in the Tone-free protocol. In addition, there is a significant difference of mean firing rate during the air-puff stimulation between these two protocols ( $p < 0.001$ , paired t-test) (Fig. 4-3C). This result indicates that the preceding auditory input reduces the neuronal activity in AN2 evoked by the following air-puff stimulus.

#### *Auditory effects on auditory and mechanosensory components of AN2 firings*

Although the air-puff stimulus mainly activated the mechanosensory receptors on the cerci, that stimulus was accompanied with the sound-noise of airflow belting from a nozzle, which might also stimulate the tympanal organs on the frontal legs. In other words, the AN2's firing response to an air-puff possibly contained two components: auditory response to the sound-noise detected by the tympanum organ and mechanosensory response to the airflow detected by the cercal organ (Someya and Ogawa, 2018). The previous study revealed that the auditory and mechanosensory responses are linearly summed when both sound and air-puff were simultaneously applied with their intensities greater than AN2's spike threshold (Someya and Ogawa, 2018). Based on the assumption

of this linear summation of auditory and mechanosensory inputs, I separated these two components from the response to the air puff and examined the auditory modulations during the unimodal stimulation, in which the air nozzle was turned upward so that the air puff did not activate the mechanoreceptors (6 neurons, Fig. 4-2B and see also Materials and Methods). In this case, the wind-evoked firing should result from only the auditory response of the tympanum. To estimate the putative mechanosensory response to the air puff, this auditory response to the unimodal stimulation was subtracted from the response to bi-modal stimulation for each sample (Fig. 4-4A).

There was a significant difference in the mean firing rate during air-puff applied as bimodal stimulation between Tone-free and 15-kHz tone protocols ( $N = 6$ ,  $p < 0.001$ , paired t-test) (Fig. 4-4B). In addition, Tone-free protocol was significantly different in the auditory response to the air puff applied with the upward nozzle from the 15-kHz protocol ( $N = 6$ ,  $p = 0.002$ , paired t-test) (Fig. 4-4C). In contrast, there was no significant difference in the putative mechanosensory response between these two protocols ( $N = 6$ ,  $p = 0.551$ , paired t-test) (Fig. 4-4D). These results suggest that the preceding 15-kHz tone reduces the auditory responses to the airflow but may not affect the mechanosensory responses in the AN2.

#### *Auditory modulation of AN2 firings induced by electrical stimulation for the cercal nerve*

As shown in Fig. 4-4, the AN2' response to the air-puff stimuli was induced by auditory inputs mediated by the tympanum organ and by mechanosensory inputs mediated by the cercal organ. The wind-elicited escape behavior is directly triggered and regulated by the cercal sensory system, because the ablation of the cercus or wind-sensitive giant interneurons impairs the escape behavior (Kanou et al., 1999; Oe and Ogawa, 2013). The

results of the previous experiments suggested little effect of the preceding tone on the ‘putative’ mechanosensory responses of AN2. However, it remained possible that the preceding auditory inputs modulate the cercal-system-mediated activities of AN2 because the ‘putative’ mechanosensory response were subtracted based on the assumption of linear integration. If the auditory and mechanosensory inputs are nonlinearly integrated in the multimodal stimulation I used, ‘pure’ air-puff stimulus without sound artifacts will be required to record the substantial mechanosensory responses of AN2 mediated by the cercal system. Since my airflow stimulator was difficult to remove the sound artifacts completely, I used an electrical stimulation of the cercal nerve instead of the air-puff. The electrical stimulation of the cercal nerve induced the firing responses in AN2 in Tone-free protocol (Fig. 4-5). In contrast, it was hard for us to discriminate the electrical-stimulation-induced firings in the Delay protocol because the high-frequency firing evoked by the 15-kHz tone masked the cercal responses (Fig. 4-5). Thus, I used the Trace protocols instead of the Delay protocol because the Long-trace protocol using the 10-kHz tone also modulated the wind-elicited escape behavior as described in Chapter 2 (Fig. 2-1 and 2-5),

First, I counted the number of spikes for 200 ms after the first stimulation pulse. Distributions of the spike number indicated little difference in the responses to the weak stimulation (stimulation pulses = 1, 2 or 5) between the Tone-free and Trace protocols. In the stronger stimulation (stimulation pulses = 10 or 20), however, the Tone-free protocol evoked more spikes than the Trace protocol (Fig. 4-6A). Two-way repeated-measures ANOVA revealed that the main effect of stimulation protocol was significant ( $p = 0.043$ ) (Fig. 4-6B). The main effect of number of stimulation pulses was not significant ( $p = 0.312$ ), but the interaction between stimulation protocol and stimulation

pulse number was significant ( $p = 0.037$ ) (Fig. 4-6B). These results suggest that the preceding sound decreases the AN2 response to the strong cercal stimulation. Probably, the preceding sound suppressed the increase in the cercal-induced response depending on the stimulus intensity.

To examine whether the suppression of the AN2's activity resulted from the adaptation to the preceding sound, I measured the spike latency of the electrically-evoked responses. There were little differences in the distribution of the spike latency between these two protocols (Fig. 4-7A). A two-way repeated-measures ANOVA, indicated the significance for the main effect of number of pulses ( $p < 0.001$ ), but there were any significances neither the main effect of stimulation protocol nor the interaction ( $p = 0.421$  for protocol and  $p = 0.604$  for the interaction) (Fig. 4-7B). These results demonstrated that the response latency becomes shorter depending on the stimulus intensity. In addition, the preceding sound had no impact on the spike latency, suggesting that the decrease of the AN2's responses to the cercal stimulation did not simply result from the auditory-induced adaptation. Taken together, it was supposed that the previous sound reduced the sustained firings in the AN2 evoked by the high-frequency stimulation.

