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Responses to relaxed and reverse selection in strains artificially selected for duration of death-feigning behavior in the red flour beetle, *Tribolium castaneum*

Kentarou Matsumura[1] · Takahisa Miyatake[1]

Abstract

Divergent lines selected artificially for many generations make it possible to answer two questions: (1) whether genetic variation still exists within the selected population; and (2) whether the selection itself is costly for the selected strain. In previous studies, the red flour beetle *Tribolium castaneum* was divergently selected artificially for duration of death-feigning, and strains selected for longer (L-strain) and shorter (S-strain) durations of death-feigning have been established (Miyatake et al. 2004, 2008). Because the selection experiments have been conducted for more than 27 generations, genetic variation may be eroded. Furthermore, because another previous study reported physiological costs to L-strains, the L-strains selected artificially for longer duration of death-feigning may have suffered more costs than the S-strains. In the present study, therefore, we relaxed the selection pressure after the 27th or 30th generation of S- and L-strains. We also carried out reverse selection during the most recent eight generations of S- and L-strains. The results showed that each strain clearly responded to relaxation of selection and reverse selection, suggesting that (1) additive genetic variation still existed in both strains after long-term selection, and (2) selection for shorter and longer duration of death-feigning was costly. These results suggest that anti-predator behavior is controlled by many loci, and longer or shorter duration of death-feigning is costly in a laboratory without predators.

Keywords Artificial selection · Quantitative trait · Relaxation of selection · Reverse selection · Tonic immunity ·
Tribolium castaneum

Introduction

Selection pressures influence animal traits such as activity, risk-taking, and antipredator behaviors. Artificial selection experiments provide useful information about selection pressures and associated responses to selection, including trade-offs between traits, genetic variation in traits, and interactions between traits and environmental variables (van Oers et al. 2003; Brodin and Johansson 2004; Wund et al. 2015).

Many animal behavioral traits have a quantitative genetic basis (Boake 1994), and artificial selection is an important tool for investigating the inheritance of these traits (Falconer and Mackay 1996). Long-term artificial selection makes it

possible to answer two questions (Hill and Caballero 1992): (1) whether genetic variation still exists within a selected strain; and (2) whether the selection pressure is costly for the selected strain.

Long-term artificial selection often results in a limited response to selection pressure, termed a "selection limit" (Robertson 1960). The selection limit may occur when additive genetic variation has been eroded in the selected strain (Falconer and Mackay 1996). To identify whether genetic variation still exists within the selected strain, artificial selection intended to reverse the direction of change in the selected strain, termed "reverse selection," is often used. For example, in *Drosophila melanogaster*, two-way artificial selection for length of the thorax was carried out, and up- (increased thorax length) and down-strains (decreased thorax length) were established; subsequently, these selected strains underwent artificial selection in the reverse direction (Robertson 1955). The results revealed that the up-strains responded to reverse selection,

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

1   Graduate School of Environmental and Life Science,
    Okayama University, Okayama City, Japan

whereas the down-strains did not, suggesting that additive genetic variation still existed in the up-strains, but not in the down-strains (Robertson 1955). Similarly, two-way artificial selection for body mass was conducted in mice (*Mus domesticus*), and after establishment of the selection strains, reverse selection was carried out (Roberts 1966). In this case, the down-strain (reduced body mass), but not the up-strain (increased body mass), responded to reverse selection, suggesting that only the down-strain had additive genetic variation (Roberts 1966). These previous selection experiments focused on morphological traits. However, responses to artificial selection often differ between morphological traits and behavioral traits. For example, heritability for selection of morphological traits is higher than behavioral traits in some *Drosophila* (Roff and Mousseau 1987) and other animal species (Mousseau and Roff 1987). To the best of our knowledge, few studies have carried out reverse selection on behavioral traits (but see Cunningham and Siegel 1978; Bult and Lynch 1996).

