Tonically immobilized selfish prey can survive by sacrificing others

Takahisa Miyatake[1,*], Satoshi Nakayama[1], Yusuke Nishi[1,2] and Shuhei Nakajima[3]

[1]*Graduate School of Environmental Science, and* [3]*Graduate School of Natural Science, Okayama University, Okayama 700-8530, Japan*
[2]*Okayama Prefectural Agricultural Experiment Station, Okayama 709-0801, Japan*

Death-feigning, also called tonic immobility, is found in a number of animal species across vertebrate and invertebrate taxa. To date, five hypotheses have been proposed for the adaptive significance of tonic immobility. These are that tonic immobility is effective for prey because (i) avoiding dead prey is safer for predators, (ii) immobility plays a role in physical defence, (iii) immobility plays a role in concealment and/or background matching, (iv) predators lose interest in unmoving prey, and (v) the characteristic immobilization posture signals a bad taste to predators. The fourth and fifth hypotheses have been considered suitable explanations for tonic immobility of the red flour beetle against its predator, the jumping spider. In the present study, we used chemical analyses of secretions by the red flour beetles under attack by the jumping spider to reject the fifth hypothesis for this system. More importantly, we tested a selfish-prey hypothesis for the adaptive significance of death-feigning as an anti-predator strategy, in which individuals adopting tonic immobility survive by sacrificing neighbours. Findings showed that survival rates of feigners were higher when in the presence of non-feigners or prey of a different species, compared to when alone, thus confirming our selfish-prey hypothesis. In summary, our results suggest that immobility following a spider attack is selfish; death-feigning prey increase their probability of survival at the expense of more mobile neighbours.

Keywords: death-feigning; jumping spider; benzoquinone; selfishness; thanatosis

1. INTRODUCTION

Many animal species, including humans, enter a state of natural paralysis when they are threatened (e.g. Miyatake *et al.* 2004, 2008; Ruxton *et al.* 2004; Gerald 2008). This immobile state is variously called tonic immobility, hypnosis, thanatosis, catatonia, playing possum, playing dead or death-feigning. Catatonic animals can conceal their presence not only from predators (Miyatake *et al.* 2004), but also from harassing mates (Dennis & Lavigne 1976) and from aggressive conspecifics (Lawrence 1992; van Veen *et al.* 1999; Bilde *et al.* 2006; Cassill *et al.* 2008). However, until recently, research into the adaptive significance of tonic immobility has been bypassed in evolutionary studies (but see Miyatake *et al.* 2004, 2008; Bilde *et al.* 2006; Honma *et al.* 2006; Ruxton 2006; Ohno & Miyatake 2007; Cassill *et al.* 2008; Gerald 2008; Hansen *et al.* 2008).

To date, five hypotheses for the usefulness of death-feigning as a defence against predators have been proposed:

(i) The 'avoiding dead prey' hypothesis: if a predator recognizes that a prey is dead, it will avoid the dead prey because of the possibility of disease acquisition. This behaviour of prey should be termed as death-feigning in the narrow sense only in cases in which a predator can recognize whether a prey is dead or alive.

(ii) The 'concealment and/or background matching' hypothesis: often an insect dropped from a perch will freeze tonically on the ground. If the dark body colour of the insect blends into the ground, the predator may lose sight of it (Ohno & Miyatake 2007). In this case, tonic immobility plays a role in concealment and/or background matching.

(iii) The 'physical defence' hypothesis: a tonically immobile grasshopper can adopt a characteristic posture that enlarges its functional body size by stretching each of its body parts, thereby making it difficult for frogs to swallow the grasshopper; thus, in this case tonic immobility constitutes a physical defence (Honma *et al.* 2006).

(iv) The 'loss of predator's interest' hypothesis: immobility is adaptive for defence against hunters who respond to moving prey (Miyatake *et al.* 2004). A predator's interest may be lost when the prey is immobilized, and the predator may search for other prey.

(v) The 'chemical alarm signalling' hypothesis: the posture of prey during tonic immobility may signal its unpalatability (Ruxton 2006). For example, predators can learn to avoid eating organisms armed with a strong chemical defence, and immobility can signal this defence to predators.