#### *Auditory effects on cercal-stimulation induced extracellular signals in the ventral nerve cord*

Finally, I extracellularly recorded the neural activity of VNC evoked by the electrical stimulation of the cercal nerve to examine the possibility that the primary cercal sensory processing within TAG was directly modulated by the preceding sound. The relative response magnitude increased depending on the stimulus intensity regardless of the stimulation protocols. A two-way repeated-measures ANOVA indicated no significant

main effect of protocol ( $p = 0.704$ ) and the interaction ( $p = 0.108$ ), whereas significance for the main effect of number of stimulation pulse ( $p < 0.001$ ) (Fig. 4-8, see also Materials and Methods in this chapter). These results suggest that the preceding sound had no effect on the primary stage of the cercal-sensory processing in the TAG.

#### 4.4 Discussion

##### *Contextual modulation begins at early stage of sensory processing*

The aim of research in this chapter was to test the key neuron that was involved in the auditory-contextual modulation of wind-elicited escape behavior. AN2 has multimodality to respond to both auditory and airflow stimuli (Someya and Ogawa, 2018). Then, I focused on AN2 as the first candidate and found that the wind-elicited response in AN2 was decreased by the preceding high-frequency (15 kHz) sound. This result suggests that AN2 would contribute to the auditory modulation of wind-elicited escape behavior.

My findings also indicate the possibility that the multisensory integration underlying the contextual modulation of escape is achieved at the early stage of sensory processing at lower CNS such as prothoracic ganglion. AN2 directly receive the synaptic inputs from the auditory receptor neurons (Hennig, 1988; Wohlers and Huber, 1982). The previous study revealing that ablation of the cerci diminished AN2's responses to the airflow (Someya and Ogawa, 2018) and my result that electrical stimulation of the cercal nerve evoked action potentials in AN2 demonstrate the multisensory integration by AN2. The cercal ascending pathways from TAG would provide the mechanosensory signals of airflow to AN2, which would be mediated by the giant interneurons (GIs). GIs project their long axons from TAG to the brain through all segmental ganglia including the prothoracic ganglion, in which GIs also arborize axon colaterals (Hirota et al., 1993; Jacob et al., 2008). Thus, it is possible that GIs directly or indirectly have synaptic contacts with AN2. In addition, the preceding sound did not affect the cercal-sensory-driven ascending signals from the TAG (Fig. 4-8). Taken together, it is supposed that AN2 would be one of the key neurons for the contextual modulation of behavior.



### *Function of AN2 for the wind-elicited escape behavior*

My results were consistent with the previous study that the AN2 also responds to wind stimuli (Someya and Ogawa, 2018). However, it was unknown whether the AN2 contributes to wind-elicited walking behavior. Although the burst firing of AN2 is sufficient to trigger avoidance steering behavior during flight, the AN2's firing causes no specific behavioral response during walking or standing (Marsat and Pollack, 2012; Nolen and Hoy, 1984; ter Hofstede et al., 2009). This means that the burst firing of AN2 did not contribute to direct triggering the wind-elicited escape behavior alone. In addition to auditory inputs from the tympanum and mechanosensory inputs from the cercal sensory system, it has been reported that AN2 also receive mechanosensory inputs from the mechanoreceptors on the legs. For instance, the auditory response of AN2 is inhibited by mechanical vibration stimuli applied to the legs (Kühne et al., 1984). Therefore, AN2 integrates multiple sensory inputs and may have an auxiliary role for modulating wind-elicited escape behavior.

### *What mechanism reduces AN2 response to wind stimuli?*

Since the air-puff stimuli I used were accompanied with the sound noise, the putative mechanosensory-component response was extracted by subtracting the auditory-component response from the air-puff-evoked response. The auditory component contained by the wind-evoked response decreased by the preceding sound, but the putative mechanosensory component was not affected (Fig. 4-4). This result suggests that the reduction in the AN2's response to the air-puff stimuli would arise from sensory adaptation in the auditory receptor neurons (Givois and Pollack, 2000). The gradual

decrement in the late phase of AN2' response to the preceding sound would be due to the receptor-cell adaptation (Fig. 4-3, 4-4A), but it cannot be asserted that the preceding sound never modulate the mechanosensory-component response. The reason is that the putative mechanosensory component was estimated based on the assumption of linear summation of the both component inputs, which is not always applicable to neuronal multisensory integration. Multimodal neurons possibly have the nonlinear summation properties: the cross-modal response is often larger than the linear summation of unimodal responses (superadditive) and often smaller than the linear summation (subadditive) (Stein and Stanford, 2008). Someya and Ogawa (2018) reported that AN2 showed the linear additivity when the air-puff of 0.17 m/s and the 15-kHz tone of 50–90 dB SPL were applied simultaneously. However, it is unclear whether the AN2 had the linear summation in my experiment using the air-puff with 0.90 m/s and the wind-noise sound, both of which were largely different in the intensity and frequency from those used in the previous study.

