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ORIGINAL ARTICLE

Behaviour and metabolism during tonic immobility (death-feigning) in *Eucryptorrhynchus scrobiculatus* and *E. brandti* (Coleoptera: Curculionidae)

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Abstract. The antipredator behaviour, tonic immobility (TI) is a valuable defence that enables insects to increase their chance of survival and is a trade-off between fleeing and protection. How the TI strategies of insects respond to environmental factors, however, remains a largely understudied subject. In this paper the effect of four factors (mechanical stimulation, light, sound and temperature) and metabolic responses were used to evaluate TI behavioural and physiological adaptions in *Eucryptorrhynchus scrobiculatus* (ESCR) and *Eucryptorrhynchus brandti* (EBRA). In the behavioural experiment, the metaventrite, which is the stimulus-sensing region in ESCR and EBRA, was subjected to mechanical stimulation. Light lengthened the TI duration in ESCR males, while sound had the opposite effect in ESCR and EBRA. The effect of temperature on the duration of TI was variable: in ESCR, the duration was shorter at low (15°C) and high (32°C) temperatures, but in EBRA, it was longer at the low (15°C) temperature. In the metabolism experiment, ESCR and EBRA metabolic rates (MR), was significantly dependent on whether they were in a state of TI or not. The TIMR declined to 76.90% in ESCR and 71.40% in EBRA. These results indicate that TI in ESCR and EBRA differed under different external conditions and contributes to the understanding of the physiological regulation of ecological traits of insect TI.

INTRODUCTION

When some insects are disturbed by predators they curl up and remain motionless or fall from their original resting places and behave as if dead, and in the absence of further stimulation recover and resume activity after a few minutes (Humphreys & Ruxton, 2018). This is called tonic immobility (TI), death feigning, thanatosis, animal hypnosis, playing dead, immobilization catatonia, playing possum or quiescence (Ruxton, 2006; Acheampong & Mitchell, 2010; Li et al., 2019) and is widely used by animals to limit injury and as a means of escape.

Previous papers report there is a trade-off between fleeing and defence (Ohno & Miyatake, 2007), because individuals control the duration of TI when threatened by a predator or disturbed by an environmental factor (Lima, 1998). How the TI strategies of insects respond to environmental factors including touching, light, sound and temperature has recently attracted more attention. For example, the TI of *Callosobruchus chinensis*, *C. maculatus*, *Tribolium castaneum* and *Timema cristinae* is induced by touching or dropping (Nakayama & Miyatake, 2009; Farkas, 2016; Matsumura et al., 2016), of *Leptionotarsa decemlineata* by shaking or light flashes (Metspalu et al., 2002) and of larvae of *Gryllus bimaculatus*, *Apis mellifera*, *Agriotes fuscicollis* by specific sounds (e.g., bird song) (Little, 1962; Nishino & Sakai, 1996; Acheampong & Mitchell, 2010; Ritter et al., 2016). However, not all factors induce TI directly. For example, specific sounds (alarm calls) can increase the duration of TI in *Gallus gallus* (Pochron & Thompson, 2019) and in two species of *Callosobruchus*, *C. chinensis* and *C. maculatus* (Miyatake et al., 2008a), high incidence of TI is associated with low temperatures (15°C and 20°C) and its duration is correlated negatively with increase in ambient temperature when TI is induced by touching. Thus, the variation in TI in different species associated with environmental factors is worthy of further study.

Recent studies indicate that TI behaviour also depends on the sex and weight of an insect (Li et al., 2019; Konishi et al., 2020). For example, TI in *C. chinensis* stimulated by touching, is longer in heavy females than males (Hozumi & Miyatake, 2005), in *T. castaneum*, it is shorter in females than males (Miyatake et al., 2008b) and in *L. decemline-*

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ata, it is not dependent on sex (Acheampong & Mitchell, 2010). Given that the variation associated with sex and weight differ between species or individuals, it is necessary to consider the condition of insects when investigating and comparing the duration and frequency of TI under different environmental conditions.

