**Excretion initiates walking in the cricket *Gryllus bimaculatus***

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**Abstract**

Feces contain information of donors and potentially attract not only conspecific animals but also predators and parasites. Excretory system must link with behavior initiation in insects. We here found that crickets start walking forward followed by excreting feces. Most of intact crickets walked around in the experimental arena and stopped and raised up their body with slightly drift backward to excrete feces. After it dropped feces on a floor, it started walking with random gait pattern away from the excreted position, and then changed the gait pattern to tripod gait. Headless cricket also showed after excretory walking. More than half of excretion, headless cricket walked backward before excreting feces. The posture of excreting feces was similar to that of intact cricket, and after-excretory forward walking was also observed. The occurrence rate of after-excretory walking was more than that of intact crickets. The gait pattern of forward walking was random and never transit to tripod gait in the headless crickets. The specimens whose abdominal nerve cord were cut at any position did not showed before- and after-excretion walking in both intact and headless crickets, although they excreted feces. These results indicate that the ascending signals from the terminal abdominal ganglion initiate leg movement through the neuronal circuits within thoracic ganglia and that descending signals from the brain must regulate leg motor circuit to express sophisticated walking.

## Introduction

Particular external stimuli can be trigger signal to initiate particular stereotyped behaviors in insect. Escape behavior is one of the stereotyped response to threatening stimulus. Insects respond with quick walk, jump, flight etc. to threatening stimulus like visual stimuli, air movement and so on (Card, 2012). In arthropod animals, sensory signals are locally processed and integrated to initiate escape movements (Nagayama et al., 1994; Tauber and Camhi, 1995; Strausfeld, 1999). Ascending information from abdominal ganglia to thoracic ganglia activate leg motor circuit in insects to initiate escape walk in insects (Gal and Libersat, 2008).

Initiation of exploratory behaviors is initiated by signals generated in the brain. It has demonstrated that descending signals from the brain is necessary in initiation of voluntary walking in not only in vertebrate animals but also in invertebrate animals (Kagaya and Takahata, 2011). Chemical cue is one of major signals to initiate exploratory behavior in insects. Insects are attracted by the components of chemical substances of foods or pheromones (Dethier, 1947). Male silk moth explores the source of female. Female pheromones are processed and integrated in the brain and initiates zigzag motor program in the male moth to detect the source of pheromones (Kanzaki, 1998). Sound signals are another kind of cue that insects used to recruit conspecific insects. Female crickets show phonotaxis to calling song stridulated by males (Alexander, 1961). Sound signals are processed and integrated in the central nervous systems in crickets (Schildberger, 1984).

The center of leg motor control is within thoracic ganglia in insect. Not only descending signals from brain but also ascending signals from abdominal ganglia must

regulate leg motor circuit to initiate walking. Most of insects show tripod gait pattern where the one side of foreleg, hind leg and another side of mid leg move synchronously (Wilson, 1966). In the thoracic ganglia, oscillatory neuronal activities so-called central pattern generating networks (CPGs) are observed (Borgmann et al., 2009). Descending information from head ganglia is necessary to generate coordinated gait movement of legs (Heinrich, 2002). Neuronal activities in the central complex of the brain is linked to initiating locomotion in insects (Strausfeld, 1999; Bender et al., 2010). Subesophageal ganglion is thought to play important role in locomotion related task (Knebel et al., 2018).

Internal signals as well as external signals function to initiate stereotyped behaviors in insects. Cricket for example stridulate courtship song every 1hr (Nagao and Shimozawa, 1987). It is known that cricket whose head is surgically removed (headless cricket) can still stridulate calling and courtship song (Howse, 1974). Internal clock located at the terminal abdominal ganglion function to initiate stridulating the courtship song every 1hr (Ureshi and Sakai, 2001). Starvation and thirst increase motivation to initiate exploratory behavior for foods and water. This indicates that food digestion and excretion system must link with initiating particular behavior in insect.

