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Title
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 2
     Artificial selections for death-feigning behavior in beetles show correlated responses
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     in amplitude of circadian rhythms, but the period of the rhythm does not
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     Short running title
     Death feigning and circadian rhythm
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### 36 Abstract

37 One of the most important survival strategies of organisms is to avoid predators. 38 Studying one of such strategies, namely death-feigning behavior, has recently become more common. The success or failure of this anti-predator strategy will 39 40 be affected by the circadian rhythms of both prey and predator, because death feigning sometimes has a diurnal rhythm. However, few studies have analyzed 41 42 the effects of differences in circadian rhythms on predator avoidance behavior 43 at the genetic level. Recently, the relationship between genes relating to 44 circadian rhythm and death-feigning behavior, an antipredator behavior, has been established at the molecular level. Therefore, in this study, we compared 45 46 three circadian rhythm-related traits, the free-running period of rhythms, amplitude of circadian rhythms, and total activity of strains of three Tribolium 47 48 species that were artificially selected for the death-feigning duration: short (S-49 strains) and long (L-strains) durations. As a result, the amplitude of circadian 50 rhythms and total activity were significantly different between S and L strains, but there was no difference in the free-running periods of the rhythm between 51 52 the strains in T. castaneum, T. confusum, and T. freemani. Although the 53 relationship between death-feigning behavior and activity has been reported for 54all three species, a genetic relationship between the duration of death feigning 55 and the amplitude of circadian rhythms has been newly found in the present study. It is important to investigate the relationship between anti-predator 56 strategies and circadian rhythms at the molecular level in the future. 57

58

#### 59 **KEYWORDS**

60 biological clock, Coleoptera, death feigning, thanatosis, tonic immobility

## 62 **1. INTRODUCTION**

Almost all organisms have a circadian rhythm (Saunders 2002). The clock genes that drive circadian rhythms affect various behavioral and life-history strategies of organisms. For example, circadian rhythms affect traits such as seasonal adaptation, mating behavior, feeding and foraging behaviors, and offspring nursing behavior (e.g., Sakai and Ishida 2001; Stokkan et al. 2001; Fuchikawa et al. 2009; Saunders 2020; Helfrich-Förster 2020; Fujioka et al. 2021).

69 Studying predator-prey relationships is one of the most important topics of 70 ethology (Ruxton et al. 2018). If the circadian rhythms of the predator and its 71 prey are different, they might not meet. Previous studies have shown a 72 phenotypic relationship between activity rhythmicity and survival strategies 73 [coyote: Higdon et al. 2019, buffalo: Tambling et al. 2015, spider: Jones et al. 742011, snail: Voutilainen 2010]. Genes involved in circadian rhythms have been 75 reported to affect predator avoidance in squirrels (DeCourcey et al. 1997, 2000). 76 In studies of chipmunks, DeCourcey et al. (1997, 2000) showed that surgical 77 removal of a region in the suprachiasmatic nucleus (SCN-X) thought to control 78 circadian rhythm caused them to become more active during times when their 79 predators are active as well. As a consequence, they had a higher risk of being 80 predated than sham-operated individuals.

One predator-avoidance behavior is death feigning, also known as tonic immobility (Humphreys and Ruxton 2018; Sakai 2021). Death feigning is affected by circadian rhythms and that the frequency of this behavior changes over the course of a day in rats (Hennig and Dunlap 1997a), domestic fowls (Roovee et al. 1976, 1977), toads (Ternes 1977), lizards (Hennig and Dunlap 1997b), and

beetles (Miyatake 2001). However, all of these studies observed death feigning
at the phenotypic level, and to date no study has examined the genetic
relationship between death-feigning behavior and circadian rhythms.

Using Tribolium castaneum, whose genome has been sequenced (Tribolium 89 90 Genome Sequencing Consortium 2008), two-way artificial selections on duration 91 of death feigning were performed to create strains with a long duration of death 92 feigning (L-strains) and strains without or with short duration of death feigning 93 (S-strains) (Miyatake et al. 2008, Matsumura and Miyatake 2018). In the recent 94 studies, RNA-seq analyses and DNA resequencing of genes expressed in long and 95 short strains showed that many mutant variations exist in the clock genes *Per*, 96 Cyc, dCLK, and Sgg in strains that feigned death longer, and were larger in Vri and 97 *Pdp* in strains that did not feigning death (Uchiyama et al. 2019; Tanaka et al. 98 2021). In addition, changes in activity with regard to death-feigning behavior 99 have been found to have a phenotypic link in the domestic fowl (Rovee et al. 100 1976) and some beetle species (Miyatake 2001; Li and Wen 2022). These studies, 101 therefore, motivated us to survey rhythm-related traits of strains with long and 102 short durations of death feigning in *Tribolium* species.