An authentic-looking prey "death" might deceive a predator into believing that the prey is inedible and giving up. What is the difference between prey that exhibit TI and those that do not? Several studies report that physiological changes associated with TI are unique. During TI in rabbits, the respiratory rate is close to zero and heart rate decreases by 33% (Giannico et al., 2014). In *L. decemlineata*, the metabolic rate during TI is approximately 50% of the normal rate (Metspalu et al., 2002). This indicates that during TI energy consumption is lower and there is a tradeoff between metabolic rate and TI, but it is unclear how prevalent this relationship is among insects. We suppose that the differences in energy consumption and metabolic rate before and after TI might help us understand how it is regulated physiologically in insects and, therefore, more investigations are needed.

Eucryptorrhynchus scrobiculatus (Motschulsky, 1854) (Ji et al., 2017) and *Eucryptorrhynchus brandti* (Harold, 1880) (Coleoptera: Curculionidae) are the most destructive pests of the tree of heaven (*Ailanthus altissima*) and are highly host-specific (Herrick et al., 1938). Both exhibit TI behaviour when the metaventrite (the metathorax between the middle and hind legs) is touched (Li et al., 2019). Concerns about *E. scrobiculatus* (ESCR) and *E. brandti* (EBRA) are numerous and mainly about their bioecology, chemical ecology, prevention and control (Wen et al., 2018; Guo et al., 2019; Yang et al., 2019). Because the TI behaviour of ESCR and EBRA has not yet been described in terms of ecology and physiology we propose to do this for these two closely related species.

In this study, the extent to which the TI of ESCR and EBRA is regulated by touch, light, sound or temperature and the metabolic differences before and after TI were determined. The associations between weight and sex and the intensity of TI in terms of duration and frequency in different environments are discussed.

METHODS

Species and rearing conditions

This study was carried out from May to July 2018. Adults of the two species of weevil were collected in Haizi Village, Ningxia, China (106.34°N, 38.10°N) and reared at the Lingwu Forest Quarantine Station. Plots of pure stands of tree of heaven were sampled and average temperatures in the plots ranged from 15°C to 32°C and the photoperiod was 13L : 11D (Wen et al., 2017).

Weevils climb up tree trunks for feeding between 9:00 and 12:00 and descended in order to hide between 17:00 and 20:00 (Ji et al., 2018). Because both weevils are diurnal, the weevils were collected using forceps between 9:00 and 12:00 and experimented on between 12:01 and 20:00.

To prevent habituation to laboratory conditions, weevils were kept in the laboratory for no longer than 48 h before being tested. ESCR and EBRA were both sexed and the species and sexes kept separately in stock culture buckets (0.15 m in diameter and 0.10 m in depth) with fresh branches from the tree of heaven for 24 h. The weights of experimental weevils were 0.11–0.51 g for ESCR and 0.03–0.13 g for EBRA.

Laboratory conditions were maintained at 25°C and 60% relative humidity. Twelve hours before the test, each individual was transferred to a culture container (2 cm in diameter and 3.5 cm in depth) in which the trials (mechanical stimulation, light, sound and temperature) were carried out. Each individual was tested only once and then placed in a different culture box.

Experimental conditions and TI

Here, the TIs of ESCR and EBRA are described as the spontaneous behaviour in which antennae and proboscises are completely contracted and appendages are strongly folded and pressed close to the abdomen when these weevils are subjected to external stimulation (Fig. 1). To induce TI behaviour, forceps were used to touch the metaventrite (the metathorax between the middle and hind legs) (Fig. 2) (Li et al., 2019). If a weevil did not respond, the same stimulus was applied a second or third time; if there was no response after three stimuli, the duration of TI was zero. If they entered a TI state, its duration was recorded using a stopwatch until the first visible movement. If the duration was longer than 1 s (Matsumura et al., 2016), it was recorded as a TI, which ranged from 1–3,600 s.

Behavioural responses

Mechanical stimulation

This test focused on whether weight and sex affected TI induced by mechanical stimulation (touch). There were four groups of EBRA of different weights: [0.03–0.05 g (I), 0.06–0.08 g (II), 0.09–0.11 g (III) and 0.12–0.14 g (IV)]. Similarly, for ESCR: [0.11–0.20 g (A), 0.21–0.30 g (B), 0.31–0.40 g (C) and 0.41–0.50 g (D)]. Insects in all the weight categories (I, II, III and IV; A, B, C and D) and both sexes (male and female) were sampled at random and used to determine the duration and frequency of TI (ESCR females n = 100; ESCR males n = 100; EBRA females n = 100; EBRA males n = 100).