Excretory system in insects has investigated to understand the physiology of food digestion (Maddrell, 1981). It has demonstrated that food digestion and excretion in insect are controlled by peripheral nervous system (Hartenstein, 1997). However, excretion induced locomotion still remains unclear in insect. Feces contains information of donor insect. Chemicals evaporated from feces potentially attract not only conspecific insects but also predators and parasites. Some insects excrete pheromones

with feces. Feces of *Drosophila* adults contains pheromones to attract conspecific flies and increase feeding (Keesey et al., 2016). In locusts, a grangerization pheromone is also excreted (Obeng-Ofori et al., 1994; Gillett and Phillips, 1977). Increasing population density accumulates excreted grangerization pheromone, which trigger change in body size, color and behavior of the locust, i.e. solitary phase to gregarious phase (Heifetz et al., 1996). A seed harvesting ants mark nest-area with feces, which allows nest mate colony recognition (Grasso et al., 2005). On the other hand, parasitic nematode are also attracted by the chemicals form insect feces (Schmidt and All, 1979). Therefore, excretion must function as one of the important signals to initiate locomotive behavior in insects.

We here focus on excretory behavior of the cricket. Crickets shows walking followed by excretion. The gait pattern of the after-excretion walking changed from random to tripod. As a first step to understand excretion related locomotion in insect, we here surgically cut abdominal nerve cord at different position. Our results suggest that feedback signal from hind gut to the abdominal terminal ganglion activates motor circuit in the thoracic ganglia to initiate walking followed by excreting feces in the cricket.

## Materials and Methods

### *Animals*

Crickets *Gryllus bimaculatus* (DeGeer) used were raised in a laboratory colony. They were reared on a 14h: 10h light and dark cycle (lights on at 6:00 h) at 28 ± 2ºC and fed a

diet of insect food pellet (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum.* Adult crickets that had molted within 2 weeks before experiments were randomly selected and used in this study.

***Behavior experiments***

A cricket was placed in an arena made by clear acrylic board (200mm x 300mm x 100mm) to observe sequence of the excretory behavior. For the floor of the arena, we used a matte floored plastic sheet and change it in every experiment. Since the inter excretion interval in the cricket is rather long and an intact cricket does not always move around during experiments, we first established a video tracking system to record the periods when cricket moving (Suppl. 1). Shortly, we used a web camera (HD Pro Webcam C910, Logicool, Japan) and windows 10 based PC to record cricket behavior. The system obtains RGB images continuously at 30 fps and each obtained image is compared with the previous frame. If obtained image is different from the previous one, the image is saved on the PC after the time stamp is inserted to the image. After recordings long time behavior, saved images are converted to MOV format movie file for latter analysis. Using this video tracking system, we succeed in automatically cropping video data where a test cricket is moving.

Recordings were started between 11:00 AM and 9:00 AM of next day. During recording, cricket in the arena was illuminated using a LED light source. We cropped excretory behavior recorded from the long lasting recorded movies. To investigate excretory behavior, we first draw ethogram of excretory behavior. We here focus on moving direction and gait pattern of the walking before and after excretion. We

categorized as “resting”, if cricket stay at the same place for more than 10min. We then made gait diagram to analyze gait pattern of walking. We measured duration when leg placed on the floor. Then gait patterns between intact crickets and headless crickets were compared. When cricket in the arena moved at the corner of the arena and it was difficult to judge the movement of the cricket, we omitted the cricket from analysis.

### *Surgical treatment of the crickets*

In order to elucidate which signals initiate excretory movement, we surgically removed the head of the cricket. Test cricket was anesthetized by exposing carbon dioxide gas for 10sec and the head was quickly cut using surgically scissors. After hemolymph from the cut end of the neck was dried, a headless cricket was placed in the experimental arena. After we examined headless crickets responded to air puff and twitching stimulus of hind leg, we started recording behavior of the cricket using the video tracking system. In some cricket, the connective of the abdominal nerve cord was cut at different position to elucidate ascending signals from the abdominal ganglion initiate excretory walking.

## Results

Excretion of feces in cricket can be observed 6-8 times in one day. Since cricket in the experimental arena does not move all time, we established video tracking system to record only when test insect moved, which enable us to save time and cost for recording media. Crickets left feces dropped on the floor of the arena. Counting number of feces

and checking excretory behavior recorded, we could evaluate if the recording system worked properly. Analyzing the results recorded, we found that crickets start walking followed by excreting feces.

