



Relationship Between the Duration of Death-Feigning and Temperature in Black and Red Elytral Types of a Polymorphic Ladybug, *Harmonia axyridis*

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Abstract Since predation is a strong selection pressure on prey, the later have evolved a variety of anti-predator behavior. Death-feigning is thought as one of anti-predator behaviors to escape from predation by temporarily stopping movement and appearing to be dead. Previous studies have shown that the duration and frequency of death-feigning varies with temperature. Body color may also affect those parameters through thermal adaptation, as body color would have a significant relationship with temperature adaptation. A ladybug, *Harmonia axyridis*, shows a polymorphism in elytral color, which is controlled by 12 alleles on a single locus. The elytral color can be divided into 1) red with black spots (Red type; R) and 2) black with red spots (Black type; B). Here, we show that the duration of death-feigning in B was correlated positively with temperature whereas that in R was correlated negatively with temperature. The evolutionary reasons for this difference require further study in terms of the trade-off between the opportunity cost associated with death-feigning and the benefit of the duration of death-feigning.

Keywords Death-feigning · Anti-predator behavior · Thermal adaptation · Polymorphism · Coccinellidae

Introduction

Prey have evolved a variety of strategies to escape predation. Death-feigning is a behavior in which an animal stops moving in response to external stimuli and has been considered as an anti-predator behavior (Humphreys and Ruxton 2018). Death-feigning is observed in a wide range of animals, from invertebrates to vertebrates (Francq 1969; Burghardt and Greene 1988; Cassill et al. 2008). Previous studies have shown that the adaptive significance of death-feigning is to reduce predation rates by distracting predators (Miyatake et al. 2004), but there is another hypothesis that it is a chemical warning of distastefulness (Ruxton 2006).

The frequency of death-feigning or its duration has been found to be influenced by several factors such as starvation, sex, and size (Miyatake 2001a, b; Hozumi and Miyatake 2005; Gerald 2008). Temperature has been considered to affect death-feigning because behaviors and physiological processes of ectotherms, including insects are strongly influenced by environmental temperature (Angilletta et al. 2006). In two weevils, *Callosobruchus maculatus* and *C. chinensis*, the duration of death-feigning was correlated negatively with temperature

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(Miyatake et al. 2008a). In insects, muscle activity is reduced when body temperature is low (Schou et al. 2013). Thus, at low temperatures, death-feigning is considered to be a better strategy to escape from predators than walking or flying, which explains the negative correlation between the intensity of death-feigning and temperature (Humphreys and Ruxton 2018). However, the weevil, *Zabrotes subfasciatus* showed a positive correlation between the frequency of death-feigning and temperature (Cardoso and dos Santos Mendonça 2019).

An example of thermal adaptation in insects is melanism, the darkening of body color (Clusella Trullas et al. 2007). It has been predicted that darker phenotypes are advantageous under low temperature because they can absorb solar energy compared to light individuals, enabling them to raise their body temperatures faster than light ones (Kuyucu et al. 2018). The above hypothesis has been supported by studies using a variety of animals (Brakefield and Willmer 1985; Goulson 1994; Forsman 1997). However, this advantage may turn into a disadvantage under high temperature where there is a risk of overheating (Clusella Trullas et al. 2007).

Elytral color in *Harmonia axyridis* is controlled by 12 alleles on a locus (Tan 1946; Gautier et al. 2018). There is a phenotypic plasticity in the size and number of black spots expressed in the red elytral type (Red type; R), which increases under lower temperatures during larval and pupal stages (Michie et al. 2010). It is not clear why *H. axyridis* exhibits such an extreme color polymorphism, but it has been suggested that thermoregulation is related to body color of *H. axyridis* (Komai 1956; Osawa and Nishida 1992; Wang et al. 2009; Noriyuki and Osawa 2015). In another ladybug, *Adalia*

bipunctata, which shows a color polymorphism similar to *H. axyridis*, the black elytral color has been considered to be an adaptation to low temperatures (Brakefield 1984; Brakefield and Willmer 1985; De Jong et al. 1996).