When an artificial selection protocol is stopped, the selected strains may be vulnerable to natural selection pressure. Predation pressure is absent in a laboratory environment as in the present experiment. Therefore, we can investigate whether artificial selection for the target traits is costly through relaxation of selection (Falconer and Mackay 1996). Several studies have reported the effects of relaxation on artificially selected strains in some animals. For example, in *D. melanogaster*, researchers used artificial selection to increase the number of bristles on the abdomen, and the strain showed a clear response to this selection (Yoo 1980). However, Yoo (1980) showed that the number of bristles decreased after relaxation of selection, and concluded that selection for an increase in the number of bristles was costly in *D. melanogaster*. However, to our knowledge, few studies have investigated the effects of relaxation of selection on behavioral traits in the selected strains (but see Dobzhansky and Spassky 1969).

Animals living under natural conditions encounter selection pressures by predators; similarly, many kinds of selection pressure act upon the antipredator behaviors and life-history traits of every wild population, as noted in other animals (see van Oers et al. 2003; Brodin and Johansson 2004; Wund et al. 2015). Examining the relationship between selection pressures and antipredator behaviors in wild populations is important in behavioral ecology. Death-feigning, which is considered an antipredator behavior, is observed in many animal taxa (Ruxton et al. 2004; Miyatake et al. 2004). A previous study (Miyatake et al. 2004) showed that two-way artificial selection successfully bifurcated the duration of death-feigning behavior in the red flour beetle (*Tribolium castaneum*), establishing strains with longer (L-strain) and shorter (S-strain) durations of death-feigning. These strains are ideal to investigate whether genetic variation is retained in the selected strains after long-term selection experiments.

Another previous study reported that individuals of the L-strains had significantly decreased survival rates compared to those of the S-strains, under high and low temperatures and vibration stress (Kiyotake et al. 2013). Therefore, L-strains pay a physiological price under selection for longer duration of death-feigning. If an individual with a longer duration of death-feigning has greater physiological costs, duration of death-feigning may decrease in subsequent generations in a predator-free environment such as a laboratory.

In the present study, we tested two hypotheses: (1) if no genetic variation exists in L- and S-strains, neither strain should respond to reverse selection; and (2) if longer or shorter duration of death-feigning is costly, individuals of the L- or S-strain should show a change in the duration of death-feigning under relaxation of selection. To test these two hypotheses, we relaxed the artificial selection process, and then carried out reverse selection experiments while continuing selection in controls strains.

Materials and methods

Insects and culture

Tribolium castaneum has been maintained in the laboratory for more than 15 years (about 90 generations) according to the rearing method described by Suzuki and Nakakita (1991). The population has been maintained at about 100 individuals during that time. The beetles are reared on a mixture of whole meal (Nisshin Seifun Group, Tokyo, Japan) enriched with brewer's yeast (Asahi Beer, Tokyo, Japan) in an incubator (Sanyo, Tokyo, Japan) maintained at 25 °C under a 16L:8D photoperiod (lights on at 07:00, lights off at 23:00). The developmental period for *T. castaneum* under optimal conditions is about 35 days (Sokoloff 1974). They become sexually mature at 2 days post-eclosion (Dawson 1964). In the laboratory, they are sexed in the pupal stage by distinct morphological characters of the abdomen, and stored in single-sex groups in petri dishes (90 mm in diameter, 15 mm in height) until the experiments.

Artificial selection for duration of death-feigning

Strains selected for longer (L-strains) and shorter (S-strains) durations of death-feigning were established by two-way artificial selection, as described in Miyatake et al. (2004). In total, 100 virgin males and 100 virgin females (7–15 days post-eclosion) were randomly collected from a stock culture and placed with food in individual wells of 48-well tissue culture plates (Falcon; Becton–Dickinson and Co., Franklin Lakes, NJ, USA) to avoid disturbance by other individuals

(Miyatake 2001). To examine the duration of death-feigning, an adult was gently moved onto a small white china saucer (140 mm diameter, 15 mm deep). Death-feigning was induced by touching the abdomen of the beetle with a wooden stick. A trial consisted of provoking the death-feigning behavior and recording its duration with a stopwatch (the duration of the behavior was defined as the length of time between touching the beetle and detecting its first visible movement). If the adult did not become immobile, the touch was repeated up to 3 times. If the adult was unresponsive to stimulation, its death-feigning time was recorded as zero. The 10 males and 10 females with the longest death-feigning times were selected to propagate the L-strains. Similarly, the 10 males and 10 females with the shortest death-feigning times were selected to propagate the S-strains. Two replicate strains were produced for each treatment. The details of the selection experiments, including direct responses and realized heritability, were described in Miyatake et al. (2004). All trials were conducted in the incubator between 12:00 and 17:00. In this protocol, the artificial selection was repeated for more than 27 generations.

