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SHORT COMMUNICATION

Aggregation pheromone interrupts death feigning in the red flour beetle *Tribolium castaneum*

Motoya Ishikawa[1] · **Kentarou Matsumura**[1] · **Takahisa Miyatake**[1]

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Abstract
Death feigning is a behavior in which a prey is rendered motionless due to stimulation or threat by a predator. This anti-predator defense mechanism has been observed across a wide range of animal taxa and is considered adaptive. However, long durations of death feigning can decrease opportunities for feeding and reproduction, and therefore is a fitness cost as compared to environments without predators. Because death feigning is thought to be affected by the balance between survival and other fitness costs, selection pressure may drive individuals who are capable of plastic changes in the intensity of death feigning. Pheromones, which are important semiochemicals that affect foraging and reproductive success, may be one of the factors influencing the intensity of death-feigning behavior. In this study, we investigated the effect of an aggregation pheromone on the death-feigning behavior of the red flour beetle *Tribolium castaneum*. We found that beetles exposed to the pheromone showed a significantly shorter duration of death feigning than beetles that were not exposed to the pheromone. Therefore, our results suggest that an aggregation pheromone can plasticly alter the death-feigning behavior in *T. castaneum*.

Keywords Anti-predator strategies · Death feigning · Aggregation pheromone · Sexual selection · *Tribolium castaneum*

Introduction

Predation is one of the key selection pressures that affect the evolution of anti-predator strategies (Lima 1998; Lima and Dill 1990; Sih 1992). Behaviors characterized by immobility, such as death feigning or thanatosis, which as a response to an external stimulus, are widely recognized as anti-predator defense mechanisms across a wide range of animal taxa (e.g., Miyatake et al. 2004; Humphreys and Ruxton 2018; Ruxton et al. 2018; Sakai 2021). The act of death feigning can lead to the loss of visibility or interest by predator, thereby reducing the risk of additional attacks by the predator. Indeed, empirical studies indicate that individuals with longer durations of death feigning exhibit higher survival rates when they encounters predators (e.g., Miyatake et al. 2004, 2009; Konishi et al. 2020). Consequently, selection pressure may drive a prey towards longer death-feigning durations in environments with higher predation pressure.

Nevertheless, lengthy and extended death-feigning durations may also increase fitness costs. For example, individuals with longer durations of death feigning exhibit lower tolerance to physical stress (Kiyotake et al. 2014). Moreover, individuals with longer death feigning remain inactive for longer periods of time, which adversely affects the number of encounters they can have with potential mates (Nakayama and Miyatake 2010a, b). It is predicted that the duration of death feigning is regulated by the balance between various selection pressures, and individuals capable of plastic changes in the intensity of any factors of death feigning may be favored by selection pressure. Several previous studies reported factors that affect the death-feigning duration in the *Tribolium* flour beetles. For example, previous studies revealed that immobile beetles awaken from death feigning when subjected to certain vibrational stimuli (Miyatake et al. 2019; Ishihara et al. 2021). Thus, death feigning may be plastically altered by multiple factors. One candidate for the factor that could plastically change death-feigning duration could be pheromones. It may be possible to forage efficiently by sensing aggregation pheromones released by individuals in a feeding area, or to encounter breeding partners efficiently by flocking in large numbers, regardless of sex (Parrish and Edelstein-Keshet 1999). Therefore, because

✉ Takahisa Miyatake
miyatake@okayama-u.ac.jp

[1] Graduate School of Environmental, Life, Natural Science and Technology, Okayama University, Okayama, Japan

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pheromones are important semiochemicals in foraging and reproduction (Carde and Minks 2012), individuals that perceive pheromones may alter their death-feigning duration briefly to forage or reproduce. Indeed, it has been suggested that pheromones may be a factor in shortening the death-feigning duration of the sweet potato weevil *Cylas formicarius* (Miyatake 2001a, b). However, to our knowledge, no study has investigated the effects of pheromones on death-feigning behavior.

Here, we examined the effects of pheromone on the death-feigning behavior of the red flour beetle *Tribolium castaneum*. *Tribolium castaneum* males releases an aggregation pheromone (4,8-dimethyldecanal: DMD), that attracts both sexes (Suzuki 1980). Because responding to this pheromone leads to successful foraging and mating, sensing DMD has a significant effect on fitness (Fedina and Lewis 2008). A previous study conducted artificial selection for death-feigning duration in *T. castaneum* (Miyatake et al. 2004), and populations with a genetically long duration of death feigning (L-population) have been established (Miyatake et al. 2004). In this study, we examined the effects of DMD on the death-feigning duration of *T. castaneum* beetles from L-populations.