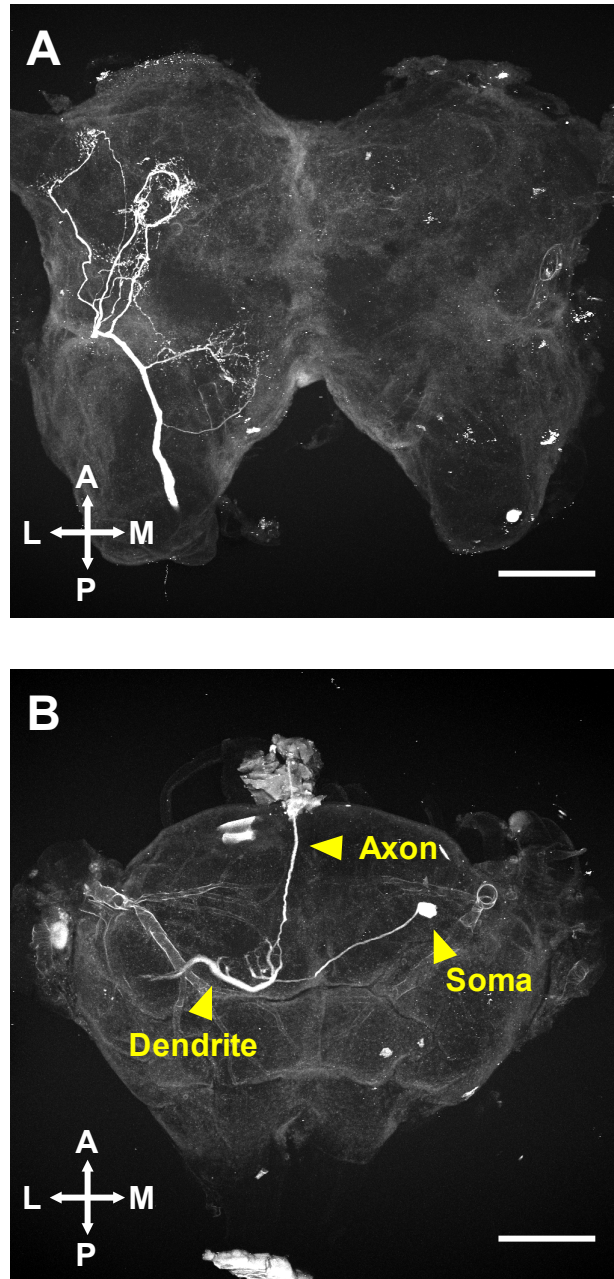
In the experiments using electrical stimulation for the cercal nerve, I could purely stimulate the cercal sensory system without the sound artifacts. The results of these experiments demonstrated that the preceding sound also reduced the cercal-stimulation-induced AN2 response (Fig. 4-6). This finding suggests that the preceding auditory input can also reduce the AN2's responses evoked by mechanosensory inputs. In addition, the preceding sound did not change the latency of the spike response to the cercal-stimulation (Fig. 4-7). This fact implies that the reduction of cercal-stimulation-induced activity in AN2 did not arise from a simple sensory adaptation or decrease of the excitability in AN2 because a sensory adaptation should generally be observed as elongation of the response latency and decrease in the firing rate (Coro et al., 1998; Givois and Pollack,

2000). Therefore, any other mechanisms such as local inhibition of the mechanosensory pathways to AN2 might cause the continuous or sustained suppression of the firing activities response to cercal stimulation in AN2.

The results in this chapter suggest the auditory and mechanosensory responses to the air-puff stimulus in AN2 were reduced by the preceding sound stimulation. However, it remains unclear that this reduction of AN2's responses would be involved in the auditory modulation of the wind-elicited walking behavior described in the previous chapters. In future, the causal relationship between the response reduction in AN2 and the modulation of wind-elicited escape behavior will need to be tested.

## 4.5 Figures

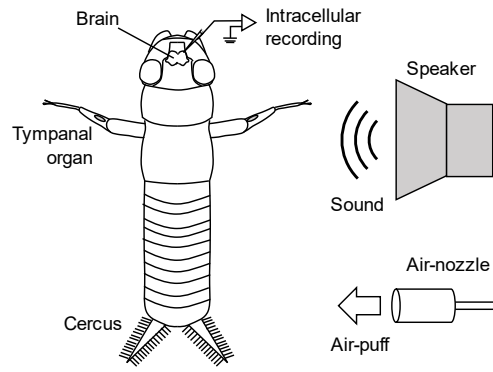
(Figure 4-1)



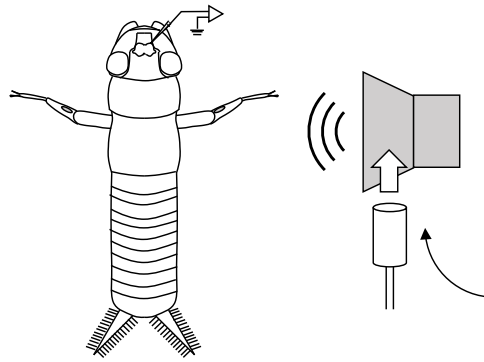
**Figure 4-1. Morphology of the ascending neuron 2 (AN2).** (A) Axon terminals of the AN2 within the brain. (B) The dendrite and soma within the prothoracic ganglion. Horizontal scale bars indicate 200  $\mu\text{m}$ .

(Figure 4-2)