Relaxation of selection

The artificial selection experiments were continued for 29 generations for replicate A of the L-strain, 27 for replicate B of the L-strain, 30 for replicate A of the S-strain, and 27 for replicate B of the S-strain. Then, these lines were maintained without selection for 5 years (about 30 generations), i.e., relaxation of selection. During this period, 10 males and 10 females randomly collected from each strain were allowed to copulate to propagate successive generations. The
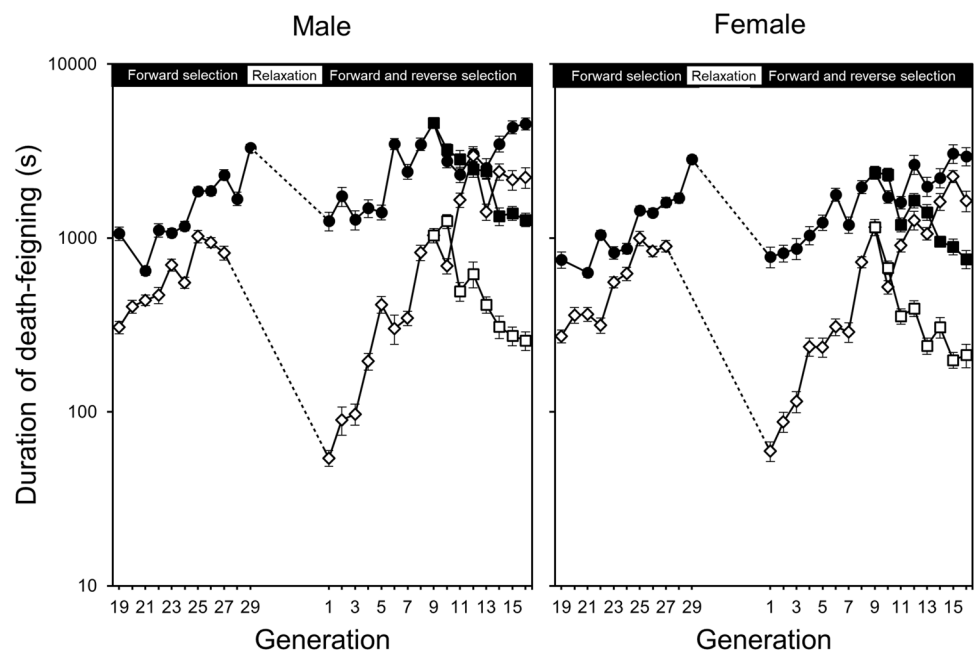
strains were maintained at 25 °C under a 16L:8D photoperiod (lights on at 07:00, lights off at 23:00).
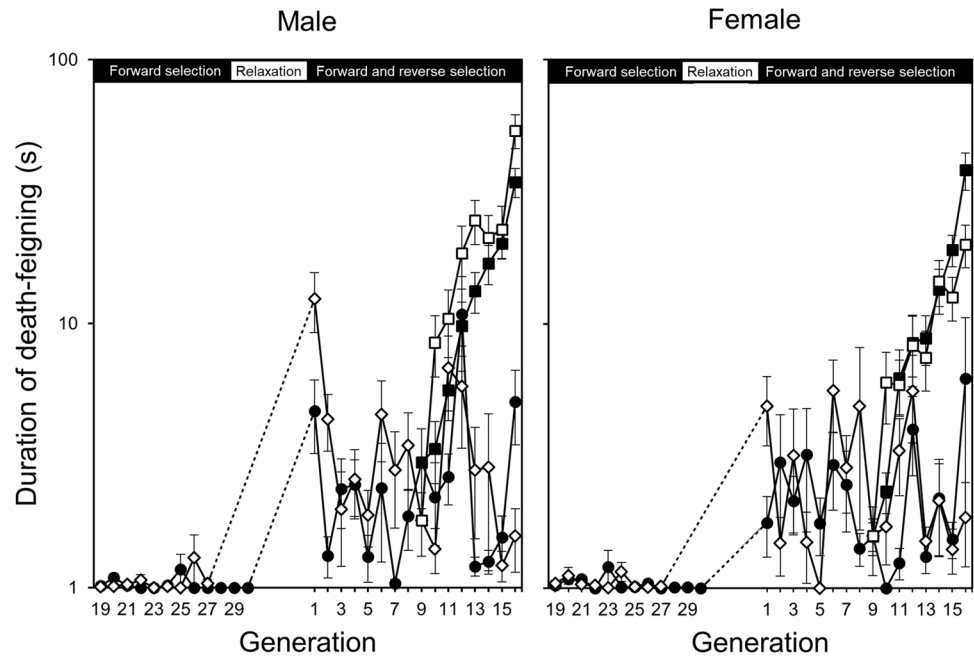
Reverse selection