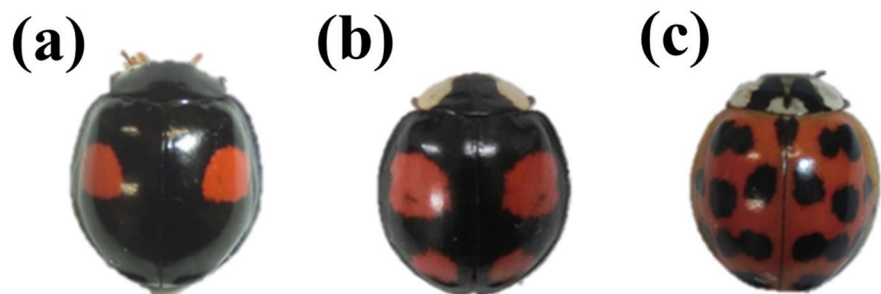
In this study, we examined the relationship between temperatures and the duration of death-feigning in *H. axyridis*. We measured the duration of death-feigning and frequency of R and B at 15 °C, 20 °C and 25 °C. Based on the results, we discuss the possibility that the observed difference is an adaptive strategy to temperature in each color morph.

Materials and Methods

Materials

H. axyridis adults were collected from an overwintering population in Shikaoui, Hokkaido, Japan on May 21, 2020 and in Higashikawa, Hokkaido, Japan on November, 2021. We classified them by elytral color and the number of spots based on Tan (1946). We used three phenotypes of *succinea* (R; with 0–19 black spots), *conspicua* (B2; with 2 red spots) and *spectabilis* (B4; with 4 red spots) for the following experiments (Fig. 1). These alleles have been observed commonly in wild populations (Michie et al. 2010). They have a dominant-recessive relationship in the order *conspicua* > *spectabilis* > *succinea* (Tan 1946). All individuals were kept together in a Petri dish (diameter: 70 mm, height: 20 mm) at 20 °C and insects were fed with an artificial food for turtles (Leptomin®, Tetra, Melle, Germany) every 2–3 days.

Fig. 1 The three common color phenotypes in *Harmonia axyridis*; (a) B2, (b) B4, (c) R



Experiment

Several days before the measurement of the intensity of death-feigning, the individuals were placed in the Petri dishes (diameter: 70 mm, height: 20 mm) and maintained at different temperatures of 15 °C, 20 °C or 25 °C. The sex of each individual was also recorded. We defined death-feigning as an immobile state with legs folded to the ventral surface of abdomen. Death-feigning was induced by turning over each individual on a Petri dish (the same product with the above dish for rearing). The duration of death-feigning was the time in sec between the start of death-feigning and the first visible movement. If an individual did not stop moving when turned over, the same experimental procedure above was repeated three times and if the focal individual did not stop moving in all the three trials, the duration was recorded as 0 s. The upper limit of the duration of death-feigning was defined at 300 s (durations longer than 300 s were recorded as 300 s). We measured the duration of death-feigning for 60 individuals (= ten individuals of each sex for three phenotypes) for each temperature, but we used 8 males and 12 females of R at 25 °C. All the individuals were used only once in this experiment.

Statistical Analysis

All statistical analyses were performed in R (ver. 4.0.0). The duration of death-feigning was compared by Mann-Whitney U test for ties correction because most of the distributions deviated from normality (confirmed using Shapiro-Wilk test). Correlation between temperature and the duration of death-feigning of each color type was examined by Kendall’s rank correlation test. For analysis of the effect of temperature on the frequency of death-feigning, we used

a generalized linear model (GLM) with binomial distribution and logit link function because the dependent variable was binomial. We set temperature as the independent variable and a dummy variable (1: did show the death-feigning, 0: did not show the death-feigning) as the dependent variable. We used three-way ANOVA (analysis of variance) for the data of the individuals that showed the death-feigning to test the effects of type (B or R), temperature, and sex on the duration of death-feigning.

Results

There was no significant difference between B2 and B4 at all temperatures, so we treated them as one type, B (Mann-Whitney U test; 15 °C: $W=151, n=40, P=0.184$; 20 °C: $W=241, n=40, P=0.252$; 25 °C: $W=161, n=40, P=0.295$). At 15 °C, the duration of death-feigning of R was significantly longer than that of B (B: 26.1 ± 6.94 s (mean \pm SE), R: 94.5 ± 23.2 s; Mann-Whitney U test: $W=562, n=60, P=0.0095$; Table 1); at 20 °C, there was no difference in the duration of death-feigning between B and R (B: 105.0 ± 20.9 s, R: 71.7 ± 21.2 s; Mann-Whitney U test: $W=379, n=60, P=0.741$; Table 1); at 25 °C, the duration of death-feigning of B was significantly longer than that of R (B: 107.1 ± 17.5 s, R: 43.2 ± 16.7 s; Mann-Whitney U test: $W=241, n=60, P=0.0110$; Table 1).