Materials and methods

Insect

Red flour beetles (*T. castaneum*), the model prey in the present study, were reared in an incubator maintained under 25 °C and a 16L:8D (lights on at 7:00, lights off at 23:00) light cycle with food (whole meal flour:beer yeast at a ratio of 16:1). We have artificially selected these beetles for duration of death feigning for over 40 generations (Matsumura and Miyatake 2018; Miyatake et al. 2004). A total of 100 males and 100 females were randomly collected from the stock population, and their death-feigning duration was assessed using artificial stimuli. We selected 10 males and 10 females with the longest durations of death feigning and placed them in a plastic container to propagate as a population with a longer duration of death feigning (L). Two replicate lines for the L-population were prepared (i.e., L1 and L2). This procedure was repeated over 40 generations. Details of this method and the direct response results are described in Miyatake et al. (2004) and Matsumura and Miyatake (2018).

Experimental design

Experiments were conducted in a petri dish (90 mm in diameter and 15 mm high). The pheromone lures (20 mm in diameter and 5 mm in height) for the commercial Torios traps (Fuji Flavor Co., Ltd., Tokyo, Japan, see https://www.fjf.co.jp/en/ecomone/product/torios.html) were used as the source of DMD for *T. castaneum*. We randomly collected virgin males and females of *T. castaneum* (21–35 days old) from L-populations (L1 male: $n=20$, L1 female: $n=20$, L2 male: $n=20$, L2 female: $n=20$). First, the death-feigning duration was measured in an environment without a pheromone lure. The next day, the death-feigning duration was measured again in the presence of pheromone lure (4,8-dimethyldecanal). We also treated the same sample size of beetles with the opposite experimental schedule (with pheromone on the first day and without pheromone on the second day).

In the presence of the pheromone, death-feigning behavior was induced by placing the pheromone in the center of a petri dish and stimulating the abdomen of a beetle placed 1 cm away from the pheromone. A transparent plastic container was placed over it to prevent dispersion of the pheromone, and observations were made. The same treatment was applied to the beetles in the control without pheromone. The time taken for the beetles to awaken from death feigning was measured as the death-feigning duration.

After each experiment was completed, the entire laboratory was ventilated so as not to affect subsequent observation experiments. In addition, the containers used in the experiments were rinsed and dried after each experiment. All experiments were conducted between 12:00 and 18:00 in a laboratory maintained at 25 °C.

Statistical analysis

Death-feigning duration was analyzed by a generalized linear mixed model (GLMM) with gamma distribution, treatment, sex, order, and interaction among these factors as explanatory variables, replicate line of the selection regime, and ID (i.e., individual identification number), and order as random effects. All analyses were conducted in R ver.4.1.0 (Core 2017) and using the statistical packages *lme4* (Bates et al. 2015) and *car* (Fox and Weisberg 2018).

Results

Figure 1 shows death-feigning duration in absence (mean ± s.e. = 2899.60 ± 288.41 in male; 2144.03 ± 287.11 in female) and presence (mean ± s.e. = 2154.83 ± 279.66 in male; 1558.65 ± 201.89 in female) treatments, respectively. Table 1 shows the result of GLMM for the effect of pheromone treatment on the death-feigning duration. We found that beetles in the presence of the pheromone showed significantly shorter duration of death feigning than beetles without the pheromone (Fig. 1, Table 1). Significant effects were not found for sex or interaction between treatment and sex (Table 1).

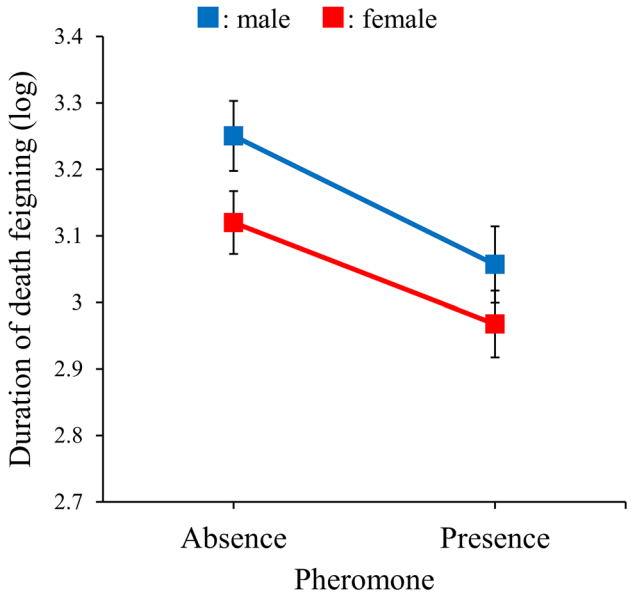

Fig. 1 Duration of death-feigning behavior in *T. castaneum* in the presence or absence of DMD, an aggregation pheromone for this beetle. Blue and red symbols showed male and female data, respectively. Error bars showed standard error