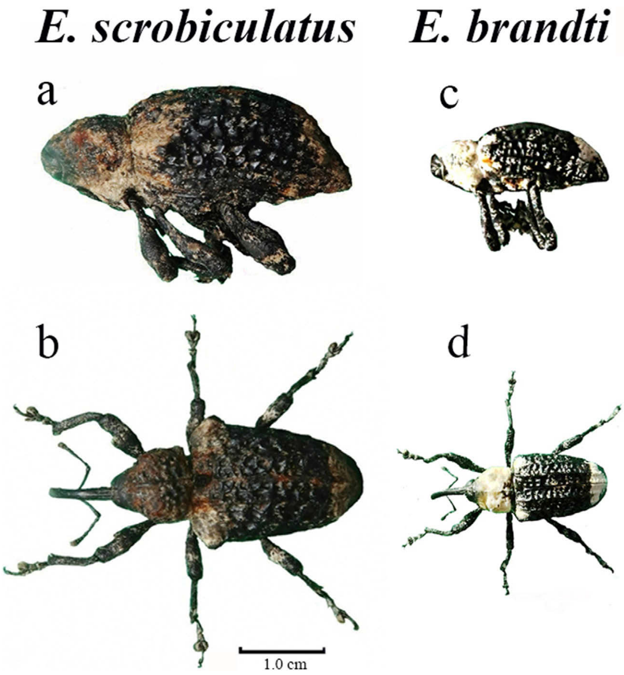

Fig. 1. Photographs of adult female *E. scrobiculatus* and *E. brandti* in the curled tonic immobility (TI) posture (a, c) and normal posture (b, d). Photo by Huijuan Li.

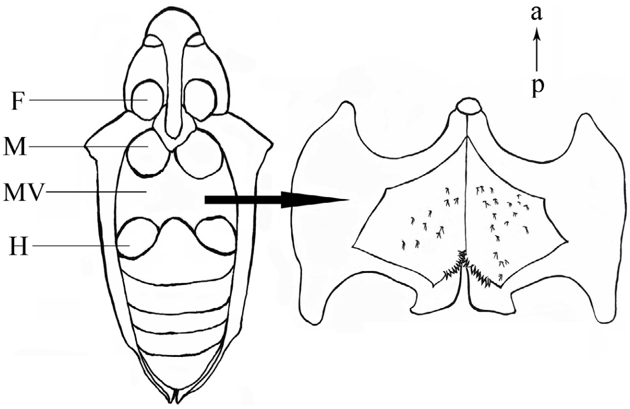

Fig. 2. Diagram of the ventral view of a male of *E. scrobiculatus*. Bold black arrow indicates a magnified view of the stimulus-sensing region. a – anterior; F – foreleg; H – hind leg; M – middle leg; MV – metaventrite (metathoracic venter between the middle and hind legs); p – posterior. Figure drawn by Huijuan Li.

Light conditions

To evaluate the effect of light on behaviour weevils were exposed to light for 6 h and its effect on TI was recorded (ESCR females n = 30; ESCR males n = 30; EBRA females n = 30; EBRA males n = 30). The light source was a pure white LED tube (4,000 lx, 3 W) and the control weevils were subjected to dark conditions for 6 h and then to red light (660 nm, 3 W) (ESCR females n = 30; ESCR males n = 30; EBRA females n = 30; EBRA males n = 30). Durations and frequencies of TI were recorded to determine the influence of light.

Response to simulated sounds

Similar to Michelsen (1992) and Lou (2013), we established four levels of sound: 2 kHz (Low), 6 kHz (Mid), 11 kHz (High) and a control group (CK, no sound) to which both species of weevils were exposed.

SineGen 2.5 software (http://www.huacolor.com) was used to create sine wave signals of the four sound levels. Synthetic sounds were produced by electricity vibrating through a loud speaker at a mean sound intensity level of 50 dB. All tests were carried out on a laboratory bench and the distance of the loud speaker from the culture box was 30 cm. A diagram of this device is shown in Fig. 3.

Weevils in a TI state induced by touching them with forceps were subjected to either 2 kHz, 6 kHz or 11 kHz sound for 1 min and the duration and frequency of TI recorded. The control group were not subjected to sound (ESCR females n = 30; ESCR males n = 30; EBRA females n = 30; EBRA males n = 30).