There is a correlation between the duration of the death-feigning and temperature in both B and R (B: $n=120, \tau=0.211, P=0.0044$; R: $n=60, \tau=-0.207, P=0.0488$). We detected no significant effect of temperature on the frequency of death-feigning in both B and R (GLM; B: estimate = 0.0591, $z=1.205, n=120, P=0.228$; R: estimate = -0.116, $z=-1.651, n=60, P=0.0987$).

Table 1 Frequency and duration of death-feigning of B and R at three temperatures

| Temperature | Freq. of death-feigned individuals | | | Duration of death-feigning (mean \pm SE sec) | | | |
|-------------|------------------------------------|------|----------|--|-----------------|----------|-----------|
| | B | R | <i>P</i> | B | R | <i>W</i> | <i>P</i> |
| 15 °C | 0.675 | 0.8 | 0.375 | 26.1 ± 6.94 | 94.5 ± 23.2 | 562 | 0.00954** |
| 20 °C | 0.6 | 0.65 | 0.783 | 105.0 ± 20.9 | 71.7 ± 21.2 | 379 | 0.741 |
| 25 °C | 0.8 | 0.55 | 0.0677 | 107.1 ± 17.5 | 43.2 ± 16.7 | 241 | 0.0110* |

The duration of death-feigning between B and R in each temperature was compared by Mann-Whitney U test and the frequency was compared by Fisher’s exact test. The statistics and *P* value are shown. *, $P < 0.05$, **, $P < 0.01$

Table 2 Results of ANOVA for the duration of death-feigning ($n=123$)

| Factor | df | Sum Sq | <i>F</i> | <i>P</i> |
|----------------------|-----|-----------|----------|----------|
| Type | 1 | 68,439 | 6.15 | 0.0146* |
| Temperature | 1 | 14,715 | 1.32 | 0.253 |
| Sex | 1 | 2630 | 0.236 | 0.628 |
| Type*Temperature | 1 | 72,054 | 6.48 | 0.0123* |
| Type*Sex | 1 | 282 | 0.0253 | 0.874 |
| Temperature*Sex | 1 | 3819 | 0.343 | 0.559 |
| Type*Temperature*Sex | 1 | 1845 | 0.166 | 0.685 |
| Residuals | 115 | 1,279,492 | | |

*, $P < 0.05$

When individuals that did not exhibit death-feigning (the duration of death-feigning = 0 s) were excluded, the result of ANOVA showed significant effects of type ($F = 6.15$, $n = 123$, $P = 0.0146$; Table 2) and the interaction between temperature and type was significant ($F = 6.48$, $n = 123$, $P = 0.0123$; Table 2) on the duration of death-feigning. None of the other factors and interactions was significant (Table 2).

Discussion

The duration of death-feigning in B was correlated positively with temperature whereas that in R was correlated negatively with temperature (Fig. 2). There are several possible hypotheses for the cause of these results. The first is that the balance of the trade-off between the two anti-predator behaviors, move away or death-feigning changes with temperature (Ohno and Miyatake 2007). Previous studies have suggested that a trade-off exists between these two strategies (Ohno and Miyatake 2007; Miyatake et al. 2008b; Cardoso and dos Santos Mendonça 2019). In general, there is an optimal temperature for muscle activity (Schou et al. 2013), and the success rate of escape behavior is expected to be maximized near the optimal temperature. Thus, if the observed relationship between elytral colors and the duration of death-feigning is a result of temperature adaptation, we would expect that muscle activity negatively correlates with temperature in B and positively in R. This remains to be confirmed to determine if it can explain the difference between B and R in the observed relationships.