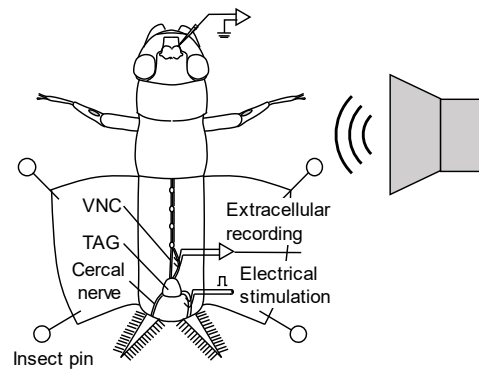
**A** Normal bimodal stimulation



**B** Unimodal stimulation

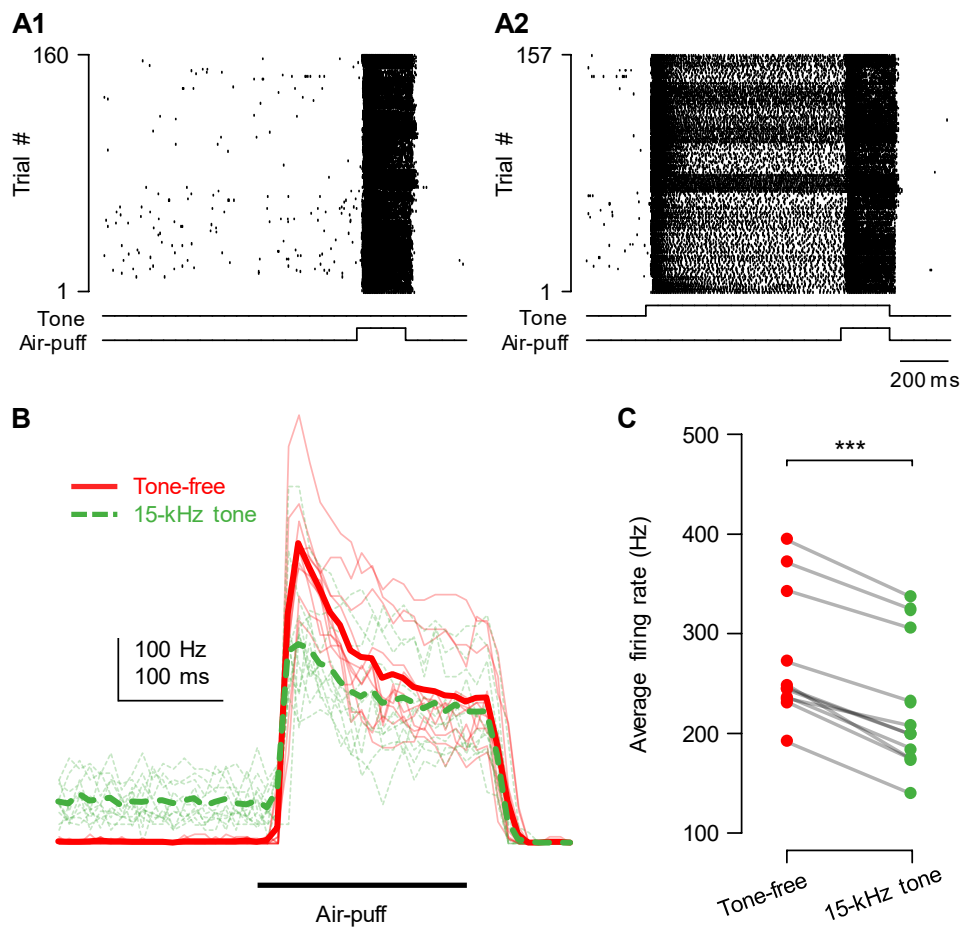


**C** Electrical stimulation for the cercal nerve



**Figure 4-2. Experimental setups for intracellular recording.** (A) Schematic diagram of the normal bimodal stimulation. An air-puff stimulus (0.90 m/s) and a 15-kHz tone stimulus (70 dB) are applied from the right side of the cricket during intracellular recording. (B) Schematic diagram of the unimodal stimulation in which the air-puff nozzle was turned upward. The air-puff stimulus is not applied to the cricket but produce the sound artifact accompanied by the belching an air-puff. (C) Schematic diagram of the electrical stimulation of the cercal nerve. The ascending neural activities are extracellularly recorded from VNC between the 4th and the terminal abdominal ganglia with a pair of hook electrodes.

(Figure 4-3)

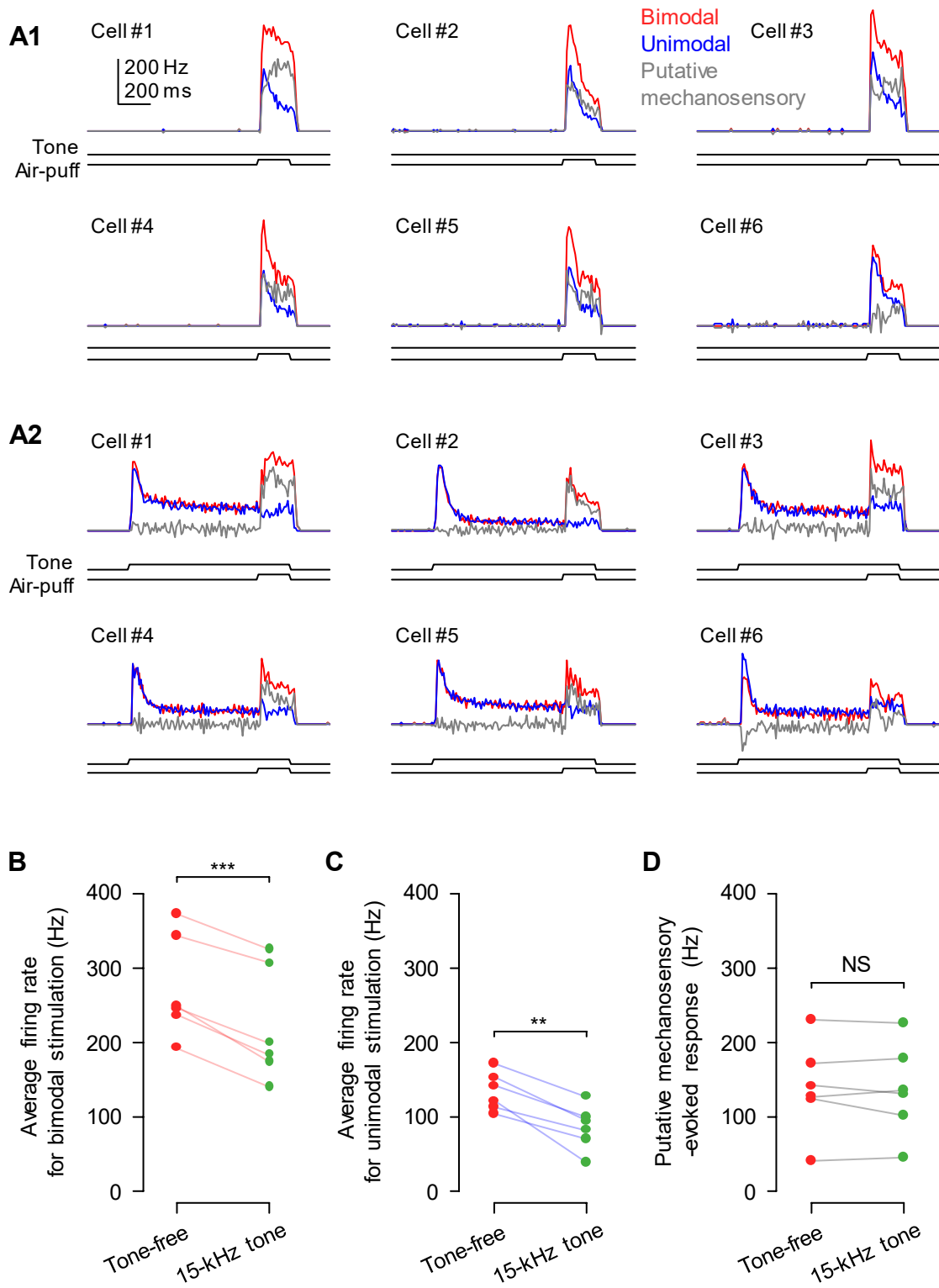


**Figure 4-3. Modulation of AN2 responses to an air-puff stimulus by the preceding 15-kHz tone.** (A) Raster plots of spikes evoked by the bi-modal stimulation with Tone-free stimulation protocol (A1) and 15-kHz tone stimulation protocol (A2). (B) Time course of mean firing rate in the response to air-puff. The red traces indicate the responses for Tone-free protocol and the green traces indicate response for 15-kHz tone protocol. The thin and light traces show averaged response for each sample. The thick and dark traces show the averaged responses across all samples for each protocol. The lower black line indicates the duration of the air-puff stimuli. (C) Pooled data of the mean firing rate

during air-puff stimulus. Gray lines denote individual cells ( $N = 11$ ). \*\*\*  $p < 0.001$  (paired t-test).

