



Arousal from Tonic Immobility by Vibration Stimulus

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Abstract

Tonic immobility (TI) is an effective anti-predator strategy. However, long immobility status on the ground increases the risk of being eaten by predators, and thus insects must rouse themselves when appropriate stimulation is provided. Here, the strength of vibration causing arousal from the state of TI was examined in strains artificially selected for longer duration of TI (L-strains: long sleeper) in a beetle. We provided different strengths of vibration stimuli to the long sleepers in *Tribolium castaneum*. Although immobilized beetles were never awakened by the stimuli from 0.01 to 0.12 mm in amplitude, almost of the beetles were aroused from immobilized status by the stimulus at 0.21 mm. There was a difference in sensitivity of individuals when the stimuli of 0.14 mm and 0.18 mm were provided. F2 individuals were also bred by crossing experiments of the strains selected for shorter and longer duration of TI. The arousal sensitivity to vibration was well separated in the F2 individuals. A positive relationship was observed between the duration of TI and the vibration amplitude, suggesting that immobilized beetles are difficult to arouse from a deep sleep, while light sleepers are easily aroused by even small vibrations. The results indicate a genetic basis for sensitivity to arousal from TI.

Keywords Beetle · Crossing experiment · Death feigning · Selection experiment · Thanatosis · *Tribolium*

Introduction

Tonic immobility (TI; sometimes called death-feigning behaviours, or thanatosis) has been examined in many animal species (e.g., Ruxton et al. 2004; Miyatake et al. 2004; Cassill et al. 2008; Rogers and Simpson 2014; Humphreys and Ruxton 2018; Skelhorn 2018). TI is thought to be an adaptive defense against predators (Miyatake et al. 2004;

Honma et al. 2006; Ohno and Miyatake 2007), a tactic to avoid sexual harassment (Khelifa 2017), and a strategy to avoid individuals of conspecific species in a social ant species (Cassill et al. 2008).

The relationships between TI and the physiological status of animals who adopt this strategy have also been investigated (Rogers and Simpson 2014). Previous studies have focused on the factors instigating TI, including physical touching (Miyatake 2001a, b; Hozumi and Miyatake 2005; Gregory and Gregory 2006), predator attack (Miyatake et al. 2004), and vibration (Acheampong and Mitchell 1997). For example, Acheampong and Mitchell (1997) caused TI in Colorado potato beetles, *Leptinotarsa decemlineata*, by dropping weights on the leaves where they were resting or by acoustic vibration, and quantified the strength of vibrations causing immobility in the beetle.

However, to our knowledge, no research has investigated the stimulation that causes the arousal from TI in animals, except a study reporting that a grasshopper trapped in the mouth of a frog was aroused after being spit out by the frog (Honma et al. 2006). If the predator uses olfactory cues for detecting preys, the prey has to switch its tactic from TI to running to escape due to a

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strong vibration of substrate, because the greater strength of vibration stimuli might alert an insect to the arrival of larger animals compared to smaller predators.

Some insects are immobilized tonically for very long durations when they are stimulated. For example, a sweet potato beetle, *Cylas formicarius*, continues a pose of TI more than 30 min (Kuriwada et al. 2011), and some West Indian sweet potato weevils, *Euscepes postfasciatus*, freeze for more than 1 h (T. Miyatake, unpublished data). Data concerning which types of stimulation cause arousal from TI may give a new perspective on the phenomenon. Individuals showing ultra-long duration of TI may suffer predation risk: for example, rats may distinguish a tonically immobilized prey by its smell (Miyatake 2001a, b), and ants may bring tonically immobilized insects as prey to the nest: the first author (TM) actually observed an ant bringing to a tonically immobilized *Cylas formicarius* weevil as bait to the nest (T. Miyatake, unpublished observation).

Therefore, experiments on arousal from TI need individuals who continue to freeze stably for a long duration. If we use wild insects, variations in the depth of TI (Miyatake et al. 2001a) may make it difficult to determine quantitatively the arousal sensitivity of each individual. To resolve the problem, it is best to use beetle strains selected for longer duration of TI and thus fixed for the duration. The L-strain selected for longer duration of TI (Miyatake et al. 2004, 2008a, b; Matsumura and Miyatake 2018) is ideal to visualize and quantify the arousal sensitivity to stimulation.