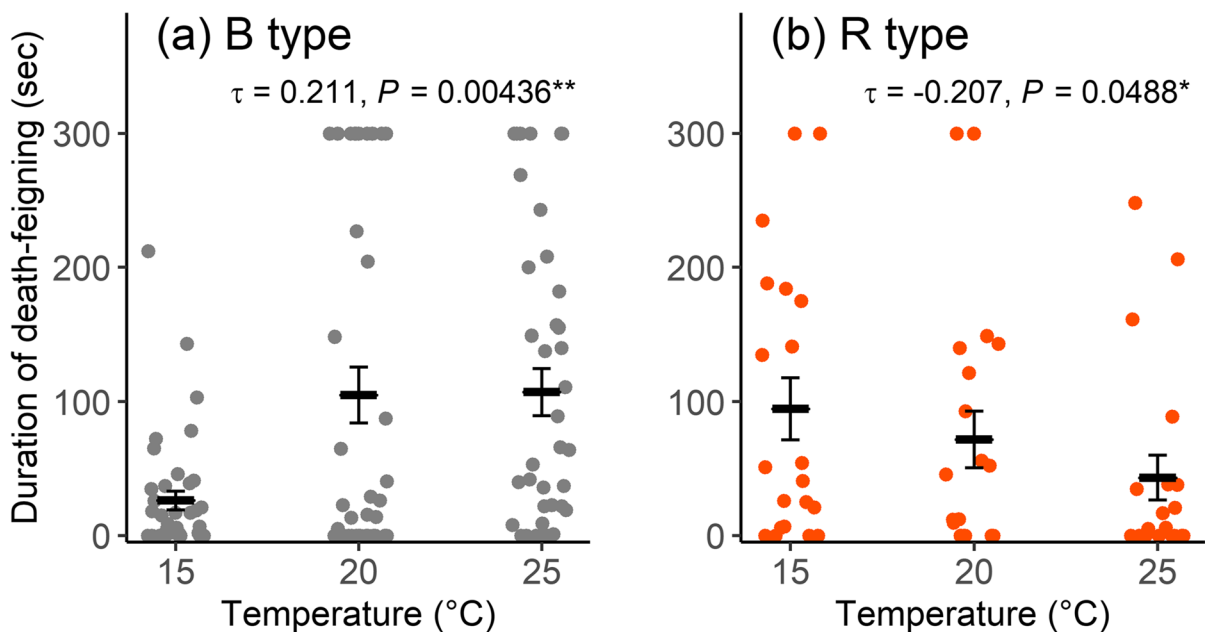


Fig. 2 The relationship between temperature and the duration of death-feigning for (a) B type ($n=120$) or (b) for R type ($n=60$). Kendall's rank correlation coefficients (τ) and P val-

ues were shown (*, $P < 0.05$, **, $P < 0.01$). Bars and whiskers indicate mean \pm SE. The plots were slightly shifted horizontally to increase understandability

The other hypothesis is that the duration of death-feigning is determined by a trade-off between predation avoidance and opportunity costs (Humphreys and Ruxton 2018). The longer duration of death-feigning is considered to lead to greater success in avoidance of predators, but greater loss of foraging and reproductive opportunities (Nakayama and Miyatake 2010). Therefore, individuals with high consumption rate may reduce the loss of foraging opportunities by shortening the duration of death-feigning. In general, the consumption rate of insects increases with body temperature up to a certain temperature (= optimum temperature for foraging) and decreases above that temperature (Angilletta et al. 2002). The black body color absorbs solar energy more efficiently than other colors and heats up faster, so black individuals may have lower optimum temperature for foraging. The darker phenotype *nigra* of *H. axyridis* has been found to have maximum consumption rate at a temperature 3.7 °C lower than the lighter phenotype *aulica* (Soares et al. 2003). Although the phenotypes, *nigra* and *aulica*, are different phenotypes from the B and R that we used, this study suggested that black body coloration is more likely to result in a metabolic maximum at lower temperatures in *H. axyridis*. To demonstrate this hypothesis, it would be necessary to measure the relationship between the duration of death-feigning and the loss of foraging and reproductive opportunities for B and R at each temperature.

Sex did not affect the duration of death-feigning (Table 2), contradicting a previous study of the weevil, *Cylas formicarius*, which shows that the duration of death-feigning decreases at night in both sexes, but the degree of decrease was larger in males than in females (Miyatake 2001b). This has been interpreted as a result of mating that occurs during night, and males having more incentives for multiple mating than females (Miyatake 2001b). In *H. axyridis*, about 14% of individuals copulate multiply, and the frequency of mating is not significantly different between sexes (Osawa 1994). This implies that the incentive for multiple mating is not different between males and females of *H. axyridis*, and this may be the reason there was no significant difference in the duration of death-feigning between sexes.

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Author Contributions EH and KT designed the study, collected samples, analyzed the data, and wrote the manuscript.

Data Availability We will deposit all the data used in this study to Figshare.

Declarations

Conflicts of Interest/Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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