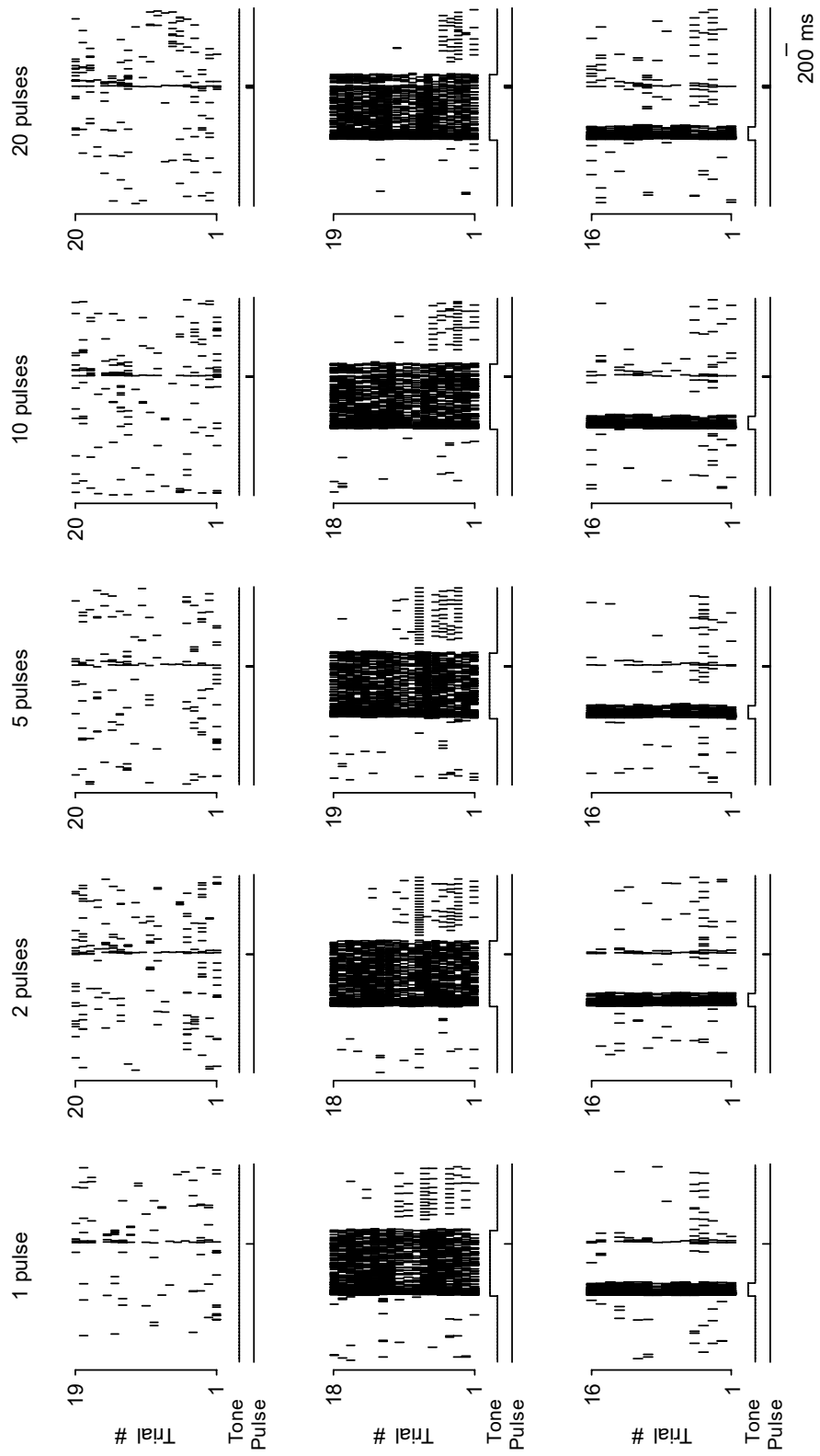


(Figure 4-4)