**Excreting feces in the cricket**

Cricket excreted feces every 3-4hr (mean ± SD: 197±93 min, N=7, n=12). Excretory behavior of the cricket was stereotyped (Fig. 1). Crickets walked around or stayed at the same place in the arena for a while and then it stopped locomote before excretion. When they excreted feces, it raised up the body with slightly moving backward and then bend the abdomen toward the ground, so that excrement drops on the ground (Fig. 2, Suppl. 2). About 30% of excretion in intact crickets, they showed backward walking. They usually walked a few cycles of steps and the distance of walking was usually short (2.5 ± 1.4 steps, N=2, n=5) (Fig. 3, Suppl. 3).

Excretory behavior was also observed in headless cricket. Inter excretion interval in headless cricket was similar to that in intact (206±147 min, N=7, n=26). There was no significance between intact and headless cricket in inter excretion interval (Mann-Whitney U test, p=0.78). While, the condition of feces was different. Intact crickets excreted solid feces. On the other hand, headless cricket excreted liquid feces. although all crickets used in this study were from same colony and fed same foods and water. We found that backward movement followed by excretion of headless crickets was different from that of intact crickets. Headless crickets showed backward walking followed by excretion more than intact crickets (p= 0.0008 with U verification) (Fig. 4, Suppl. 4). The number of steps of hindlegs in backward walking was 13.8± 16.5 steps

(N=6, n=20). The gait pattern of the backward walking in the headless cricket was random (Fig. 4 B).

**After excretion walking in the cricket**

Most of crickets started walking after excreting feces in intact cricket. In about 70% of excretion, the cricket walked forward to leave from the feces position. In about 30 %, crickets stayed at the same position as excreted (Fig. 1 A). The gait pattern of the cricket is characterized as tripod gait. Intact crickets showed ordinary tripod gait before excretion (Fig. 3 B). However, the gait pattern just after excreting was random (Fig. 3 C). After a few steps with random gait, the cricket changed its gait pattern to ordinary tripod gait. Once the cricket changed walking to tripod gait, it kept sophisticated gait pattern. Headless cricket, on the other hand, never showed tripod gait walk after excretion. In more than 90% of excretion, headless crickets showed forward walking with random gait until it stopped (Fig. 4 C).

To examine if excretion initiating walk is regulated by abdominal nervous system, we cut abdominal nerve cord at different position and observed behavior expressed. Crickets whose nerve cord were cut at the position between 2nd and 3rd abdominal ganglion excreted feces (Table 1). However, they did not show after excretory walking with random gait pattern. We then examined using headless cricket, since after excretory walking is clearer than intact crickets. We cut the connective of the nerve cord between 2nd and 3rd abdominal ganglion and between 6th and terminal ganglion. In all specimens, excreting feces were observed. Similar to ordinary crickets, headless crickets did not show backward walking followed by excretion if the nerve cord was cut

at the position between 2nd and 3rd abdominal ganglion. Backward walking just after excretion also disappeared if abdominal nerve cord was cut at the position between the 2nd and 3rd abdominal ganglion ant between 6th and terminal abdominal ganglion. For control, we performed sham operation by cutting only cuticle of the abdomen. Headless crickets whose cuticle of abdomen was cut showed backward walking followed by excretion and forward walking after excretion.

## Discussion

Feces contain information of donor insects. To leave away, or not to leave away after excreting feces, that must be the strategy of insects. Locust excretes grangerization pheromones with feces (Obeng-Ofori et al., 1994; Gillett and Phillips, 1977; Heifetz et al., 1996). Accumulation of the pheromone recruit conspecific locusts and increasing population density introduces to change locusts from solitary phase individual to gregarious phase. Gregarious locusts have long wings to move long way. On the other hand, cricket change body size smaller, if population density increases (Iba et al., 1995). For crickets, leaving away from feces position would prevent suffering from density effect. The cricket is solitary insect and live in a burrow to make a territory (Phillips and Konishi, 1973). Excretory walking could prevent from risks of predator and parasites.