In the present study, we examined the relationship between arousal from TI and the strength of vibration stimuli necessary to do so using these strains. To clarify the presence of genetic background in the relationship between duration of TI and arousal, we also investigated the strength of stimuli causing arousal from TI using individuals with a genetic base for duration of TI. We bred an F2 population by crossing experiments using the strains selected for shorter (S-strains) and longer (L-strains) duration of TI. As a result of long-term artificial selection in the red flour beetle, *Tribolium castaneum*, S-strain beetles never feign death regardless of the tactile stimuli provided, whereas L-strain beetles immobilize for more than one hour even when provided small tactile stimuli (Matsumura and Miyatake 2018). Because the duration of TI showed dominance in the shorter direction for crossed F1 populations (Miyatake et al. 2008a, b), we could not measure the sensitivity to vibration causing TI in the S-strain and F1 population. In the F2 population, the depths of TI could be separated, and thus the arousal sensitivity to vibration could be investigated. Therefore, we provided different strengths of vibration to immobilized beetles of the L-strains and F2 population, and recorded the arousal from TI in the present study.

Materials and methods

Insects and artificial selection

We used strains artificially selected for duration of TI in *T. castaneum*. The protocol of the artificial selection experiment was described in Miyatake et al. (2004, 2008a, b) and Matsumura and Miyatake (2018). Briefly, 100 males and 100 females (15 days old) were randomly collected from the stock culture, and the TI duration was observed. Males and females (10 each) with the shortest duration of TI were selected to propagate the short-duration strain (S-strains); similarly, 10 each with the longest duration were selected to propagate the long-duration strains (L-strains). The males and females of each strain were placed in a plastic cup (65 mm diameter, 25 mm height) with 20 g of medium and allowed to lay eggs for 1 week. The pupae from the eggs were stored in separate-sex groups in other plastic cups and allowed to emerge. When the adults reached 10–15 days of age, 100 males and 100 females were randomly collected from each line and their TI was observed again. The same procedure was carried out for each generation. Two selection replicates for the short and long strains (S-A and L-A; and S-B and L-B) initiated at the same time were tested and maintained in the chamber. The selection regimes were continued for more than 27 generations for each line. The details of the selection experiments, including direct responses and realized heritability, were described in Miyatake et al. (2004) at generation 10 and in Matsumura and Miyatake (2018) at generation 27.

In the present experiment, we used selected beetles at generation 27. These L-strain beetles (A and B) immediately show TI if touched even slightly and continue to be immobilized more than 10,000 s (Matsumura and Miyatake 2018). On the other hand, beetles derived from S-strains (A and B) never show TI no matter how touched. Therefore, L-strain beetles are ideal subjects to measure arousal from TI.

The beetle culture used in this study has been maintained in the laboratory of Okayama University according to the rearing method described by Suzuki and Nakakita (1991). The beetles were reared with a mixture of whole-meal (Nisshin Seifun Group, Tokyo, Japan) enriched with brewer's yeast (Asahi Beer, Tokyo, Japan) as the rearing medium and kept in a chamber (Sanyo, Tokyo) maintained at 25 °C and 60% RH under a photoperiod of 16:8 (L:D) h (lights on at 0700, light off at 2300).

Measurement of vibration stimuli

A beetle was gently placed at the center of the bottom of a petri dish (90 mm in diameter, 15 mm in height) that

had a vibrator (Vp408, Acouve Laboratory Inc., Tokyo, Japan) attached to the bottom. Each beetle was poked with a stick, which caused TI. On achieving this status, we conveyed vibration stimuli to the petri dish by generating a continuous sine wave made by a sound editorial system (Audacity 2.05, Softonic, Barcelona, Spain) as an input signal and amplifying the wave by a digital amplifier (Lepy LP-2020A, Kyohritsu Electronic Industry Co., Ltd., Osaka, Japan). Only one petri dish was used throughout the present experiment. Four carrier frequencies, that is, 25 Hz, 40 Hz, 50 Hz, and 60 Hz, were provided to immobilized beetles to give different strengths of vibration. We also provided a 100 Hz to some beetles of the L-strains.

Because actual amplitude of vibration stimulus depends on the frequency characteristic of the vibrator and the substrate, we measured and identified vertical displacement while vibrating the petri dish using a triangle measuring instrument displacement meter (LK-G155A, Keyence Corp., Osaka, Japan). Each vibration in the vertical direction of the petri dish was identified as follows: 25 Hz: input signal, 0.1 → 0.01 mm, 0.3 → 0.03 mm, 0.5 → 0.08 mm, 40 Hz: 0.1 → 0.07 mm, 0.3 → 0.14 mm, 0.5 → 0.21 mm, 50 Hz: 0.1 → 0.18 mm, 0.3 → 0.34 mm, 0.5 → 0.38 mm, and 60 Hz: 0.1 → 0.12 mm, 0.3 → 0.48 mm, 0.5 → 0.53 mm. We also confirmed that the vibration amplitude of 100 Hz with an input signal of 0.5 measured as 0.08 mm.