Temperature conditions

Considering the temperatures at which both species of weevil were active in Ningxia (Wen et al., 2017) the effects of 15°C, 25°C and 32°C were evaluated. The weevils used for the temperature experiments were transferred to constant-temperature incubators at 15°C, 25°C or 32°C for 24 h without feeding and then mechanically stimulated, and the frequency and duration of TI recorded. Five females and five males were included in each group and replicated five times (ESCR females n = 25; ESCR males n = 25; EBRA females n = 25; EBRA males n = 25).

Metabolism

To determine weevil metabolism the normal metabolic rates for both species (NMR, the carbon dioxide released by resting weevils) and their TI metabolic rates (TIMR) were recorded. Metabolic rate (MR) was measured using a closed flow respirometer (RES-1/RES-2; Sable Respirometry Systems, North Las Vegas, NV, USA) at 25°C ± 1°C. The incubators were controlled at a constant temperature and the 15 ml respiratory chamber was placed in one of the incubators. The carbon dioxide and moisture in the chamber was absorbed by calcium hydroxide and anhydrous calcium sulphate, respectively, and the gas flow used for the measurements was 100 ml/min. Before each experiment weevils were starved for 24 h, and then one weevil was placed in the chamber for 10 min to acclimatize and then it's NMR was measured for 15 min and replicated five times (ESCR females n = 5; ESCR males n = 5; EBRA females n = 5; EBRA males n = 5). After the NMR test, the weevils were first placed in the culture box to acclimatize for 30 min and then induced to adopt TI before placing them individually in the chamber and measuring their TIMRs. The MR formula is as follows:

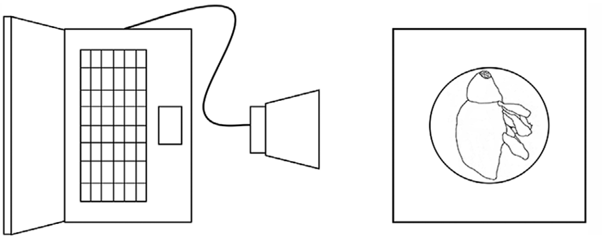

Fig. 3. Diagram of the device used to produce sound. Synthetic sounds were produced by a computer via a loud speaker (left of diagram) and TI weevils were placed on the laboratory bench close to the source of the sound (right of diagram).

$$MR = \frac{SVPa}{8.314 \times 10^{11} WT}$$

In the formula, S is the slope of the change in CO_2, V is the volume of the chamber (ml), Pa is the atmospheric pressure (kPa), W the weight of the adult tested (g), T the temperature in the chamber (K) and 8.314 is the model parameter.

Data analysis

To test for the effects of light, sound, temperature and MR on the duration of TI, the statistical significance was analysed using a single-factor analysis of variance (one-way ANOVA) followed by Tukey's HSD multiple comparison tests ($P < 0.05$) (R-i386 3.3.2). To test for the effects of mechanical stimulation on the duration of TI, the sex and body weight were considered to be variables of the weevils and a General Linear Model was constructed (SPSS Statistics v. 17.0). Assumptions about the normality and homogeneity of variances were verified, and all results in the text are mean values and standard errors (mean ± SE).

RESULTS

Mechanical stimulation

The duration and frequency of TI induced by mechanical stimulation were associated with weight (EBRA: n = 25, $P = 0.789$; ESCR: n = 25, $P = 0.697$) and sex (EBRA: n = 25, $P = 0.196$; ESCR: n = 25, $P = 0.782$) (Table 1, Fig. 4). When considering the interactions between weight and sex, no significant differences were detected (EBRA: n = 100, $P = 0.180$; ESCR: n = 100, $P = 0.139$). However, TI in ESCR was longer (163.62 ± 14.43 s) than in EBRA (123.88 ± 15.26 s) (n = 200, $P = 0.040$).