## Excretion of feces

Cricket showed excretory behavior every 3-4hr in both intact and headless specimens. This indicates that the cycle of excretion would be independent from brain control. Headless cricket excreted liquid feces in this study suggesting that the rectum absorb water must be dysfunction of the excretion system. Removing fore gut system might affect to disturb rectum absorb water in headless crickets. At moment, how feces condition (solid or liquid) affect excretory behavior is unclear. However intact cricket sometimes excreted liquid feces in this study and show ordinary excretory behavior. Feces condition might not affect excretory behavior. Intact and headless crickets whose abdominal nerve cord was cut also excreted feces every 3-4hrs suggesting that the timing of excretion could be controlled by digestion system in the mid- and hind gut. Indeed, isolated hind gut of the insect is known to move and acetylcholine, amino acids and biogenic amines modulate the movement of the gut (Kanehisa, 1966). Efferent neurons were found innervating from terminal abdominal ganglion to hind gut in cricket (Hustert and Topel, 1986; Klemm et al., 1986; Elekes et al., 1987; Nässel, 1988). In the cockroach, the sensory afferents that detect movements of gut innervate terminal abdominal ganglion (Ishida and Engelmann, 1983). These studies indicate that the information of the movement of hind gut must be processed in the terminal abdominal ganglion.

**Excretion induced movement**

We found that headless crickets showed backward walking before excretion feces and forward walking with random gait pattern after excreting feces. Although backward walking was not dominant movement in intact crickets, some of intact

crickets showed shorter backward walking. When excreting feces, both intact and headless crickets raised up and drifted their body backward. The backward walking followed by excreting feces would be a reflex movement and could be inhibited by the information from head ganglia including brain and subesophageal ganglion. In the arthropod animals, information from head ganglia is known to inhibit all reflex activities (Bethe, 1898). Since cricket bended the abdomen to drop feces, the movement of drifting the body backward would be the preparation movement for excretion. To initiate this movement, the cricket need to move legs so that the body move backward. Hence, backward walking followed by excretion would be a part of the preparation movements for the excretory posture.

The backward walking disappeared if abdominal nerve cord was cut at any position between the 6th and terminal abdominal ganglion. This suggest that backward walking must be initiated by the information from the terminal abdominal ganglion. The afferents from hind gut must feedback the information of the condition of hind gut to the terminal abdominal ganglion. It was demonstrated that volumetric feedback information detected by hind gut afferents regulates feeding in the locust (Simpson, 1983). Our results suggest that the feedback information of the excretion is processed in the terminal abdominal ganglion and transfer into the thoracic ganglia to initiate leg movements.

Forward walking after excreting feces was observed in both intact and headless crickets. Foreword walking can be divided into random gait walk and tripod gait walk. Since headless crickets only expressed random gait walk, tripod gait walk must be coordinated by the descending information from the head ganglia. To control

coordinated leg movement in walking, function of the central pattern generating networks (CPGs) is thought to be important. The CPGs are thought to be mediated by descending influences from the brain to initiate, to maintain, to modify and to stop motor output for walking (Bidaye et al., 2017). Subesophageal ganglion is thought to modulate interactions between sensory inputs from legs and motor output (Knebel et al., 2018). Random gait walks observed after excretion must be a kind of a reflex activities that is initiated by ascending information from the terminal abdominal ganglion. In intact crickets, the ascending information from the terminal abdominal ganglion to the thoracic ganglia initiates walking and the descending information from the head ganglia mediates leg movements to be coordinated. Crickets detect air currents using filiform hairs arranged on the surface of cerci of abdomen and respond with quick avoidance movement (Edwards and Palka, 1974). The information of air current is processed and integrated in the terminal abdominal ganglion and the signal transfer to the thoracic ganglia to initiate avoidance walking (Aonuma et al., 2008; Mendenhall and Murphey, 1974; Yono and Aonuma, 2008). This study demonstrated that the feedback information from the hind gut must be other signals processed in the terminal abdominal ganglion to initiate walking response in the crickets.

We concluded that feedback information from hind gut would processed in the terminal abdominal ganglion to initiate sequence of excretory movements in the cricket. The information would initiate backward movement to express excretory posture and then forward walking. We now need to investigate neuronal pathway underlying sequence excretory movements, which help us gain understand excretory behavior of insect, and also neuronal mechanisms underlying walking and gait pattern tradition in insects.

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## Figure legends

**Figure 1.** Ethograms for excretory behavior of the cricket. These ethograms indicate behavior of specimens before and after excretion. The thickness of each arrow corresponds to the appearance rate. (A) Ethograms for excretory behavior in intact crickets (n=18). (B) Ethograms for excretory behavior in headless crickets (n=33). WB: walking backward, WF: walking forward, Re: resting

**Figure 2.** Stereotyped posture of excreting feces of the cricket. (A) Snap shots of excreting feces of an intact cricket. (B) Schematic illustration of excreting.