The experimental protocol was as follows. First, we provided vibration stimuli to a tonically immobilized beetle for 5 s, and then we observed the beetle's mobility for 3 s. Next, we provided a stronger vibration to the beetle and then observed 3 s. After repeating this protocol, if the beetle woke up from immobility, we recorded the strength of vibration and considered the sensitivity value of arousal from TI. Each individual was gradually progressed to a high vibration frequency from a low vibration frequency, and we recorded the amplitude as each individual was awakened from TI.

Experiment 1: F0 populations

Virgin L-strain beetles aged from 30 to 60 d were used for the experiment. Twenty males and females were randomly selected from A and B lines of the L-strains. One day before the experiment, these beetles were isolated in a well of a 48-well cell plate (Greiner Bio-One, Frickenhausen, Germany) to avoid disturbance by other beetles (Miyatake 2001a). A mixture of 5 g wholemeal (Nisshin Seifun Group, Tokyo, Japan) enriched with brewer's yeast (Asahi Beer, Tokyo, Japan) as food to avoid the effect of starvation during TI (Miyatake 2001b). The experiment was conducted between noon and 6 pm because TI shows diurnal periodicity in insects (Miyatake 2001a). All the experiments were conducted in the laboratory at 25 ± 2 °C.

Experiment 2: F2 individuals

Ten males and ten females from the strains selected for longer (L-strains: A and B) and shorter (S-strains: A and B) durations of TI more than 40 generations (Matsumura and Miyatake 2018) were crossed and thus made F1 populations with each replicated A and B. A short duration of TI is dominant compared to a long duration, and thus almost all F1 cross individuals did not immobilize (Miyatake et al. 2008a, b). This makes it difficult to measure the sensitivity of arousal to the strength of vibration stimuli. Therefore, we measured the sensitivity of F2 populations because Mendel's law of separation in F2. Ten pairs from each F1 population were crossed, and we got F2 individuals ($N=160$). Because of the difference in duration of TI, we expected variations in the duration of TI and the sensitivity to arousal from TI as shown in the response to vibration stimuli in the F2 populations if both traits have a heritable base.

We picked 20 virgin males and 20 virgin females aged 30 d to 60 d from each F2 population (LA males × SA females, SA males × LA females, LB males × SB females, and SB males × LB females), and placed each beetle in a well of a 48-well culture plate with a bit of food (5 g graham whole meal) a couple of days before the experiment. Between noon and 6:00 pm, we measured the strength of vibration stimuli that shown by the sensitivity to arousal from TI. All experiments were conducted in the laboratory maintained under 25 ± 2 °C.

For the F2 population, we measured the duration of TI for all individuals as well as the strength of vibration stimuli causing arousal from TI. The measurement method of TI is same as the measurement of F0 population described above. To measure the duration, each beetle was gently placed on its back in the white china saucer described above (140 mm diameter, 15 mm height). We induced TI in the beetle by touching the abdomen of it with a wooden stick. A trial consisted of provoking TI and recording its duration with a stopwatch. The duration of TI was defined as the length of time between the onset of TI and detection of its first visible movement. If the beetle did not respond, the touch was repeated. When the beetle was immobilized, the duration was recorded. All the trials were conducted between noon and 6 pm in the laboratory described above.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP Ver. 12.2 (SAS Institute Inc. 2015). We used ANOVA to analyze the effect of the vibration amplitude for the beetle's awakening. To test the significance of correlation between duration of TI and vibration amplitude, we used analysis of covariance (ANCOVA).

Results

No beetle awoke from TI at 25 Hz vibration frequency (from 0.01 to 0.08 mm) (Fig. 1a, Video 1), while 80% of beetles were awakened from TI at 40 Hz (from 0.07 to 0.21 mm) (Fig. 1), and all were awakened by 50 Hz (from 0.18 to 0.38 mm) and 60 Hz (from 0.12 to 0.53 mm) of vibration frequencies (Fig. 1, Video 2). However, all immobilized beetles ($n = 20$ from the LA strain) were only sliding around on the bottom of the petri-dish when