Influence of light

As shown in Fig. 5-1, the duration of TI in ESCR males was longer than that of females when exposed to light (n

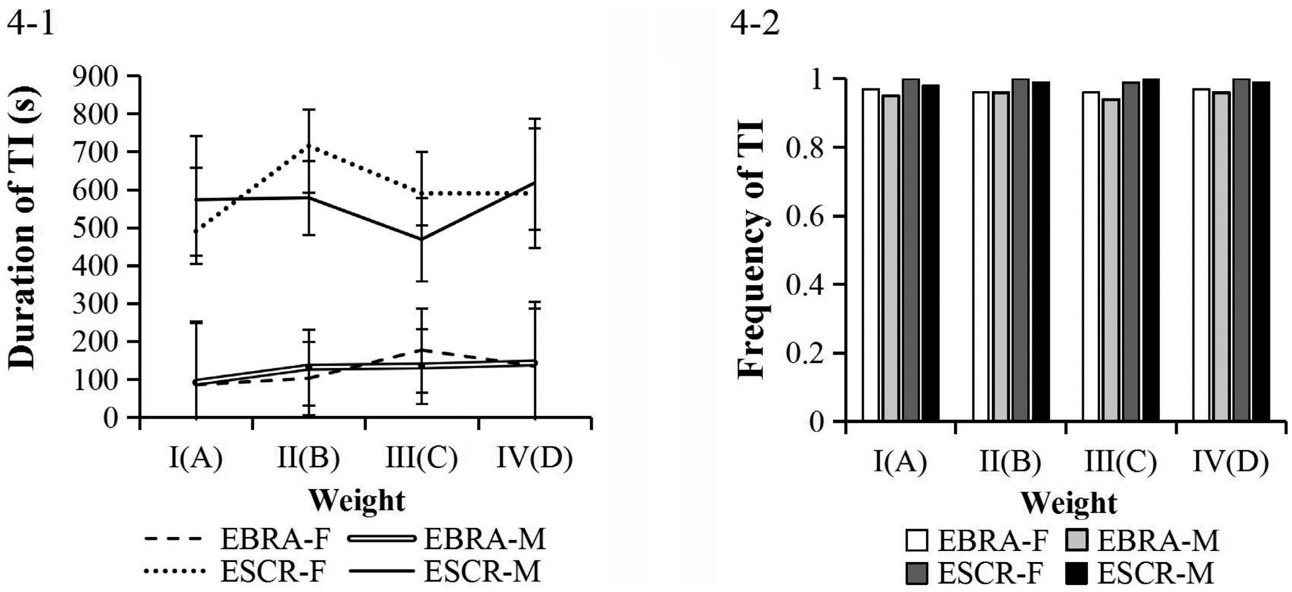

Fig. 4. Duration (4-1) and frequency (4-2) of TI of different weights (I, II, III and IV) for EBRA and (A, B, C and D) for ESCR when stimulated mechanically (touched). ESCR – *Eucryptorrhynchus scrobiculatus*; EBRA – *Eucryptorrhynchus brandti*; M – male; F – female. Error bars – standard errors (SE).

= 30, $P < 0.001$; n = 30, $P < 0.001$). However, we did not detect similar differences in EBRA (n = 30, $P = 0.400$; n = 30, $P = 0.400$). This indicated that only the duration of TI in ESCR was influenced by light. No differences were recorded in the frequency of TI in EBRA and ESCR (Fig. 5-2).

Influence of sound

The trends in variation in the TI of ESCR males and females in the different sound conditions were very similar (Fig. 5-3). Tukey's HSD indicated that low-frequency sound (2-kHz) reduced the duration of TI in ESCR males and females (n = 30, $P = 0.010$; n = 30, $P = 0.020$). At high frequencies, the duration of TI in EBRA females was longer than that recorded for males (n = 30, $P = 0.040$; n = 30, $P = 0.010$). Low- and mid-frequency sounds reduced the duration of TI in EBRA males (n = 30, $P = 0.030$; n = 30, $P = 0.040$), but not significantly so in females. No differences were recorded in frequency of TI (Fig. 5-4).

Influence of temperature

Temperature affected ESCR and EBRA differently, and there was no significant difference between the sexes in the duration of TI (Fig. 5-5). The duration of TI in EBRA at the low temperature (15°C) was significantly longer than at the optimal (25°C) and highest temperature (32°C) (n = 25, $P = 0.020$; n = 25, $P = 0.030$). However, the duration of TI in ESCR was shorter at 15°C and 32°C than at 25°C (n = 25, $P = 0.040$; n = 25, $P = 0.040$). No differences were recorded in the frequency of TI (Fig. 5-6).

Table 1. Statistics of the effects of weight and sex on the duration of TI in *E. scrobiculatus* and *E. brandti* when stimulated mechanically.