**Figure 3.** Excretory behavior of an intact cricket. (A) Snapshots of excretory behavior. Cricket stopped moving at an excreting place. It raised up and drifted its body backward just before excreting feces. Some of crickets stepped backward before they raised up the body. After excretion, it walked forward to leave away from the excreting place. Arrow indicates feces of the cricket. Time 0 sec indicates excreting feces. (B) Gait diagram of an intact cricket. Cricket walked with tripod gait before stopping at an excreting place. (C) Gait diagram of an intact cricket just after excreting feces. The cricket started walking with uncoordinated (random) gait just after excretion. After a few second, the gait pattern changed to ordinary tripod gait. L and R in the gait diagrams indicate left and right side. F, M and H indicate foreleg, middle leg and hind leg respectively.

**Figure 4.** Excretory behavior of a headless cricket. (A) Snapshots of excretory behavior. The headless cricket walked backward and then stopped moving at an excreting place. It raised up and drifted its body backward just before excreting feces. Then after excreting feces, it started walking forward away from the excreting place. Arrow indicates feces of the cricket. Time 0 sec indicates excreting feces. (B) Gait diagram of the headless cricket. Cricket walked backward with random gait pattern before stopping at an excreting place. (C) Gait diagram of the headless cricket just after excreting feces. The headless cricket started walking with random gait until it stopped. L and R in the gait diagrams indicate left and right side. F, M and H indicate foreleg, middle leg and hind leg respectively.

**Supplementary files**

**Suppl. 1** Source code of the program for video tracking system.

**Suppl. 2** An intact cricket excreting feces.

**Suppl. 3** (A) Excretory behavior of intact cricket. (B) Excretory behavior of headless cricket.

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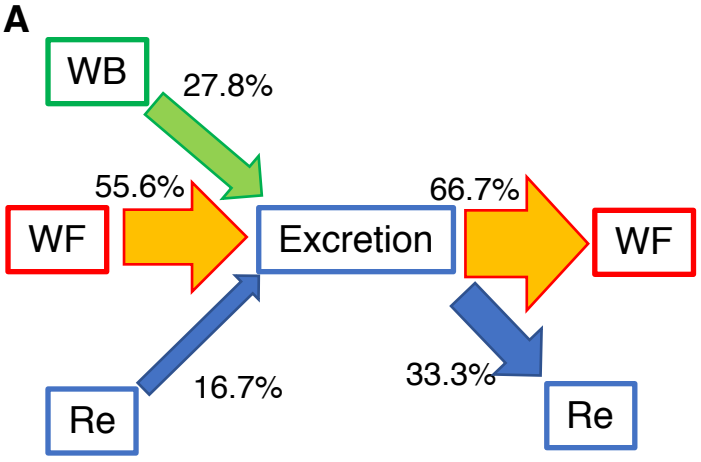
Table 1. Expression of backward walking followed by excreting feces in the crickets. Backward walking was observed in both intact and headless crickets but did not if abdominal nerve cord was cut between 2nd and 3rd abdominal ganglion or between 6th and terminal ganglion. N: number of specimens, n: times of excretion, Cut: Position of nerve cord cut, A2-A6: 2nd-6th abdominal ganglion, TG: terminal abdominal ganglion, Sham: sham operation (only coticule of the abdomen was cut)