103 It has also recently been shown that *T. castaneum* exhibits circadian rhythms 104 and that the amplitude of these rhythms varies in field populations, i.e., there is 105 genetic variation (Abe et al. 2021). For these reasons, we consider *T. castaneum* 106 an optimal model organism for studying the relationship between the duration 107 of death feigning and traits related to circadian rhythms.

A recent study indicates that large variations in the numbers of alleles were found in circadian genes of the strain selected for longer death-feigning duration than the strain selected for shorter death-feigning duration (Tanaka et al. 2021). Also, a survey of field populations of *T. castaneum* revealed geographic variation in rhythm-related traits, resulting in clines (Abe et al. 2021). We, thus, hypothesized that there is a difference in the free-running period of the rhythm (that is the length of time it takes for an organism's endogenous rhythm to return to the same phase in the absence of environmental time cues) and the amplitude of circadian rhythms between the strains selected for long and short deathfeigning duration.

118 To test this hypothesis, we used ideal insect strains which had been artificially 119 selected for shorter and longer durations of death feigning. These two-way 120 artificial selection lines have been conducted for males and females in Tribolium 121 castaneum (Miyatake et al. 2004; Miyatake 2009), T. confusum (Nakayama et al. 122 2010), and T. freemani (Konishi et al. 2020) for more than 10 generations. In the 123 present study, we measured free-running periods and compared the circadian 124 rhythm traits, total activity, free-running period of the rhythm, and amplitude of 125 circadian rhythms of the short and long strains of the three *Tribolium* species.

126

# 127 2. MATERIALS AND METHODS

# 128 2.1. Insects

Three *Tribolium* species, *Tribolium* castaneum, *T.* confusum, and *T.* freemani, were used in the present study. All three of these species are considered "pest species" of stored grain pests and consume flour and rice bran (Sokoloff 1974). The three species were subjected to two-way artificial selection for death-feigning duration: long strains with a long duration of death feigning and short strains without or short death- feigning duration. The breeding and selection methods for each species have been described in detail in the following papers: *T.*  136 castaneum (Miyatake et al. 2008; Matsumura and Miyatake 2018; Miyatake et al. 137 2004, 2009), T. confusum (Nakayama et al. 2010), and T. freemani (Konishi et al. 2020). Adult females and males of two replicate lines of *T. castaneum* after 30 138 139 generations of selection, three replicate lines of *T. confusum* after 17 generations 140 of selection, and three replicate lines of T. freemani after 10 generations of 141 selection were used in the experiment. All selections were made and experiments were conducted in incubators set at 25°C and under constant 142 143 conditions with 16 hours of light and 8 hours of darkness (16L:8D).

144

## 145 **2.2 Locomotor activity**

146 To assess circadian rhythmicity, we maintained each adult beetle (about 147 20 days after eclosion) under 16L:8D conditions for more than 20 d in an 148 incubator kept at 25°C before the measurement of locomotor activity, and 149 then measured the locomotor activity of T. castaneum, T. confusum, and 150 T. freemani for 10 d in darkness. A beetle from each population was 151 placed in a clear plastic Petri dish (30 × 10 mm) in an incubator (MIR-152 153, Sanyo, Osaka, Japan) maintained at 25°C under complete darkness 153 (DD) to measure locomotion. The locomotor activity of each individual was monitored using an infrared actograph: an infrared light beam was 154 passed through a clear Petri dish, and the beam was projected onto a 155 photomicrosensor (E3S-AT11; Omron, Kyoto, Japan) that detected all 156 157 interruptions of the light beam. Signals of interruption of the infrared 158 light beam were recorded every 6 minutes for 10 days (Matsumura et al. 159 2020). Measurement methods were the same as described by Abe et al. (2021) and Matsumura et al. (2020). The sample sizes of each species 160

161 measured for circadian traits are shown in Table 1.