Source	*E. brandti*		*E. scrobiculatus*	
	F-statistic	*p*-value	F-statistic	*p*-value
Weight	0.359	0.789	0.520	0.697
Sex	2.744	0.196	0.091	0.782
Weight * Sex	1.648	0.180	1.851	0.139

General linear models were used to analyse TI behaviour. Asterisks indicate an interaction between variables.

Metabolism

There were significant associations between MR and TI (Fig. 6). During TI the MR of ESCR declined significantly to 76.90% of its normal MR and in EBRA to 71.40%. There were significant differences between the sexes in terms of TIMR in EBRA, with that of females ($6.04 \cdot 10^{-9} \pm 4.15 \cdot 10^{-10}$ mol^{-1}g^{-1}s) significantly lower than that of males ($8.891 \cdot 10^{-9} \pm 8.54 \cdot 10^{-10}$ mol^{-1}g^{-1}s) (n = 5, $P = 0.040$). No significant relationships were recorded between sex and TIMR in ESCR. In addition, EBRA had a higher MR than ESCR during TI and in the normal state (n = 10, $P < 0.001$; n = 10, $P < 0.001$).

DISCUSSION

The results define the TI traits of two species of *Eucryptorrhynchus* (ESCR and EBRA). First, these two related species have the same stimulus-sensing region (the metaventrite), which is sensitive to touch and their TI behaviour was affected by light, sound and temperature. Second, our first investigation of their behaviour and metabolism revealed that only the duration of TI varied greatly between species and individuals and, therefore, it was a more important index for evaluating TI behaviour under different environmental conditions. When comparing these results with those of previous studies on TI in *T. castaneum*, *C. formicarius* and other species (Konishi et al., 2020; Miyatake 2001), this investigation of the TI of ESCR and EBRA had referential value.

Because TI in ESCR and EBRA was induced by touching the metaventrite, we designed experiments to determine