The red flour beetle, *Tribolium castaneum* (Herbest), freezes, thereby feigning death, when it is attacked by the Adanson's house jumper spider, *Hasarius adansoni* Audouin

(Miyatake *et al.* 2004). The jumping spiders are not scavengers, but they are chase-and-capture hunters that ingest only newly killed prey. This prey–predator relationship is a suitable model system to test the adaptability of tonic immobility (Miyatake *et al.* 2004) because the jumping spiders and flour beetles cohabit on rice bran or corn flour in cereal storehouses (T. Miyatake 2007–2008, personal observation), and the beetles frequently immobilize tonically when stimulated through predation (Miyatake *et al.* 2004, 2008). A previous study (Miyatake *et al.* 2004) has shown that the avoidance rate of *T. castaneum* by *H. adansoni* is significantly higher for beetles derived from strains selected for longer duration of immobility (long strain) than for those from strains selected for shorter duration (short strain). Miyatake *et al.* (2004) have suggested that the differential survival rate between these strains when attacked might be attributed to the fourth hypothesis described above: loss of predator interest.

Here we propose another, broader hypothesis for the adaptive significance of tonic immobility: that a tonically immobilized selfish prey survives by sacrificing its neighbours, especially in group or community-living organisms. Many organisms, especially herbivorous species, live in groups and cohabit in communities (e.g. Bertram 1978; Krause & Ruxton 2002). For example, we often find many species of larvae and adult beetles, including *T. castaneum*, and many larvae of stored-product moths in wheat or rice storehouses (T. Miyatake 2007–2009, personal observation). In such situations, immobilized individuals may increase their survival by sacrificing others who do not immobilize, but instead flee, when they are attacked by predators. Predators such as spiders and sucking predatory insects are known to target moving prey more often than immobilized prey (Jackson & Pollard 1996; T. Miyatake & S. Nakayama 2008, personal observation). Therefore, immobilization may evolve as a form of selfish behaviour (see Hamilton 1971; Beauchamp 2007). In the previous study by Miyatake *et al.* (2004), the adaptive significance of tonic immobility was examined by presenting a predatory spider with an individual beetle from either the short or long strain. However, a more realistic test of the relative survival success of the two strains should examine beetles from the two strains when presented to a predator together, and also compare the survival of feigners when alone, and with non-feigners.

In the present experiment, we first compared the survival of a lone feigner with that of a feigner in the presence of a conspecific non-feigner. Second, we compared the survival of feigners and non-feigners with that of a closely related beetle species, *Tribolium confusum*. Third, we examined which beetle was attacked by a spider when two beetles, one each derived from the short and long strains, were presented to the predator. Fourth, we compared the survival rates of beetles under predation risk when five short strain and five long strain beetles were mixed in a group. Finally, we test the hypothesis (number (v), above) that, rather than feigning death, the beetles are signalling to predators their possession of a chemical defence. *Tribolium* beetles release methylbenzoquinone (MBQ; Happ 1968) when they are stimulated by predators, and this chemical is assumed to function as an external defence to deter predators (Yezerski *et al.* 2004). If predator avoidance is the result of chemical defence, rather than tonic immobility *per se*, we predict

that *T. castaneum* strains selected for increased tonic immobility might have stronger chemical defences than control strains (Ruxton 2006). Therefore, we compared internal amounts of MBQ between *T. castaneum* strains that had been bred for different durations of tonic immobility, and compared the percentage of beetles releasing MBQ when attacked by a jumping spider.

2. MATERIAL AND METHODS

(a) *Insects culture and artificial selection*
The *T. castaneum* beetle culture used in this study has been maintained in a laboratory environment for more than 25 years. The beetles were reared on wholemeal (Yoshikura Shokai, Tokyo, Japan) enriched with brewer's yeast (Asahi Beer, Toyko, Japan) and kept in a growth chamber (Sanyo, Tokyo, Japan) maintained at $25 \pm 1°C$ and 60 per cent RH under a photoperiod of 16 L : 8 D cycle (lights were switched on at 07.00, and off at 23.00). The protocol of artificial selection for duration of tonic immobility and the method for observation of tonic immobility (death-feigning) is described in detail in Miyatake *et al.* (2004, 2008). Briefly, 100 males and 100 females were randomly selected from the base stock culture and their death-feigning behaviour was observed (F0 generation). The males and females (ten each) with the shortest duration of death-feigning were selected to propagate the short strain (S strains); similarly, the ten males and females with the longest duration were selected to propagate the long strains (L strains). The males and females of each strain were placed in a small plastic cup with medium and allowed to copulate and lay eggs for one week. Pupae arising from the eggs were stored as separate-sex groups in different plastic cups and allowed to emerge. When the adults reached 10–15 days of age, 100 males and 100 females were randomly selected from each strain and their death-feigning observed again (F1 generation). Two selection replicates for the short and long strains (S-1 and L-1; and S-2 and L-2) were established in this manner and subsequently maintained in the chamber under the same conditions. The selection regimes were continued for more than 20 generations (see Miyatake *et al.* 2008). After 20 generations, S beetles never feign death; the frequencies of individuals showing tonic immobility by artificial stimuli and the durations of immobility were 0 per cent and 0.00 ± 0.00 (mean $\pm$ s.d.) seconds ($n = 100$), respectively, in S-1 strain. Whereas L beetles almost always feign death; the frequency was 99 per cent and the duration was 1490.20 ± 1228.42 s ($n = 100$) in L-1 strain.