162

## 163 **2.3. Statistical analysis**

164 To determine the circadian rhythm, the locomotor activity data were 165 analyzed looking at the relationship between period and amplitude, with period presenting the cycle length of the circadian rhythm, and 166 167 amplitude representing the relative strength of a circadian rhythm (Fig. 1). The free-running period of circadian rhythms (=period) was 168 169 established using a  $\chi^2$  periodogram test for data on locomotor activity 170 between 20 and 28 h according to Halberg (1969). Circadian rhythmicity 171 was determined using  $\chi^2$  periodogram analysis, and amplitude was used 172 as an index of the strength of the rhythms. The amplitude of circadian 173 rhythms (=power) was defined as the maximum difference between the  $\chi^2$  value and the significance threshold line at P = 0.05, that is, the size 174175 of the peak above the 5% threshold; see Figure 1 in Halberg (1969). The amplitude is high when the rhythm is clear and strong, and an amplitude 176 177 of less than 0 indicates a statistically arrhythmic state. Moreover, total 178 activity was calculated as the base-10 logarithm of the total number of 179 interruptions of the infrared light over 10 d. To analyze the relationships 180 between strains selected for duration of death feigning and activity, 181 period, and amplitude, we used a generalized linear model with a normal 182 error distribution and replication as a random effect, and ANOVA with 183 Satterthwaite's method, using R version 4.0.3 (R Development Core Team. R 2017) was used. 184

#### 186 **3. RESULTS**

The short strains were more active than the long strains in *T. castaneum* and *T. freemani*, while no relationship between the strains of *T. confusum* was seen (Fig. 2; Table 2). There was no relationship between strain and the period in the three species (Fig. 3; Table 2).

191 On the other hand, the amplitude of the circadian rhythm differed significantly 192 between strains of all three species, but the direction differed among the species: 193 the short strain showed a significantly higher amplitude than the long strain in 194 T. castaneum, whereas the long strain showed a significantly higher amplitude 195 than the short strain in T. confusum and T. freemani (Fig. 4, Table 2; see also 196 examples of actograms of the three species in Fig. 5). Larger and smaller 197 amplitude values indicated that T. freemani tended to be rhythmic, while T. 198 castaneum and T. confusum tended to be arrhythmic (also see Fig. 4 and 5). 199 Although the sexes of all three species did not differ in activity, period, or 200 amplitude (Table 2), there was an interaction between strain and sex in the 201 activity and period of *T. castaneum*, indicating the strength of the difference in 202 activity and the direction of the difference in period between strains differ in 203 males and females (see Fig. 2 and 3).

204

# 205 4. DISCUSSION

In the three *Tribolium* species examined in this study, there was a significant difference in the amplitude of circadian rhythms between long (L) and short (S) strains. In *T. castaneum*, the amplitude of the S strains was significantly larger than that of the L strains, while in *T. confusum* and *T. freemani*, the amplitude of the S strains was smaller than that of the L strains. This suggests a correlation between death-feigning behavior and the amplitude of circadian rhythm, a novel finding which to our knowledge has not been demonstrated before.

213 In a previous study of geographic variation in circadian rhythm traits among 214 field strains of T. castaneum, no geographic variation was observed in the free-215 running period, but a cline was observed in the amplitude of circadian rhythms, 216 and the amplitude was smaller in northern than in southern Japan (Abe et al. 217 2021). Combining the results of this previous study with the present results, it 218 seems as if the amplitude of circadian rhythms is a genetically variable trait in 219 *Tribolium* species. We found that whereas the amplitude of circadian rhythms 220 was significantly larger in S than in L strains in T. castaneum, but smaller in T. 221 confusum and T. freemani, there was no difference in the free-running periods of 222the rhythm according to selection regime in the three species. The reason why 223 the direction of the amplitude of circadian rhythms differed among the species 224is not known. Since we examined multiple replicate lines of selection for all three 225 species in the experiment, the possibility of genetic drift is low. To understand 226 this phenomenon, future studies should investigate the difference in the 227 molecular systems of clock genes of the three *Tribolium* species.