| Specimens | Intact | | | Headless | Headless | Headless | Headless |
|---|---|---|---|---|---|---|---|
| Cut | - | A2-A3 | A6 - TG | - | A2-A3 | A6-TG | Sham |
| Number of specimens | N (n)<br>7 (18) | 8 (34) | 8 (32) | 7 (33) | 6 (13) | 9 (20) | 10 (40) |
| Frequency of backward walking | N (n)<br>2 (5) | 0 (0) | 0 (0) | 6 (17) | 0 (0) | 0 (0) | 6 (14) |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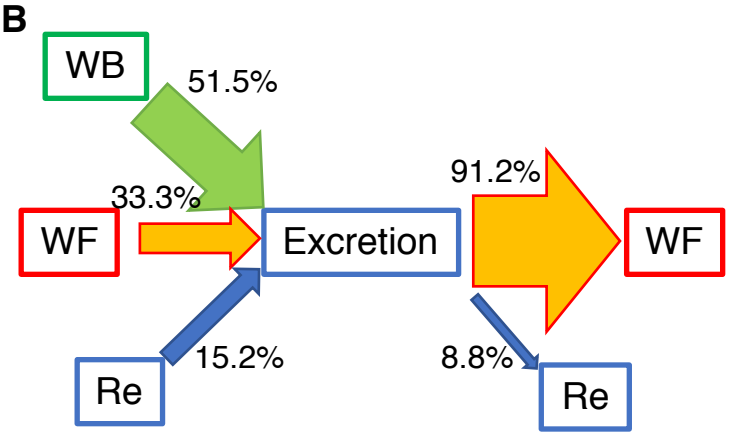