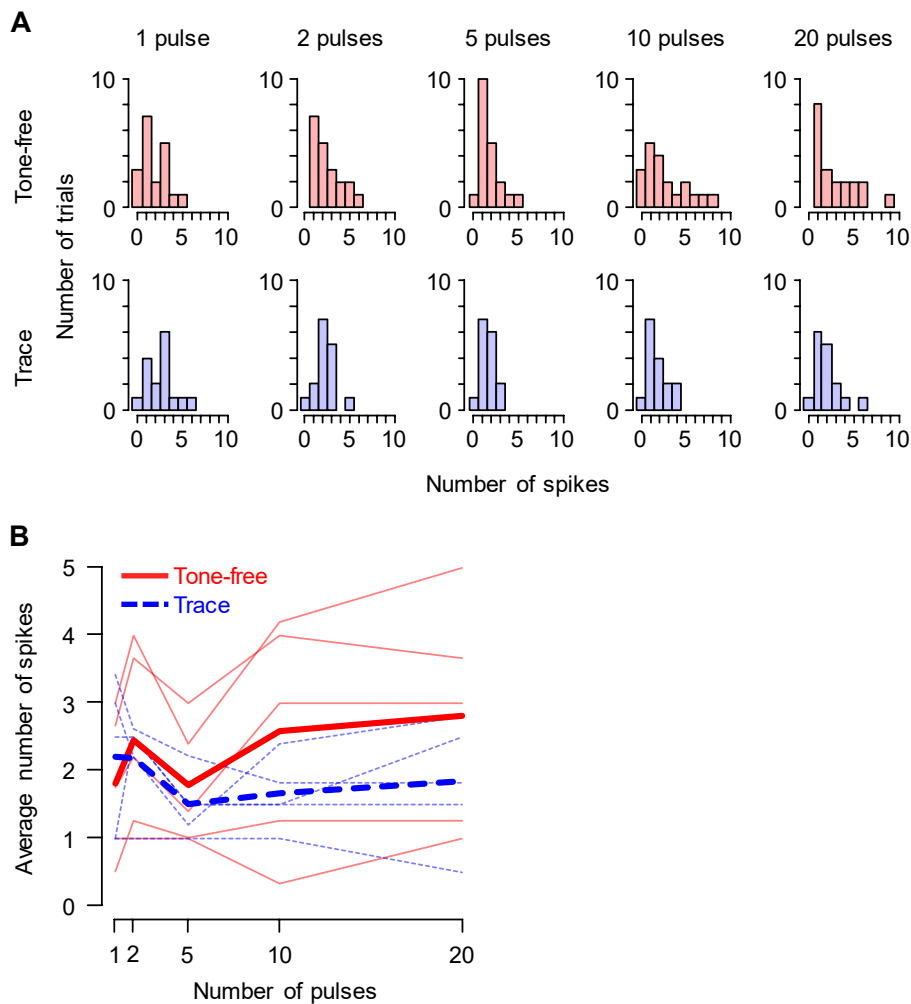
**Figure 4-4. AN2 responses to bimodal and unimodal stimulation.** (A) Time courses of mean firing rate in the responses to bi-modal (red traces) and unimodal (blue traces) stimulation for Tone-free protocol (A1) and 15-kHz tone protocol (A2) for 6 individuals. The gray traces indicate the putative mechanosensory-evoked responses estimated by subtracting the response to the unimodal stimulation from the response to the bi-modal stimulation. (B–D) Pooled data of the mean firing rate during air-puff stimulus in the bi-modal (B), the unimodal (auditory) (C) and the putative mechanosensory responses (D). Lines in the light colors denote individual cells (N = 6). \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$  (paired t-test).

(Figure 4-5)



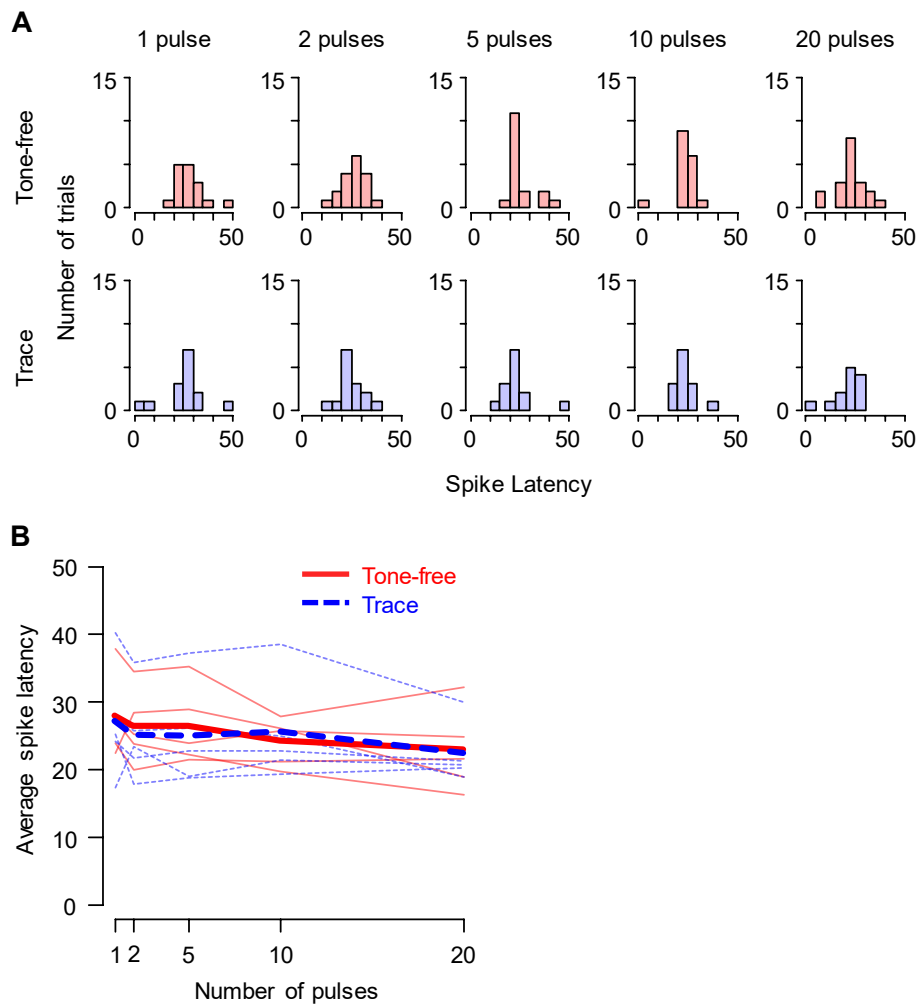
**Figure 4-5. Raster plots of spikes in AN2 responses to electrical stimulation of the cercal nerve.** The top, middle, and bottom rows show the responses for Tone-free, Delay (15-kHz tone in the previous chapters), and Trace stimulation protocols, respectively. The columns are the responses to different intensity of stimulation in the number of stimulation pulses (1, 2, 5, 10 and 20 pulses).