In this study, we found that artificial selection on the duration of death feigning changed in the amplitude of circadian rhythms as a correlated response, which is consistent with differential expressions of clock genes among strains selected for duration of death feigning at the genomic level. Mutations in clock genes may alter the amplitude of the rhythm by causing mutations in the clock gene feedback loop (Tomioka and Matsumoto 2010).

234 On the other hand, the response of the free-running period of rhythm showed

no correlation to the selection for duration of death feigning in the *Tribolium*species. This reaffirms the robustness and homeostasis of the free-running
period of rhythm.

238 In a study by Tanaka et al. (2021) in which the strains of T. castaneum were 239 analyzed for DNA re-sequencing, six clock genes, Per, Cyc, dCLK, Sgg, Vri, and Pdp, 240 mutated at different rates between short and long strains. Genetic factors that 241 affect circadian rhythms are traits comprising many genes in the feedback loop 242 of the circadian clock (Tomioka and Matsumoto 2010). Comparisons of the DNA-243 seq data between the strains revealed mutations in the clock genes, but the 244 mutations did not change the period of the circadian rhythm. On the other hand, 245 a comparative study at the molecular level and its relationship with the 246 amplitude of the circadian rhythm and the level of activity is a subject for future 247study.

248 In an experiment that explored the genetic background of death-feigning 249 duration in crossing experiments of the short and long strains used in this 250 experiment, it was found that death-feigning duration is also a trait that is 251 controlled by quantitative inheritance (Matsumura and Miyatake, bioRxiv). RNA-252 seq analysis also showed that many genes, such as tyrosine metabolism, stress 253 response genes, and insulin signaling pathways, are differentially expressed in 254the short and long strains (Uchiyama et al. 2019). In addition to these genes, the 255 relationship between the circadian clock and the duration of immobility is 256 expected to become clearer as genomic analysis of the duration of death feigning 257 in organisms proceeds in the future.

In contrast, for the activity measured in this study, the short strains showed
significantly higher values than the long strains in *T. castaneum* and *T. freemani*.

The activity value is the same as the results of previous studies that showed short-time locomotor activity in *T. castaneum* (Miyatake et al. 2008) and *T. freemani* (Konishi et al. 2020), while the results differ between activity in the present study and short-term locomotor activity in *T. confusum* (Nakayama et al. 2010). Therefore, the amplitude of the circadian rhythm and total activity in *Tribolium* species are traits with a high potential for genetic variation, i.e., evolvability (Kirschner and Gerart 1998).

Larger and smaller amplitudes (Fig. 4) indicate that *T. freemani* tends to be rhythmic, while *T. castaneum* and *T. confusum* tend to be arrhythmic (see Fig. 5). The reason why the relationships were different among species is not known. It may depend on the history of the strains used for artificial selections, such as differences in the number of breeding individuals or genetic bottlenecks in the three species. Also, it is a possibility that there is no common genetic background between the death-feigning period and the circadian rhythm among species.

The appearance of death-feigning behavior is believed to be related to the switching on and off of the active and inactive modes of the organism (Miyatake 2021). Therefore, the correlated responses to selection for duration of death feigning on the amplitude of circadian rhythm observed in this study are interesting, and it will be important to investigate in detail the relationship between the genome regions controlling the amplitude of the circadian rhythm and activity and predation avoidance tactics in the future.

In summary, when we artificially selected for long and short durations of deathfeigning, we did not observe a correlated response in the periods of circadian rhythms among the strains, but we did observe a common correlated response in the amplitude of the circadian rhythm and total activity among the three

species. The period of circadian rhythms was not affected by genetic changes in anti-predator strategies, but the amount and amplitude of activity have important genetic effects on anti-predator strategies that are closely related to the active or inactive phase of organisms.

289

# 290 **CONFLICT OF INTEREST**

- 291 The authors declare no conflicts of interest for this study.
- 292

# 293 Ethical statement

294 The beetles (Tribolium castaneum, T. confusum and T. freemani) used in this study 295 are invertebrates and therefore have not been subjected to animal ethics review. 296 The study was conducted in a manner that avoided or minimized discomfort or 297 distress to the laboratory animals, and efforts were made to ensure that the 298 animals did not suffer unnecessarily at any stage of the experiment. The T. 299 castaneum beetle culture has been maintained in the laboratory at Okayama 300 University. This population has been maintained on whole meal flour with yeast. 301 We reared this population at 25-28°C, which is similar to the natural conditions 302 for this insect. All animals in the study were handled carefully.