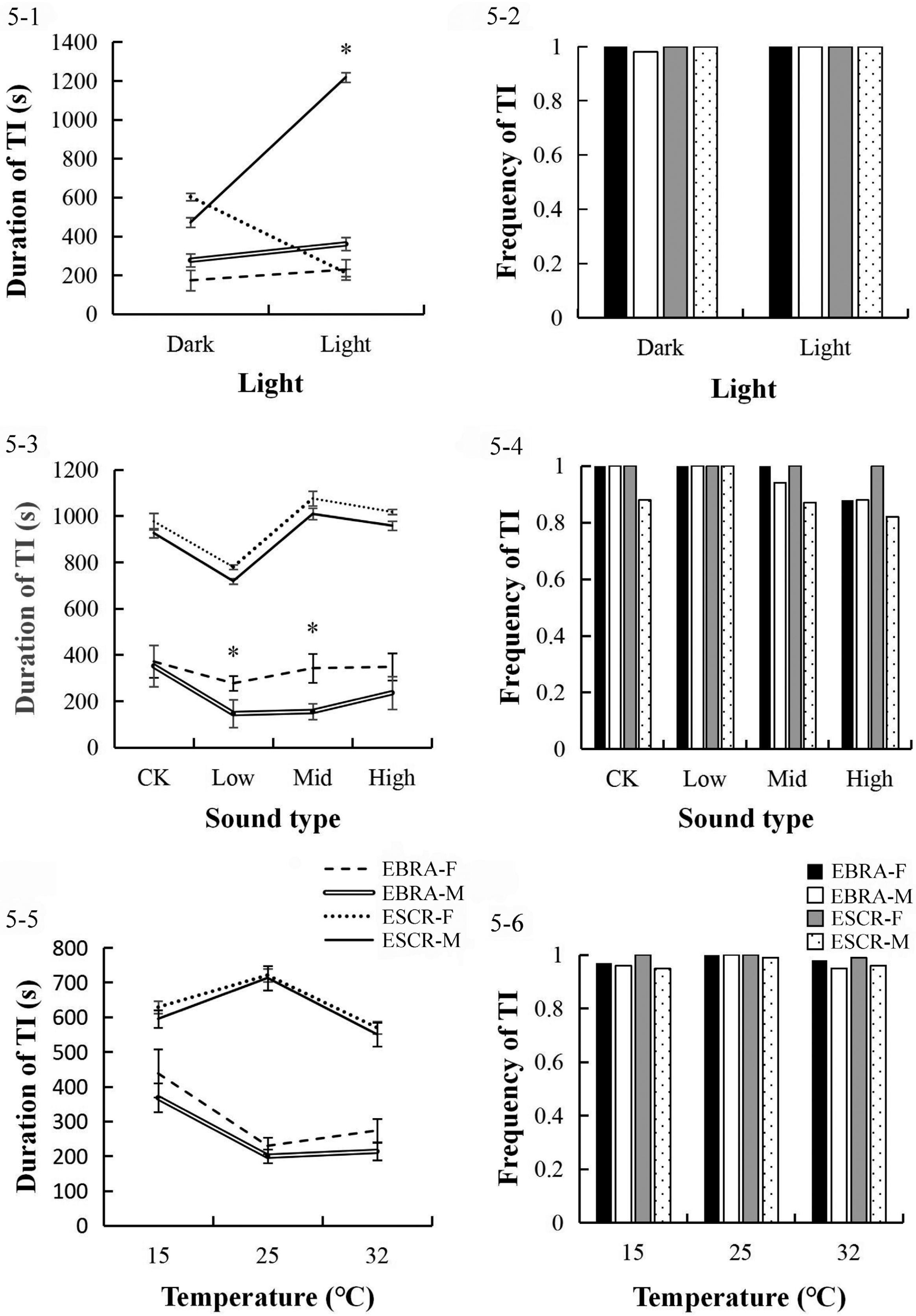

Fig. 5. Duration and frequency of TI recorded in the dark and light, different levels of sound and different temperatures. ESCR – *Eucryptorrhynchus scrobiculatus*; EBRA – *Eucryptorrhynchus brandti*; M – male; F – female. 5-1: duration of tonic immobility (TI) in light and dark conditions. Asterisks indicate significant differences for ESCR-F and ESCR-M. 5-2: frequency of TI under light and dark conditions. 5-3: duration of TI at different levels of sound. Asterisks indicate significant differences for EBRA-F and EBRA-M. 5-4: frequency of TI at different levels of sound. 5-5: duration of TI at different temperatures. 5-6: frequency of TI at different temperatures. Error bars – standard errors (SE). * $P < 0.05$.

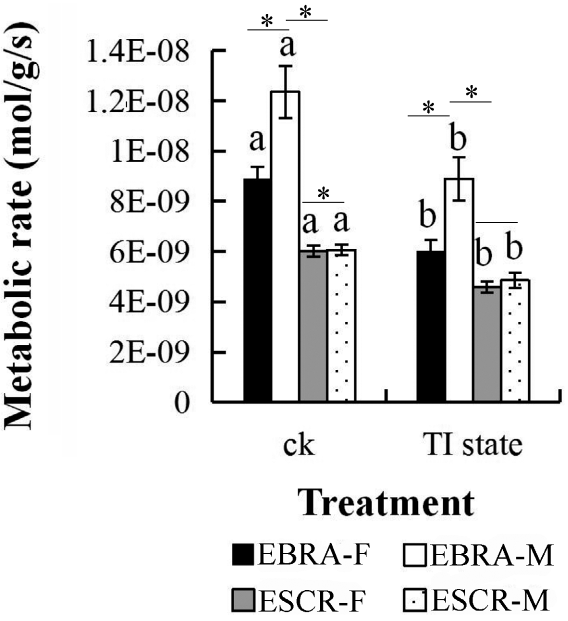

Fig. 6. Normal (CK) and tonic immobility (TI) metabolic rates recorded for ESCR and EBRA. ESCR – *Eucryptorrhynchus scrobiculatus*; EBRA – *Eucryptorrhynchus brandti*; M – male; F – female. Error bars – standard errors (SE). * P < 0.05.

whether body weight and sex affected TI, which is the case for *C. chinensis*, *C. maculatus* and *T. freemani* (Hozumi & Miyatake, 2005; Miyatake et al., 2008a; Konishi et al., 2020). In different weight groups and sexes the duration of TI in ESCR and EBRA in response to mechanical stimulation did not differ significantly. However, our results differed from those for *C. chinensis* and *C. maculatus*, which indicate that body size is significantly associated with the duration of TI and that it is significantly longer in females than in males (Hozumi & Miyatake, 2005). In some beetles, the intensity of death-feigning varies between species, and even within species, which confirms that death-feigning is more complex. Matsumura et al. (2016) and Miyatake et al. (2019) conclude that this difference in "depth of death feigning" is genetically determined. Our results indicate that a comprehensive understanding of the intensity of TI in ESCR and EBRA is worth further study.