After the relaxation of selection, we restarted forward selection as a control for reverse selection. In the forward selection experiments, L- and S-strains were artificially selected for duration of death-feigning in their initial directions for 16 generations (see black circles and white diamonds in Figs. 1 and 2). Virgin males ($N = 50$, 7–15 days post-eclosion) and females ($N = 50$, 7–15 days post-eclosion) were randomly collected from each strain, and we measured the duration of death-feigning by the method described above. In the L-strain, the 10 males and 10 females with the longest death-feigning were selected. Similarly, in the S-strain, the 10 males and 10 females with the shortest death-feigning were selected. The forward selection was carried out for 16 generations (Figs. 1 and 2). The rest of the selection protocol was the same as that for forward selection before the relaxation of selection.

We also started reverse selection for strains that had previously been selected for shorter and longer durations of death-feigning. In the reverse selection experiments, individuals of the L- and S-strains were artificially selected for duration of death-feigning in the reverse direction for eight generations (black and white squares in Figs. 1 and 2). Virgin males ($N = 50$, 7–15 days post-eclosion) and females ($N = 50$, 7–15 days post-eclosion) were randomly collected from each strain, and we measured the duration of death-feigning using the method described above. From the L-strain, the 10 males and 10 females with the shortest

Fig. 1 Response of death-feigning duration ($+1$s) in the L-strains of *Tribolium castaneum* to forward selection (replicate A: *black circles*; replicate B: *white diamonds*), reverse selection (replicate A: *black squares*; replicate B: *white squares*), and relaxation of selection (*dashed line*). *Error bars* show standard error

Fig. 2 Responses of death-feigning duration (+ 1s) in the S-strains of *Tribolium castaneum* to forward selection (replicate A: *black circles*; replicate B: *white diamonds*), reverse selection (replicate A: *black squares*; replicate B: *white squares*), and relaxation of selection (*dashed line*). *Error bars* show standard error

death-feigning duration were selected. Similarly, from the S-strain, the 10 males and 10 females with the longest death-feigning duration were selected. Reverse selection was carried out for eight generations (See Figs. 1 and 2). The remainder of the selection protocol was the same as the method used for forward selection before the relaxation of selection.

Statistical analysis

We calculated the realized heritability of forward (for the last 16 generations) and reverse selection strains (for the last eight generations) following Falconer and Mackay (1996). Heritability was calculated as the effect of the regression of the population mean on the cumulative selection differential. In the statistical analysis, we analyzed effects of selection strains and replicate strains. Moreover, we also analyzed the effect of sex, because a previous study (Matsumura et al. 2016) revealed that duration of death-feigning differed between the sexes. To test for effects of realized heritability in artificial forward and reverse selection, we used analysis of variance (ANOVA) with strain, sex, and replicate strains as explanatory variables.

To test the significance of the effect of forward selection for duration and frequency of death-feigning behavior, we used ANOVA with generation (1st and 16th since selection was restarted), replicate strains, and sex as explanatory variables. Moreover, to test significance of the effect of reverse selection for duration and frequency of death-feigning behavior, we used ANOVA with generation, replicate strains, and sex as explanatory variables. The duration of

death-feigning (+ 1s) was then log-transformed to approximate normality.