Table 1 Results of GLMM to test the effect of the aggregation pheromone on the duration of death feigning

Factors	Estimate	*d.f*	χ^2	*p*
Treatment	−0.331	1	37.61	< 0.0001
Sex	0.295	1	1.45	0.1176
Treatment × sex	−0.099	1	0.64	0.4236

Factor explanatory variable, *Estimate* parameter estimate, *d.f.* degree of freedom, χ^2 χ^2 value, *p* *p* value

Discussion

This study found that *T. castaneum* exposed to DMD exhibited significantly shorter death-feigning duration than beetles not exposed to DMD. The aggregation pheromone is an important semiochemical that affects foraging and reproductive success (Carde & Minks 2012). Indeed, adults of *T. castaneum* are strongly attracted to DMD in both sexes upon sensing it, and males that release more DMD increase mating success (Fedina & Lewis 2008). It is possible that the beetles switch their behavior to foraging and reproductive activities by sensing this signal, shortening their long death feigning. This result suggests that *T. castaneum* is able to alter its death-feigning duration when it detects an aggregation pheromone. This is the first study to demonstrate that pheromone presentation alters death-feigning behavior, and therefore this an important finding in predator avoidance behavior.

While there is much knowledge on the factors that induce death-feigning behavior, there is a lack of information on the factors that awaken the predator from death-feigning behavior. Since continuing the death feigning when the predator has lost interest in the prey is waste of time, the prey may be awakened from the death feigning by a signal. Previous studies have shown that the death feigning is ended by vibrational stimuli (Ishihara et al. 2021; Miyatake et al. 2019), and the present study suggests that aggregation pheromones are one of the factors. One adaptive significance of using aggregation pheromones as an investigative cue to terminate death feigning might be that a situation, in which death-feigning individuals can detect aggregation pheromones, can be considered a situation in which they are not dangerous to their sender. Also, if some individuals do not respond to aggregation pheromones and continue death feigning, they may not be aware of information that could be advantageous in their survival strategies, such as foraging or the presence of the opposite sex. Thus, individuals that awaken in response to aggregation pheromones will increase their chances of survival.

In our results, males showed a trend of longer death-feigning duration than females regardless of the pheromone treatment, but the difference was not statistically significant. Because *T. castaneum* larvae often cannibalize eggs (Park et al. 1965, 1974), the females lay eggs while actively migrating and dispersing (Campbell and Hagstrum 2002; Ziegler 1976). It has also been shown that the dispersal activity of *T. castaneum* is higher in females than in males, even in virgin individuals (Matsumura and Miyatake 2015). Therefore, although the females used in this study were virgins, it is possible that they may have allocated more time to dispersal by shortening their death-feigning duration (Matsumura et al. 2016). On the other hand, there was no interaction between pheromone treatment and sex, suggesting that there is no difference between the sexes in the effect of the pheromone on death-feigning behavior in *T. castaneum*.

In conclusion, although death-feigning behavior is a common predator avoidance behavior observed across a wide range of animal taxa, there are few studies focused on its arousal factors. The present study revealed that sensing the pheromone (4,8-dimethyldecanal) by *T. castaneum* shortens the death-feigning duration. It is worth discussing what cues aggregation pheromones may provide to death-feigning individuals in terms of predation risk. For example, a situation in which a death-feigning individual is able to detect aggregation pheromones can be considered a situation in which it is not dangerous to its senders (i.e., other individuals in the vicinity of the death-feigning individual). Therefore, it appears to be adaptive to terminate death feigning in this situation. In addition, individuals that awaken from

feigning death in response to aggregation pheromones when environmental factors change or new natural enemies appear will have a survival advantage over those that continue to feign death. Furthermore, the effects of other pheromones on predation avoidance behavior or on mate searching (i.e., sex pheromone) should be examined and in other species to further our understanding of the relationships between predation avoidance or mating behaviors and pheromones.

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Data availability Data in this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors have no conflict of interest and competing interest for this study.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Informed consent This article deal with insects and, thus, we did not need Informed consent.

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