(Figure 4-6)



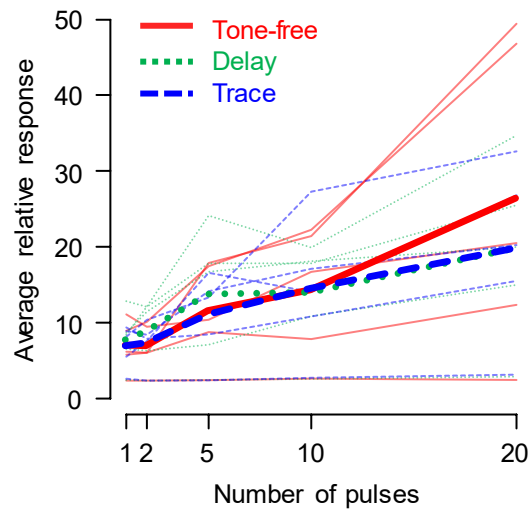
**Figure 4-6. Pooled data of the AN2 responses to the electrical stimulation of the cercal nerve in different stimulation protocols.** (A) Distributions of the number of spikes for Tone-free (upper row) and Trace (lower row) protocols across the different intensity of stimulation. The number of spikes was counted for 200 ms after the first stimulation pulse. (B) Relationships between the stimulation intensity (number of stimulation pulses) and mean number of spikes for Tone-free (red) and Trace (blue) protocols. The thin and light traces show the data for each individual cell (N = 5), and the thick and dark traces show the data averaged across all samples for each protocol.

(Figure 4-7)



**Figure 4-7. Latency of the first spike evoked by the electrical stimulation.** (A) Distributions of the first-spike latency for Tone-free (upper row) and Trace (lower row) protocols across the different intensity of stimulation. (B) Relationships between the stimulation intensity (number of stimulation pulses) and mean spike-latency for Tone-free (red) and Trace (blue) protocols. The thin and light traces show the data for each individual cell (N = 5). The dark traces show the data averaged across all samples for each protocol.

(Figure 4-8)



**Figure 4-8. Ascending spike responses to the electrical stimulation of the cercal nerve.** Relationship between the stimulation intensity (the number of stimulation pulses) and mean value of the relative response magnitude for Tone-free (red), Delay (green), and Trace (blue) protocols. The thin and light traces show the data for each individual cell (N = 5). The thick and dark traces show the data averaged across all samples for each protocol.

## General Discussion

### *Towards understanding context-dependent modulation of escape behavior*

In chapter 1, 2 and 3, it was demonstrated that the ‘preceding’ and ‘high-frequency’ sound dramatically modulate wind-elicited escape behavior in various locomotor aspects. In chapter 4, I revealed that AN2 would be a candidate neuron which plays a significant role in the auditory modulation of wind-elicited escape behavior. In this thesis, I established a novel behavioral experimental paradigm using the crickets, which allows us to understand the neural circuit underlying the context-dependent change of innate behavior.

To my knowledge, the present study showed the context-dependent modulation of escape locomotion by using two simple sensory stimuli for the first time. A previous study in fish has illustrated the neural mechanism involved in the context-dependent or cross-modal modulation of responsibility in escape behavior. Mu et al. (2012) revealed that a preceding visual input increased the response probability of sound-elicited escape behavior. They also demonstrated that M-cell response was enhanced by light-sensitive dopaminergic neurons in the caudal hypothalamus: increasing the signal-to-noise ratio of the auditory signals mediated by sensory afferents and the synaptic efficiency between the afferents and M-cell. This study elucidates how the cross-modal interaction between trigger and contextual stimuli is involved in modulation of escape response but did not discuss the contextual modulation in the locomotive performance. I found that the preceding sound modulate not only the response threshold but also various locomotor parameters including direction, distance, and variability in turning angle. My findings indicated that the cross-modal interaction could ‘shape’ the behaviors induced by the different modality of sensory inputs. However, it remains unknown how AN2 and its



downstream circuits alter the escaping locomotion. Further physiological studies should elucidate the whole of neural mechanism underlying the auditory modulation of the escape behavior.

*Contextual modulation of escape locomotion requires context preceding trigger*

The results in Chapter 1 and 2 suggests that the auditory modulation of wind-elicited escape behavior depends on the temporal relationship, not on the spatial relationship between the trigger and contextual stimuli. In other words, the context-dependent change of escape locomotion requires the context stimulus preceding, not simultaneous the trigger stimulus. This concept would be a common feature in contextual modulation of escape across animals. For example, refuges or obstacles alter animals' escape trajectories, as mentioned in General Introduction (Eaton and Emberley, 1991; Ellard and Eller, 2009; Hemmi, 2005; Ingle and Hoff, 1990; Kanou et al., 2016; Ritzmann et al., 1991; Zani et al., 2009). Those previous studies also suggest that animals somehow have to perceive the environmental context before they receive the threatening stimuli in order to choose appropriate escape strategies adapting to the context.

Furthermore, the context-dependent modulation does not always require the temporal overlap of the contexts and the trigger. Previous studies in gerbils and frogs have reported that in order to change their escape trajectory these animals continued to take account for a position of obstacle even after the obstacles were moved or removed (Ellard and Eller, 2009; Ingle and Hoff, 1990). These facts suggest that the spatial memory about the objects affects the locomotive performance, especially 'where to escape'. However, it has been completely unknown the neural mechanism that modulates escape behavior by the spatial memory. The result in Chapter 2 that the walking direction

was also modulated by the sound which terminated before the onset of the air-puff stimuli suggests the working memory of the acoustic context in the cricket. As shown by the result using the long-trace protocol, this ‘contextual memory’ was retained for at least 600 ms. In addition, electrophysiological results in Chapter 4 revealed that AN2’s response to the electrical stimulation of the cercal nerve was modulated in Trace protocol, in which the time interval between the termination of 15-kHz tone and the onset of air puff was 600 ms. As discussed in Chapter 4, it is unlikely that the modulation of AN2 response was due to adaptation. It remains unclear what and how neural mechanism preserves the memory of the acoustic context. If AN2 plays a crucial role in the auditory modulation, the auditory context could be memorized at least within the prothoracic ganglion.