To test the significance of the effect of relaxation experiments, we used ANOVA (type 2) to compare durations of death-feigning between generations before and after selection relaxation in each selected strain. Generation, replicate strain, and sex were the explanatory variables in this analysis. All statistical analyses were conducted using JMP software (SAS Institute Inc 2015).

Results

After we stopped forward selection for the duration of death-feigning, L-strains showed decreased duration of death-feigning (Fig. 1), and S-strains showed increased duration (Fig. 2). We found significant differences between the duration of death-feigning before versus after relaxation in both selected lines (L-strain: $F_{1,587} = 598.32$, $P < 0.0001$; S-strain: $F_{1,596} = 98.02$, $P < 0.0001$). There were also significant differences between replicate strains (L-strain: $F_{1,587} = 626.61$, $P < 0.0001$; S-strain: $F_{1,596} = 11.16$, $P = 0.0009$). In the S-strain, males showed longer duration of death-feigning than did females ($F_{1,596} = 5.12$, $P = 0.0241$), but no difference was found in the L-strain ($F_{1,587} = 2.63$, $P = 0.1055$). The results show that the S- and L-strains responded well to the relaxation of selection, suggesting that there were costs of forward selection for shorter and longer durations of death-feigning in *T. castaneum*.

When we restarted selection after relaxation, forward selection on the L-strains resulted in increased duration of death-feigning (Fig. 1), but the S-strains did not respond

to selection (Fig. 2). L-strains showed significantly higher realized heritability than S-strains in forward selections (Tables 1 and 2). There were significant differences among replicate strains, but no differences in sex (Table 2). Duration of death-feigning was significantly different between pre- and post-forward selection experiments in both L- and S-strains, respectively (Figs. 1 and 2, Table 3). For forward selection, there were no significant changes in the frequency of death-feigning (Figs. 3 and 4, Table 4).

Reverse selection produced L-strains that showed decreased duration of death-feigning (Fig. 1; black and white squares), whereas S-strains showed increased duration (Fig. 2; black and white squares). L- and S-strains showed a trend of higher realized heritability by reverse selection (Table 1). There were no significant differences among selected strain and other factors (Table 2). Duration of death-feigning was significantly different between pre- and post-reverse selection experiments in both L- and S-strains, respectively (Figs. 1 and 2, Table 3). Frequency of death-feigning was significantly different between pre- and post-reverse selection experiments in S-strains, but there were no differences in L-strains (Figs. 3 and 4, Table 4).

Discussion

Relaxation of selection over about 5 years caused L- and S-strains to show shorter and longer durations of death-feigning, respectively (Figs. 1 and 2). Restarting forward selection after relaxation resulted in increased duration of death-feigning in L-strains over 16 generations (Fig. 1, Table 3), but S-strains did not respond to renewed forward selection (Fig. 2). Reverse selection on the L- and S-strains resulted in a decrease and increase, respectively, in the duration of death-feigning (Figs. 1 and 2, Table 3), suggesting that these strains maintained genetic variation with respect to death-feigning duration.

Table 1 Realized heritability by forward and reverse selection for duration of death-feigning

Direction	Replicate line	Sex	Strain	
			Long	Short
Forward	A	Male	0.0611	−0.0852
		Female	0.071	−0.0786
	B	Male	0.1255	0.0878
		Female	0.1474	0.0164
Reverse	A	Male	0.3213	0.1559
		Female	0.0938	0.1786
	B	Male	0.3415	0.1164
		Female	0.4731	0.0875

Table 2 Results of ANOVA for realized heritability by forward and reverse selection

Selection	Factor	d.f.	F	P
Forward	Strain	1	22.72	**0.0089**
	Replicate line	1	17.59	**0.0138**
	Sex	1	0.11	0.7524
	Error	4		
Reverse	Strain	1	3.46	0.1363
	Replicate line	1	0.52	0.5093
	Sex	1	0.08	0.7970
	Error	4		

Bold values indicate statistically significant results ($P < 0.05$)