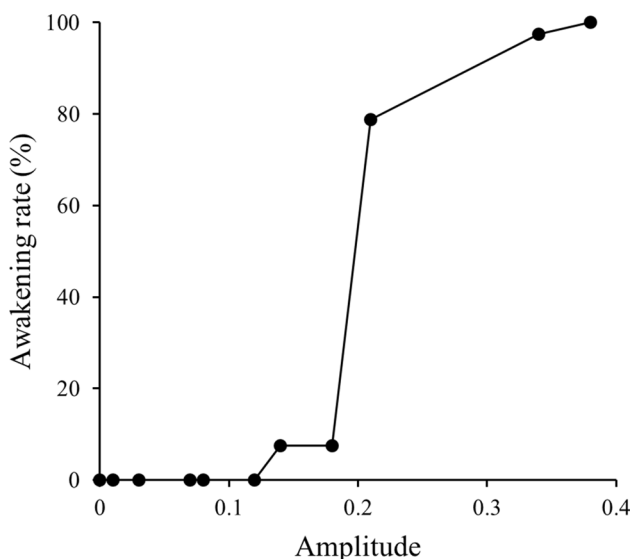


Fig. 1 Effects of amplitude on awakening beetles; each amplitude (mm) of vibration in the vertical direction of the petri dish was identified in the Materials and Method

100 Hz vibration with 0.08 mm displacement was presented, and thus never woke up at the 100 Hz vibration.

The beetles never woke under the vibration amplitude of 0.1 mm (Fig. S1). These trends are the same in the selection replicates A and B of both sexes (Fig. S1). ANOVA showed no significant effects among replicate lines and sexes in the amplitude on a beetle’s awakening (Table S3).

Figure 2 shows the segregation of F2 individuals by the awakening rate in amplitude (a) and the causal relationships between the amplitude and the duration of TI (b). F2 beetles separated in their amplitudes from 0.01 to 0.53 mm. A small percentage of beetles were not woken by the amplitude under 0.53 mm (less than 5%). These trends were the same in crossing replications of both sexes in amplitude on beetle’s awakening (Fig. S2). No significant effects were found for strains, replicated lines, sex and each interaction (Table S4). The duration of TI significantly related to the amplitude (Fig. 2b). Details of results of these analyses were described in Table S5.

Discussion

No individual from the Long strains was aroused from TI when a vibration stimulus of 25 Hz (0.01–0.08 mm in vibration amplitude) was provided, but all were aroused and stated to walk when a vibration stimulus of 50 Hz (0.18–0.38 mm) was provided. Vibration of the ground might inform beetles of an approaching predator; for example, vibration of the foundation such as leaves may be caused by an approaching predator (see Humphreys and Ruxton 2018). If the predator uses olfactory cues for detecting preys as a mouse does, the prey may have to switch tactics from TI to running to escape due to a strong vibration of the substrate because the greater

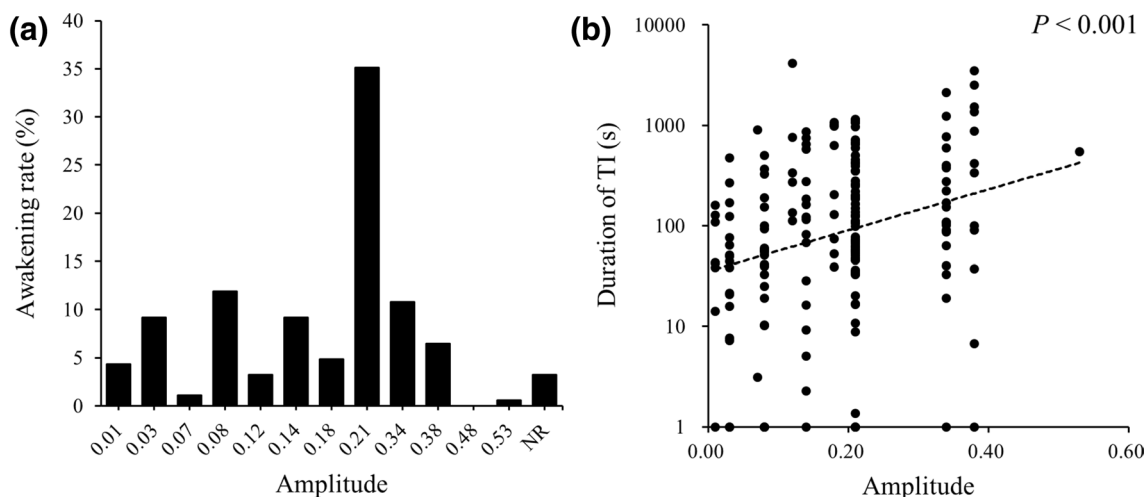


Fig. 2 Histogram of awaking rate (%) for each amplitude (mm) (a) and a causality relationship between the amplitude and the duration of TI (b) in F2 beetles. “NR” (= no response) in the graph (a) means the beetles who were not woken by the amplitude under 0.53 mm

strength of vibration stimulus might suggest the arrival of larger animals such as a mouse or bird as potential predators compared to smaller arthropod predators such like a jumping spider (Miyatake et al. 2004), predatory bugs (Matsumura and Miyatake 2015) or ants (Shaheen et al. 2016).