#### *Identical stimuli don’t always elicit the same responses*

Animals exhibit variable escape behaviors, even which is elicited by identical stimuli. This behavioral variability will be due to the context-dependent modulation, which is the main theme in my thesis. However, the escape behavior itself is also variable innately, especially in the directional control (Domenici et al., 2008, 2011a; Humphries and Driver, 1970). As discussed in Chapter 3, the variability in the escape direction is one of the crucial factors for a successful escape, which would be effective for betraying the predator’s prediction on the prey’s escape direction (Brembs, 2011; Domenici et al., 2008, 2011a; Humphries and Drive, 1970). However, the neural substrates producing the behavioral variability remains completely unknown.

The result in Chapter 3 offers a good experimental platform to address the issue. The finding that high-frequency sound increased the variability of the turn magnitude

suggest that the neural activities altered by the sound would be involved in causing the fluctuation of turning. Since the turn angle like moving direction could be regulated by the descending neurons from the brain (Oe and Ogawa, 2013), further study on the postsynaptic neurons of the AN2 and their downstream including descending neurons allow us to elucidate the neuronal mechanisms underlying the ‘capricious’ behavioral selection.

*Potential effects of sex differences in the auditory system on the auditory modulations*

Male crickets used in my study were known to exhibit both positive/negative phonotaxes as well as female (Leonard and Hedrick, 2009; Pollack, 1982; Popov and Shuvalov, 1977; Ulagaraj and Walker, 1973). In contrast, the cricket’s auditory system some sex differences in the recognition of temporal pattern of calling song. In black field cricket, *Teleogryllus oceanicus* whose calling song consists of two distinct parts, chirp and trill, the female preferred the chirp meanwhile the male preferred the trill (Pollack, 1982). In addition, the female cricket also shows more preference to the song containing longer-bout call but the male showed less selective, which indicates that the positive phonotaxis in male crickets may be to exclude the rival conspecific males (Leonard and Hedrick, 2009). The sex difference in the ecological meaning suggests that the 5-kHz sound may be perceived as distinct contexts by male and female crickets and have different impacts on the wind-elicited escape behavior in females from in males. Further study should also focus on the sex differences of auditory modulation of wind-elicited walking behavior.

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## Research Achievements

### *Publications*

1. **Fukutomi, M.** and Ogawa, H. (2017) Crickets alter wind-elicited escape strategies depending on acoustic context. *Scientific Reports* 7: 15158.
2. **Fukutomi, M.**, Someya, M. and Ogawa, H. (2015) Auditory modulation of wind-elicited walking behavior in the cricket *Gryllus bimaculatus*. *Journal of Experimental Biology* 218: 3968–3977.

### *Presentations*

#### **International** (All presentations were poster)

1. **Fukutomi, M.** and Ogawa, H. Contextual modulation of escape behavior by multisensory integration in the cricket *Gryllus bimaculatus*. *13<sup>th</sup> International Congress of Neuroethology* [P098] Brisbane, Australia. July 2018.
2. **Fukutomi, M.** and Ogawa, H. Crickets modulate wind-elicited escape behavior depending on auditory context with sound carrier frequency. *47<sup>th</sup> annual meeting of the Society for Neuroscience* [156.02/LL5] Washington, D.C., USA. November 2017.
3. **Fukutomi, M.** and Ogawa, H. Acoustic stimulus impacts on directional variability of wind-elicited walking behavior in the cricket. *46<sup>th</sup> annual meeting of the Society for Neuroscience* [160.04/TT8] San Diego, USA. November 2016.
4. **Fukutomi, M.** and Ogawa, H. (2015) Preceding auditory inputs modulate responsiveness and orientation in wind-elicited walking behavior in the cricket. *45<sup>th</sup>*

*annual meeting of the Society for Neuroscience* [637.05/DD32] Chicago, USA.

October 2015.

5. **Fukutomi, M.**, Someya, M. and Ogawa, H. (2014) Auditory modulation of wind-elicited walking behavior in the cricket. 44<sup>th</sup> annual meeting of the Society for Neuroscience [625.24/EE19] Washington, D.C., USA. November 2014.
6. **Fukutomi, M.**, Someya, M. and Ogawa, H. (2014) Preceding auditory cue modulates walking direction in wind-elicited walking behavior in the cricket. 11<sup>th</sup> *International Congress of Neuroethology & 36<sup>th</sup> Annual Meeting of Japanese Society for Comparative Physiology and Biochemistry* [PO-2063] Sapporo, Japan. July 2014.

## **Domestic**

1. 福富 又三郎, 小川 宏人. コオロギは聴覚状況に応じて気流逃避行動を変化させる. 日本動物学会第 87 回富山大会 [1G0945] 富山, 2017 年 9 月 (口頭発表)
2. 福富 又三郎, 小川 宏人. コオロギ気流誘導性歩行運動と下行性神経活動は先行する聴覚刺激によって修飾される. 第 39 回日本神経科学大会 [P3-127] 横浜, 2016 年 7 月 (ポスター発表)
3. 福富 又三郎, 小川 宏人. 先行する音刺激はコオロギ気流逃避行動を修飾する. 日本動物学会第 86 回新潟大会 [1D1600] 新潟, 2015 年 9 月 (口頭発表)
4. 福富 又三郎, 小川 宏人. 先行する音刺激はコオロギの気流誘導性逃避行動を修飾する. 第 38 回日本神経科学大会 [1P209] 神戸, 2015 年 7 月 (ポスター発表)
5. 福富 又三郎, 染谷 真琴, 小川 宏人. Preceding auditory cue modulates wind-



elicited walking behavior in the cricket. 脳と心のメカニズム第 15 回冬のワークショップ [21] 留寿都, 2015 年 1 月 (ポスター発表)

6. 福富 又三郎, 染谷 真琴, 小川 宏人. コオロギに気流誘導性逃避行動に対する先行聴覚刺激のクロスモーダル効果. 第 37 回日本神経科学大会 [P3-174] 横浜, 2014 年 9 月 (ポスター発表)
7. 福富 又三郎, 染谷 真琴, 小川 宏人. 聴覚刺激によるコオロギの気流誘導性歩行運動の変化. 日本動物学会北海道支部第 59 回大会 [O-13] 函館, 2014 年 8 月 (口頭発表)

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