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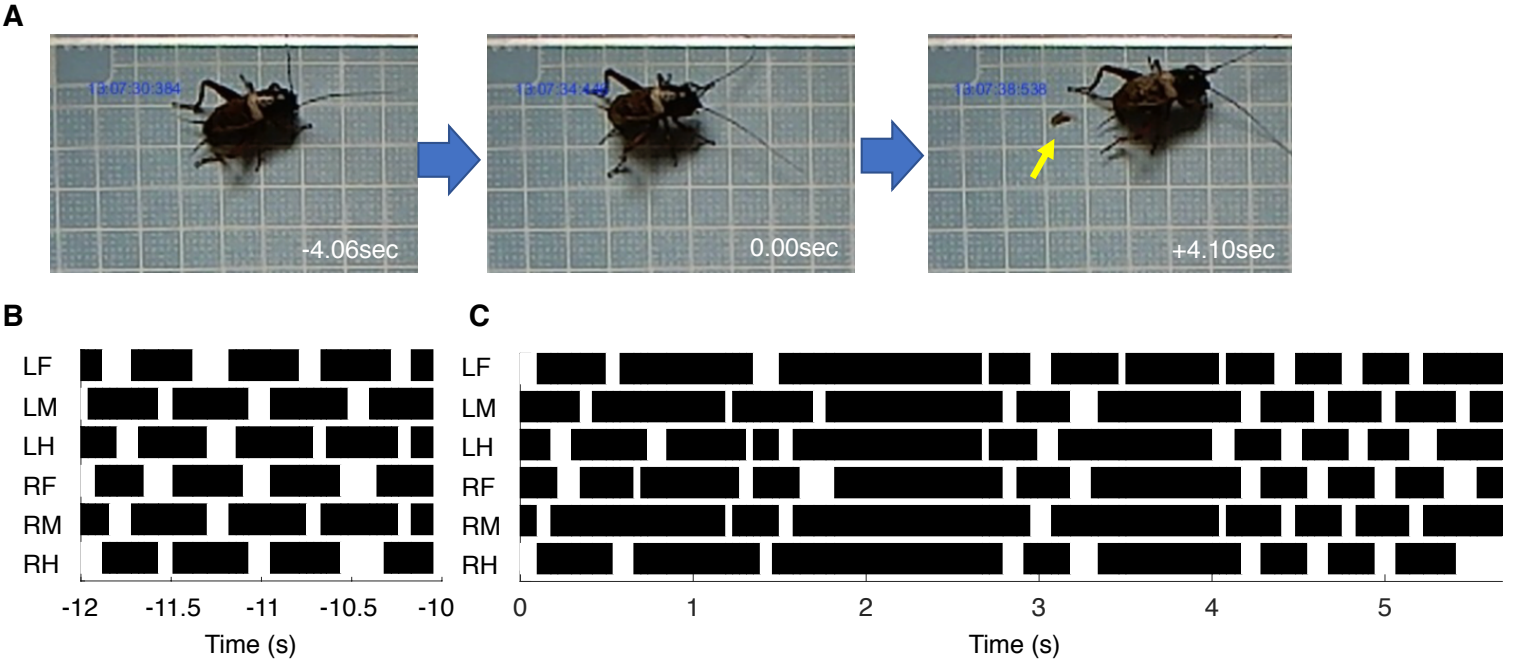
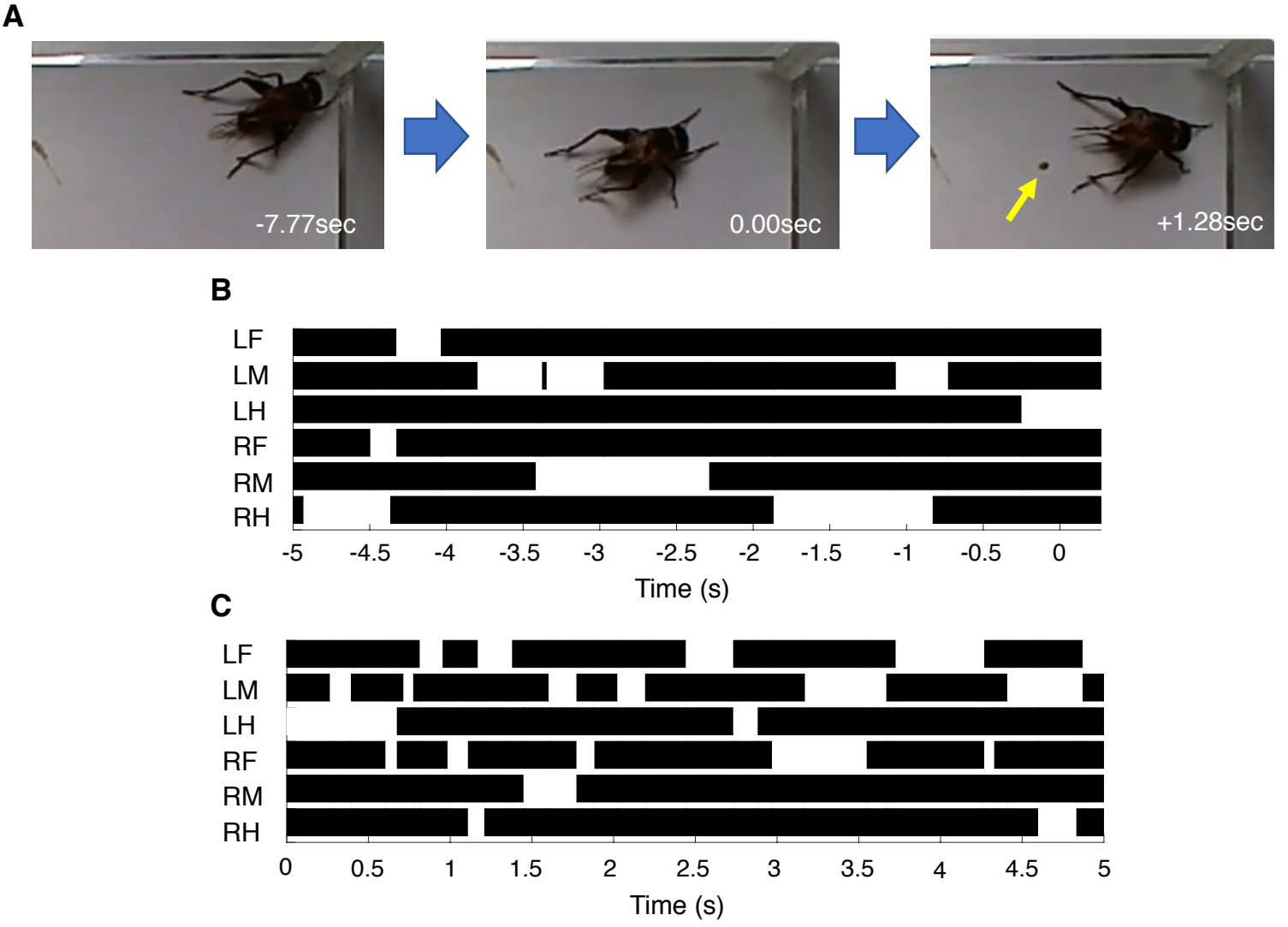

A
-4.06sec
0.00sec
+4.10sec
B
LF
LM
LH
RF
RM
RH
-12
-11.5
-11
-10.5
-10
Time (s)
C
LF
LM
LH
RF
RM
RH
0
1
2
3
4
5
Time (s)


A
-7.77sec
0.00sec
+1.28sec
B
LF
LM
LH
RF
RM
RH
-5
-4.5
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
Time (s)
C
LF
LM
LH
RF
RM
RH
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Time (s)