Dobzhansky and Spassky (1969) carried out artificial selection for positive and for negative phototaxis and geotaxis in *Drosophila pseudoobscura* over 20 generations. Furthermore, they revealed that both positive and negative strains showed throwback by relaxation of the selection in phototaxis and geotaxis, respectively. The results suggested that artificial selections for positive and negative phototaxis and geotaxis are costly in *D. pseudoobscura* (Dobzhansky and Spassky 1969). In the present study, we revealed that selected strains of longer and shorter duration of death-feigning show throwback by relaxation of the selection in *T. castaneum*. Therefore, artificial selection for longer and for shorter duration of death-feigning may be costly in this beetle.
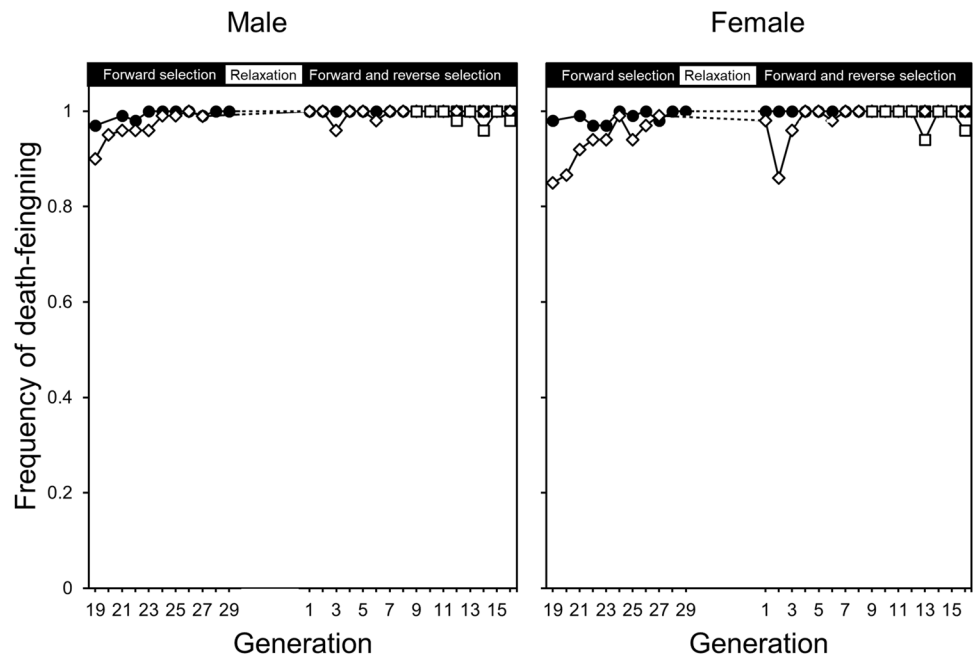
Table 3 Results of ANOVA for duration of death-feigning behavior in forward and reverse selection experiments

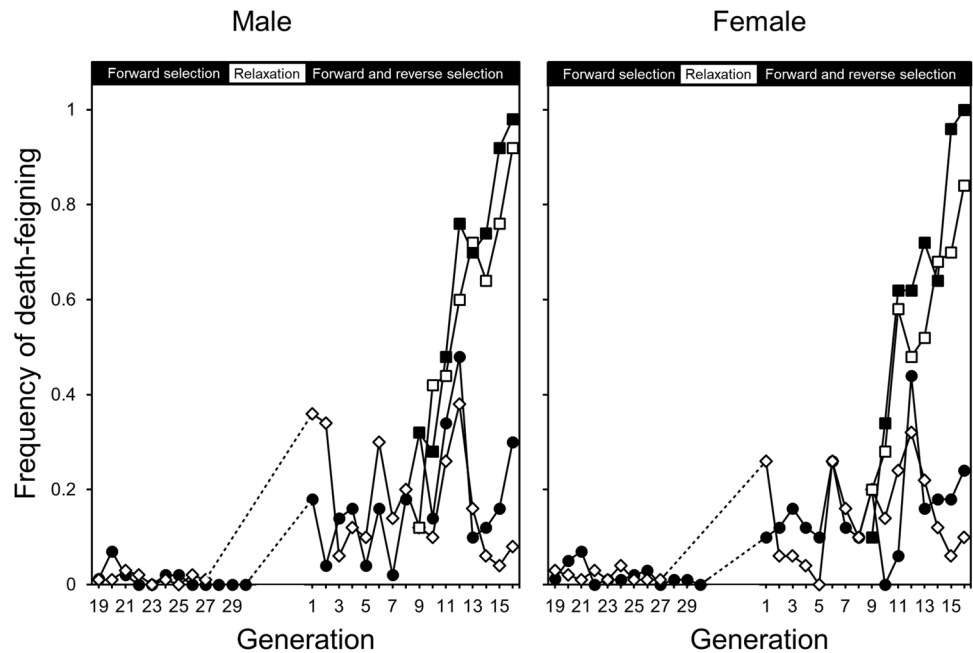| Selection	Strain	Factor	d.f.	F	P
Forward	Long	Generation	1	634.19	**< 0.0001**
		Replicate line	1	328.98	**< 0.0001**
		Sex	1	11.92	**0.0006**
		Error	396		
	Short	Generation	1	7.36	**0.0069**
		Replicate line	1	0.78	0.3774
		Sex	1	5.04	**0.0253**
		Error	396		
Reverse	Long	Generation	1	55.44	**< 0.0001**
		Replicate line	1	504.68	**< 0.0001**
		Sex	1	14.48	**0.0002**
		Error	396		
	Short	Generation	1	282.51	**< 0.0001**
		Replicate line	1	0.18	0.6755
		Sex	1	10.51	**0.0013**
		Error	396		