Because previous studies report that light affects TI (Metspalu et al., 2002), we carried out experiments to determine whether light lengthens the duration of TI. Surprisingly, our results revealed that the duration of TI in ESCR males was significantly longer in light, while that of females was significantly decreased, but neither sex of EBRA responded to light. However, the duration of TI of nocturnal *C. formicarius* in light is longer, possibly because there is a high risk of daytime predation (Miyatake, 2001; Kuriwada et al., 2009). We suspect that the diurnal rhythm might play an important role in controlling the duration of TI. In addition, light stimuli might affect vision, the nervous system, or circadian rhythms and thereby act as a signal controlling the lengthening or shortening of the duration of TI (Wen et al., 2018). For example, the duration of TI in *Cathaica fasciola* increases as light intensity increases (Zhang et al., 2009). A possible explanation of the different effects of light on the two diurnal weevils (ESCR and EBRA) studied is that the diurnal rhythm and physiological differences between species modulate the light stimuli.

In most animals, auditory cues control the duration of TI (Humphreys & Ruxton, 2018). For instance, the duration of TI in domestic chickens (*G. gallus*) increased when exposed to specific alarm calls of 1 to 4 kHz (Pochron & Thompson, 2019). Our data revealed that low-frequency sound (2-kHz) significantly reduced the duration of TI in ESCR males and females, and in EBRA males. Only EBRA females were unaffected by sound. Because the hearing systems of prey alerts them to the presence of a predator, the interference of a response to sound was not considered in this study; the results of this study do not support the theory that sound is a danger signal that induces TI behaviour instead of warning prey to run away.

Optimal temperature is essential for survival, as it is involved in regulating physiological metabolism, energy trade-offs and behavioural patterns in insects (Zhao & Chen, 1980). When exposed to a low temperature (15°C) duration of TI in EBRA was more likely to be longer, which is similar to *C. fasciola*, *C. chinensis* and *C. maculatus* (Miyatake et al., 2008a; Zhang et al., 2009), in which low temperatures increase the duration of TI. However, in ESCR the duration TI at low (15°C) and high (32°C) temperatures was shorter, similar to previous results for mantises (Holmes, 1906). Our results indicate that variations in the duration of TI recorded in different temperature conditions might reflect differences in physiology and further study is needed to reveal the relationship between temperature and the physiology of TI.

Because metabolism is a component of environmental adaptation (Xu & An, 2002; Casas et al., 2015), TI intensity might be associated with metabolism (Brooks et al., 2011; Krams et al., 2014). Our results indicate that the MR of EBRA was greater than that of ESCR and TIMR declined to 76.90% in ESCR and 71.40% in EBRA. Similarly, in *L. decemlineata* the normal metabolic rate is about twice as high as during TI (Metspalu et al., 2002). This indicates that TI is not only a defence against predators, but that the low-energy consumption is physiologically determined.

Although the mechanisms regulating TI in insects are unknown, there is evidence that the sympathetic nervous system, parasympathetic nervous system (Klemm, 1977; Alboni et al., 2008; Giannico et al., 2014), or femoral chordotonal organs (Nishino & Sakai, 1996) may be involved. The brain is active during TI as the insect actively monitors the external environment (Alboni et al., 2008). For example, central nervous system processing remains intact during TI in chickens (Gentle et al., 1989). In addition, based on optogenetic and electrophysiological studies, the subcortical pathway in the SC-pulvinar-amygdala mediates TI mechanisms in mice (Wei et al., 2015; Munch et al., 2020). Importantly, the expression of dopamine-related genes in the tyrosine metabolism pathway of *T. castaneum* differ in populations selected for long and short duration of TI (Uchiyama et al., 2019; Konishi et al., 2020). Our study also provides experimental and theoretical evidence supporting the need for further studies on the TI mechanism.

In conclusion, the TI behaviour of ESCR and EBRA in which low-energy consumption was regulated by light, sound and temperature, indicate that these two weevils are ideal research models for further studies on the ecological traits and physiological regulation of TI in all species.

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