Adult females of Adanson jumper spider, *H. adansoni*, were used for predation experiments. For each experiment, different inexperienced beetles and spiders were used. The sex of beetles was not recorded. Each female spider was maintained in a plastic Petri dish (8 cm diameter and 2 cm height) with wet cotton and was fed larvae of *T. castaneum* or adults of *Drosophila hydei* Sturtevant. Before the tests, each spider was starved for one week.

(b) *Survival rates of feigners when alone and with non-feigners*
The survival rates of feigners (L-1 strain) when alone ($n = 16$), and when with non-feigners (S-1 strain; $n = 35$), were compared under predation risk. Beetles were placed in a plastic Petri dish (diameter 9 cm and height 1.5 cm) with an individual spider, and attacking and predation behaviours

were observed for 15 min or until the spider killed a beetle. The χ^2 test was used to compare predation rates between the two treatments.

(c) Survival rates of feigners and non-feigners when with other species

The survival rates of a feigner (L-1 strain) and a non-feigner (S-1 strain) when they were with an adult of *T. confusum*, a closely related species, were examined under predation risk. Beetles were placed in a plastic Petri dish (diameter 9 cm and height 1.5 cm) with a single spider and observed until the spider had killed one of the beetles or for 15 min. Experiments were replicated 18 and 16 times for S and L strains, respectively. The *T. confusum* population used in this experiment has been maintained for several years at the National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba, Japan (Suzuki & Nakakita 1991). Prior to the start of the experiment we determined that the frequency of individuals showing tonic immobility by artificial stimuli was 37.0 per cent ($n=100$) and the duration of immobility was 11.56 ± 31.96 (mean $\pm$ s.d.) seconds ($n=37$) for this population. The χ^2 test was used to compare the two treatments.

(d) Effectiveness of tonic immobility in pairs and in groups

In a previous experiment (Miyatake *et al.* 2004), the effectiveness of tonic immobility was compared separately in short and long strains. In the present study, the effectiveness of tonic immobility was analysed when beetles derived from short and long strains were put together under two treatments. First, we compared the predation avoidance rate of beetles of short and long strains when in pairs. Two beetles, one each from the short and long strains, were put into a plastic Petri dish (8 cm diameter and 2 cm height) with a spider. Predation behaviour was observed until one of the beetles was killed by the spider. Nineteen trials each were performed for beetles from S-1 and L-1 strains.

Second, the survival rates of beetles were compared when in groups. Five beetles (3–4 weeks old) from each strain (S and L) were randomly chosen as prey and marked with a dot on their elytra with a paint marker (white: Paint Maker PX-1, Mitsubishi, Tokyo, Japan) to distinguish their strain. The 10 beetles, five S adults and five L adults, and an adult female spider which had been starved for 10 days before the experiment, were placed in a plastic Petri dish (diameter 9 cm and height 1.5 cm) and observed until one individual was attacked and killed by the spider or for 15 min. This trial was replicated seven times using different prey and predators each time (14 times in total: 2 treatments, with either S or L marked white; and 7 replications of each). For both pair and group treatments, the χ^2 test was used to compare survival rates of the two strains.