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- 421

Species	Strain	Replication	Sex	Ν	Total
T. castaneum	L	1	female	56	
	L	2	female	45	
	S	1	female	48	
	S	2	female	53	
	L	1	male	46	
	L	2	male	41	
	S	1	male	54	
	S	2	male	55	398
T. confusum	L	1	female	39	
	L	2	female	66	
	L	3	female	69	
	S	1	female	62	
	S	2	female	57	
	S	3	female	60	
	L	1	male	44	
	L	2	male	47	
	L	3	male	64	
	S	1	male	60	
	S	2	male	44	
	S	3	male	55	667
T. freemani	L	1	female	22	
	L	2	female	11	
	L	3	female	26	
	S	1	female	23	
	S	2	female	21	
	S	3	female	24	
	L	1	male	22	
	L	2	male	20	
	L	3	male	27	
	S	1	male	29	
	S	2	male	21	
	S	3	male	26	272

Table 1. Sample sizes of the three *Tribolium* beetles

Species	Traits	Factor	df	Mean Sq	F	Р
T. castaneum	Activity	Strain	1	6.2	31.9207	<0.0001
		Sex	1	0.6	3.2623	0.0717
		Strains X sex	1	1.0	5.2828	0.0221
	Period	Strain	1	24.9	0.0536	0.8171
		Sex	1	22.1	0.0477	0.8273
		Strain X sex	1	2431.0	5.2369	0.0226
	Amplitude	Strain	1	21276.9	19.9122	<0.0001
		Sex	1	77.2	0.0722	0.7882
		Strains X sex	1	1886.3	1.7653	0.1847
T. confusum	Activity	Strain	1	0.0	0.1601	0.6892
		Sex	1	0.7	2.4199	0.1203
		Strain X sex	1	0.3	1.1487	0.2842
	Period	Strain	1	192.1	0.326	0.5682
		Sex	1	180.1	0.3056	0.5806
		Strain X sex	1	684.7	1.1619	0.2815
	Amplitude	Strain	1	1953.2	11.7002	0.0007
	·	Sex	1	1156.6	0.6692	0.4033
		Strain X sex	1	3795.3	2.2945	0.1303
<b>T</b> ( <b>·</b>	A	o	1	1 5	10 0077	0.001.0
I. treemani	Activity	Strain	1	1.5	10.6877	0.0012
		Sex	1	0.1	0.5765	0.4484
		Strain X sex	1	0.1	0.4681	0.4945
	Period	Strain	1	46.3	0.1354	0.7132
		Sex	1	1168.8	3.4183	0.0656
		Strain X sex	1	339.5	0.993	0.3199
	Amplituda	Strain	1	50212.0	10 2171	<0.0001
	Ampiltude	Surain	1	53515.0	1 7111	0.102
		Strain V agy	1	5254.0	1./111	0.192
		Strain X sex	1	9.0	0.0029	0.9008

Table 2. Results of ANOVA with Satterthwaite's method for circadian rhythm traits of *Tribolium* beetles

425 Legends of Figures

426

Figure 1 Relationship between amplitude and activity. When the amplitude is large, the period is clear (A), but when the amplitude is small, it is difficult to detect a clear period (B).

430

Figure 2 Comparisons of "activity" between the long (L-female and L-male) and
short (S-female and S-male) strains in *Tribolium* species. Each bar shows
standard errors.

434

Figure 3 Comparisons of "period" between the long (L-female and L-male) and
short strains in *Tribolium* species. Each bar shows standard errors.

437

Figure 4 Comparisons of "amplitude" between the long (L-female and L-male)
and short (S-female and S-male) strains in *Tribolium* species. Each bar shows
standard errors.

441

Figure 5 Examples of actograms of *T. castaneum*, *T. confusum* and *T. freemani*.





446 Fig. 1







![](_page_22_Figure_1.jpeg)

![](_page_23_Figure_0.jpeg)

![](_page_23_Figure_1.jpeg)

![](_page_24_Figure_0.jpeg)

461 Fig. 5