Bold values indicate statistically significant results ($P < 0.05$)

Fig. 3 Response of death-feigning frequency in the L-strains of *Tribolium castaneum* to forward selection (replicate A: *black circles*; replicate B: *white diamonds*), reverse selection (replicate A: *black squares*; replicate B: *white squares*), and relaxation of selection (*dashed line*)

Fig. 4 Responses of death-feigning frequency in the S-strains of *Tribolium castaneum* to forward selection (replicate A: *black circles*; replicate B: *white diamonds*), reverse selection (replicate A: *black squares*; replicate B: *white squares*), and relaxation of selection (*dashed line*)

Kiyotake et al. (2013) reported that beetles from the L-strain of *T. castaneum* showed lower resistance to some environmental stresses than did those from the S-strain. In the present study, the duration of death-feigning in the L-strains was decreased by relaxation of selection for about 5 years (Fig. 1). This confirmed that the selection for longer duration of death-feigning was costly.

The beetles of the S-strain showed an increase in death-feigning duration following relaxation of forward selection for 5 years. This suggests that artificial selection for shorter death-feigning duration was also costly in *T. castaneum*,

although a previous study reported that the S-strain showed no physiological costs (Kiyotake et al. 2013). In any case, it is important to allow ample genetic variation to persist despite the appearance of a selection limit, as this allows genetic drift to change the mean population expression under relaxed selection due to the small population size.

Our understanding of the response of the S-strains to relaxation of the selection is as follows. Two-way artificial selection for duration of death-feigning was conducted in the adzuki bean beetle *Callosobruchus chinensis*, and L-strains and S-strains differing in the duration of death-feigning were

Table 4 Results of ANOVA for frequency of death-feigning behavior in forward and reverse selection experiments

Selection	Strain	Factor	d.f.	F	P
Forward	Long	Generation	1	1.00	0.3179
		Replicate line	1	1.00	0.3179
		Sex	1	1.00	0.3179
		Error	396		
	Short	Generation	1	1.25	0.264
		Replicate line	1	0.02	0.9012
		Sex	1	1.87	0.1724
		Error	396		
Reverse	Long	Generation	1	1.02	0.3142
		Replicate line	1	4.06	**0.0445**
		Sex	1	1.02	0.3142
		Error	396		
	Short	Generation	1	428.56	**< 0.0001**
		Replicate line	1	0.77	0.3823
		Sex	1	3.06	0.081
		Error	396		

Bold values indicate statistically significant results ($P < 0.05$)

established (Ohno and Miyatake 2007). In these *C. chinensis* strains, females of strains selected for shorter duration of death-feigning had decreased reproductive success and decreased longevity compared to females selected for longer duration of death-feigning (Nakayama and Miyatake 2009), suggesting that artificial selection for longer duration of death-feigning confers some benefit to the life history traits of females by conserving their energy through low activity (Nakayama and Miyatake 2010).