(e) Benzoquinone

The internal methyl-1,4-benzoquinone (98% MBQ: Sigma-Aldrich Japan, Tokyo) concentrations of individual adult beetles from the two selection strains were compared. Each male was put into a glass mini-vial (1.5 ml, 35 mm height and 11.75 mm diameter; MV-07, TGK Ltd., Tokyo, Japan) with 100 µl of diethyl ether, and crushed using a glass stick. One hour after crushing, 10 µl of decane ($C_{10}H_{22}$) diluted with diethyl ether (0.1 mg ml^{-1}) was added and the solution was analysed using gas chromatography (G-3000,

Hitachi Ltd., Tokyo) with a DB1 column (0.25 mm × 30 m; J&W Scientific, Folsom, CA, USA). The column was maintained at 50°C for the first 5 minutes, after which the temperature was increased to 230°C at a rate of 10°C min^{-1}; and this peak temperature was maintained for 30 min. The relative quantity of MBQ was determined by measuring the ratio of the peak area of MBQ to the peak area of decane. The measurements were performed for 20 males from each of the two replicates of each of the two selection strains, thus 80 males were assayed ($=20 \times 4$ strains) in total. The Mann–Whitney U test was used to compare the relative quantities of MBQ between selection regimes and within each selection replicate (i.e. S-1 versus L-1 and S-2 versus L-2).

In addition, the percentage of beetles that released MBQ when attacked by *H. adansoni* was compared between the short and long strains. One adult male *T. castaneum*, from either the short or long strain, and one adult female jumping spider were placed in a glass vial (upper diameter 12 mm, bottom diameter 15 mm and height 40 mm), the vial was sealed tightly with laboratory film (Parafilm, PM-996, Alcan Packaging Co., Neenah, WI, USA) and attacking behaviours were observed. When a female jumping spider is provided with a *Drosophila* fly, the spider never sets the fly free, but eats it immediately in the first attack (Miyatake *et al.* 2004). However, when the prey consists of a flour beetle, the spider usually initially relinquishes its hold on the prey, probably because of the hard cuticle of the beetle. If the prey then struggles or moves following this first attack, it is immediately attacked again and then is usually eaten. However, if the prey becomes tonically immobilized and remains immobile after the attack, the spider often loses interest and the prey may survive (Miyatake *et al.* 2004). In the present study, we pierced the parafilm with a fibre (SPME Fiber Assembly 100 µm Polydimethylsiloxane Coating for Manual Holder Red, Supelco, Bellefonte, PA, USA) attached to a SPME Holder 57330-U (Supelco) to absorb the chemicals in the air of the vial for five minutes immediately after the beetle was attacked by the spider. The materials absorbed from the air were analysed via gas chromatography using the method described above. To analyse the difference in the number of beetles releasing MBQ between the strains, we used multiple logistic regression analysis with the number of beetles releasing MBQ as the dependent variable and strains, selection replicates and their interaction as explanatory variables. If no significant effect of interaction was found, this effect was removed, and we reanalysed with strains and selection replicates as explanatory variables. The statistical package JMP v. 6.03 was used for all analyses (SAS Institute 2005).

3. RESULTS
(a) Survival rates of feigners alone and with non-feigners

The results shown in figure 1*a* indicate that 38 per cent of L strain beetles were eaten by the spider when they were alone, while only 8.6 per cent L strain beetles were killed by the spider when presented in conjunction with an individual from the S strain (figure 1*a*: $\chi^2=5.88$, $p=0.0153$).