In the present experiment, the individuals aroused from TI started to walk suddenly after arousal (Video 2). It is necessary to examine whether or not the strength of the same vibration stimulus at 50 Hz indicates a mammalian predator. Although the adaptive meaning of the arousal response to strong vibration was not determined by the present study, we clarified the existence of a strength threshold that causes arousal of tonically immobilized beetles. It has been reported that the duration of TI has a genetic base in *T. castaneum* (Prohammer and Wade 1981, Miyatake et al. 2004, 2008a, b) and other Coleoptera beetles, *T. confusum* (Nakayama et al. 2010) and *Callosobruchus chinensis* (Nakayama and Miyatake 2009). However, this is the first report to clarify that the arousal sensitivity to vibration stimuli has a genetic base because of separation in F2 individuals.

The present study also revealed a positive relationship between the duration of TI and the sensitivity to vibration stimuli causing arousal from TI in *T. castaneum*. Correlated responses to selection for TI have been reported in physiological and mating traits such as larval behavior, mating success and dopamine expression level in brain and locomotor activity of *T. castaneum* (Miyatake et al. 2008a, b) and in locomotor activity of *T. confusum* (Nakayama et al. 2010) and the adzuki bean beetle, *Callosobruchus chinensis* (Nakayama and Miyatake 2009, 2010a, b). In *C. chinensis*, positive correlations between the duration of TI and life history traits; individuals selected for longer duration of TI had shorter development time, greater longevity, higher emergence rate, bigger eggs and higher reproductive effort compared to individuals selected for shorter duration of TI (Nakayama and Miyatake 2009), and a negative correlation between the duration and flight ability (Ohno and Miyatake 2007) have been detected. In *T. castaneum*, the duration of TI correlates negatively to anti-predation strategies, i.e., escape by walking, of both sexes and mating success of males (Nakayama and Miyatake 2010a). Also, genetic correlations were observed between the duration of TI and brain expression of neurophysiological substances such as dopamine in *T. castaneum* (Nishi et al. 2010) and *T. confusum* (Nakayama et al. 2012). Therefore, the present result clearly shows that the sensitivity in immobility, or the duration of TI, shows many pleiotropic effects with other traits, suggesting a complex genome cascade causing TI that should be studied in the future.

The timing of arousal from TI, namely the duration of TI, is important from a viewpoint of fitness. A very long duration of TI has to be dangerous because ants can easily bring a tonically immobilized prey to the nest as bait (Shaheen

et al. 2016), or some predators, such as mammals, can easily detect TI prey by using their sense of smell. Although the present study did not reveal the adaptive phase of timing of arousal from TI, this study certified a genetic base for sensitivity to vibration stimuli in arousal from death feigning.

Sexual difference in the duration of TI was found in the present result; males showed longer duration of TI than females (Table S5). Effects of sex on tonic immobility have been studied, but the results are slightly complex. Also in a previous study, males had significantly longer tonic-immobility duration than females in *T. castaneum* (Matsumura et al. 2016). In a closely related species *T. confusum*, however, no sexual difference in the duration of tonic immobility was found (Nakayama et al. 2012). Another study using the both species, no sexual differences in the duration of tonic immobility were also found in *T. castaneum* and *T. confusum* (Matsumura et al. 2017). For the other Coleopteran species, *Cylas formicarius*, females showed longer duration of tonic immobility (death-feigning duration) than males (Miyatake 2001a). Also in *Callosobruchus chinensis* and *C. maculatus*, females showed longer tonic immobility (Miyatake et al. 2008a, b). Therefore, detailed investigations for inter-specific variation in the sexual differences in tonic immobility are required in the future.

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Author contributions T.M. designed the study. K.M., R.K., K.O., J.Y. T.M. collected data. T.M., KM analysed the data. TM, RF, NN interpreted the results and wrote the manuscript. All authors gave final approval for publication.

Compliance with ethical standards

Conflict of interest Takahisa Miyatake, Kentarou Matsumura, Ryota Kitayama, Keiichi Otsuki, Ji Yuhao, Ryusuke Fujisawa and Naohisa Nagaya declare that they have no conflict of interest.

Ethical approval This study was not required to complete an ethical assessment prior to conducting our research.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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