If the *T. castaneum* females in the S-strains also show costs in life history traits similar to those in *C. chinensis*, this might explain the cost of selection for shorter duration of death-feigning caused by stopping of the forward selection. Therefore, additional studies that compare life history traits of females among the selected strains in *T. castaneum* are needed.

The L-strains showed clear responses to restarting forward selection following 16 generations of relaxation, i.e., increased duration of death-feigning and higher realized heritability (Fig. 1, Tables 1 and 2). Moreover, after reverse selection for eight generations, the L-strains showed decreased death-feigning duration and higher realized heritability (Fig. 1, Table 3). The results of these two experiments suggest that genetic variation with respect to death-feigning behavior still exists in the L-strains.

In contrast, the S-strains did not show clear responses to the forward selection for shorter duration of death-feigning following 16 generations of relaxation (Fig. 2). However, the duration of death-feigning in the S-strains was increased by reverse selection for longer duration. These results suggest that genetic variation with respect to death-feigning still exists in

the S-strains, and the lack of response to forward selection may be due to phenotypic limits (i.e., death-feigning under 0 s cannot be measured; see Fig. 2). A similar phenomenon was reported in a previous study in which reverse selection was carried out on a strain of corn (*Zea mays*) that was artificially selected for a lower amount of protein (Dudley 2007). Reaching the selection limit may be a result of decreased genetic variation; therefore, the time until the selection limit is reached may depend on the number of genetic loci controlling the target trait (Hill and Caballero 1992; Falconer and Mackay 1996).

The present results suggest that the duration of death-feigning is controlled by a number of genetic loci, as genetic variation was still present in both selected strains of *T. castaneum* even after 40 generations of selection. Phillips et al. (2002) carried out two-way artificial selection for enhanced (FAST) and reduced (SLOW) sensitivity to ethanol's locomotor stimulant effects for more than 35 generations in mice (*Mus musculus*). They concluded that genetic variation remained in both selected strains, as both FAST- and SLOW-strains showed a clear response to reverse selection over 16 generations (Phillips et al. 2002). In general, a behavioral trait may be controlled by many genetic loci in animals (Boake 1994; Ritchie and Butlin 2014). Conducting both forward and reverse selection for a trait can reveal when the selection limit appears in the trait. However, few studies have carried out both forward and reverse selection on behavioral traits (but see Dobzhansky and Spassky 1969; Cunningham and Siegel 1978; Bult and Lynch 1996). Therefore, additional selection experiments for behavioral traits are required using various taxa of animals.

We are aware that the very small population size in this study (10 males and 10 females) makes it difficult to draw firm conclusions regarding the effect of selection because the population was subject to strong effects of drift and inbreeding over the course of the study (see Ödeen and Florin 2000). However, our results still show direct responses to forward and reverse selection, and thus we regard the general conclusion as acceptable for the effects of selection, but not for drift. Additional selection experiments with larger populations are needed.

Miyatake et al. (2008) revealed that beetles in the S-strains walked farther and showed higher expression of brain dopamine than did those in the L-strains. If death-feigning behavior is correlated genetically with walking ability and biogenic amines, these related traits might also be changed by relaxation of forward selection or reverse selection for duration of death-feigning. Investigations of responses to relaxation and reverse selection in related traits would also be interesting and informative.

Beetles living under natural conditions encounter selection pressures by predators; similarly, many kinds of selection pressure act upon the antipredator behaviors and life-history traits of every wild population, as noted in other animals (see van Oers et al. 2003; Brodin and Johansson 2004; Wund et al. 2015). Examining the relationship between selection pressures

and antipredator behaviors, including death-feigning and running escape, in wild populations is highly interesting. The present results further elucidate evolutionary abilities and predictable responses to selection in the domain of antipredator behavior, and the costs of antipredator behaviors in environments without predators.

Compliance with ethical standards

Conflict of interest K. Matsumura and T. Miyatake declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. We followed all of the Committee on Publication on Ethics (COPE) guidelines.

Research involving human participants and/or animals This article does not contain any studies with human participants performed by any of the authors.

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