(b) Survival rates of feigners and non-feigners with other species

The results shown in figure 1*b* indicate that 27.7 per cent of L strain beetles and 62.5 per cent of S strain beetles were eaten by the spider when they were with a *T. confusum*
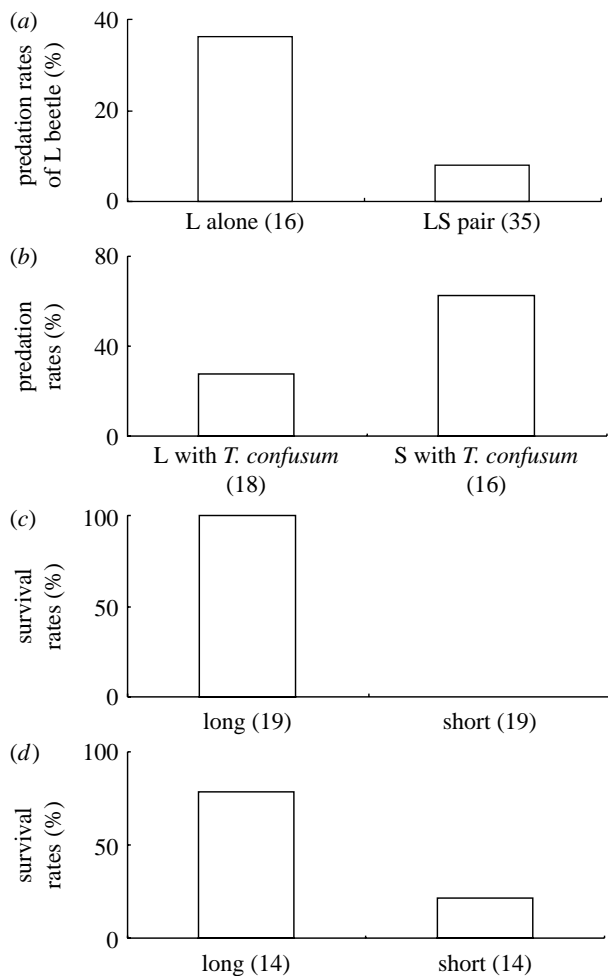
Figure 1. (*a*) Predation rates of long strain beetle when alone and with short strain beetle, (*b*) survival rates of long and short strain beetles when they are placed with *T. confusum* beetle and a spider and survival rates of long and short strain beetles when they are (*c*) in pairs and (*d*) in group under predation pressure.

beetle. A significant difference in predation rates was found between short and long strains when they were cohabited with a *T. confusum* beetle (figure 1*b*: $\chi^2 = 4.22$, $p = 0.0399$).

(c) *Effectiveness of tonic immobility in pairs and in groups*

When a pair of beetles, one each derived from the short and long strains, were placed with a spider, the beetle derived from the short strain was eaten every time (figure 1*c*: $n = 19$, $\chi^2 = 34.1053$, $p < 0.0001$). For the group experiment, no significant effect of marking was found ($\chi^2 = 0.190$, $p = 0.6625$), and therefore all replicates ($n = 14$) were pooled and reanalysed. The pooled data shown in figure 1*d* indicate that the predation rates were 78.6 per cent ($n = 14$) and 21.4 per cent ($n = 14$) in short and long strains respectively ($\chi^2 = 4.591$, $p = 0.0325$). Both comparisons, i.e. pairs and groups, indicated that long strain beetles had higher survival rates than short strain beetles when they were simultaneously placed with a spider.

(d) *Benzoquinone*

Relative internal quantities of MBQ did not differ between short and long strains, and the direction of difference differed between replicates (replicate 1: 5.9 ± 2.8 and

4.2 ± 1.6; $Z = 1.89$, $p = 0.06$; replicate 2: 4.4 ± 1.9 and 5.8 ± 3.2; $Z = 1.57$, $p = 0.12$).

The frequencies of beetles releasing MBQ when they were attacked by a spider were 33.3 per cent ($n = 12$) in S-1, 44.4 per cent ($n = 9$) in L-1, 54.5 per cent ($n = 11$) in S-2 and 30.0 per cent ($n = 10$) in L-2 strains. Because logistic regression analysis showed no significant effect of interaction (strain $\times$ selection replicate; $p > 0.1$), we reanalysed after removing this effect. The second model showed that there was no significant difference between strains in the frequency of individuals releasing MBQ ($\chi^2 = 0.2050$, $p = 0.6507$) and selection replicates ($\chi^2 = 0.1132$, $p = 0.7365$).

4. DISCUSSION

Survival rates of feigners were higher when in the presence of non-feigners or prey of a different species, compared to when alone, suggesting that the survival rate of a feigner increases when there are non-feigners around, when faced with the risk of predation. Our results suggest that immobility following a spider attack is an effective survival strategy, at the expense of more mobile neighbours.

This is the first direct investigation into the adaptive significance of tonic immobility. Previously survival rates had been compared using only one beetle from each of the short and long strains (Miyatake *et al.* 2004). In nature, *T. castaneum* lives on the surface of wheat flour or rice bran in cereal storehouses, and usually coexists with other beetles (adults and larvae) of the same and other species including *T. confusum* (J. du V.), *Palorus ratzeburgi* (Wissmann), *Palorus subdepressus* (Wollaston) and *Tenebrio molitor* Linnaleus; this environment also usually supports many other arthropod product pest species; for example, moths including *Ephestia kuehniella* (Zeller), *Cadra cautella* (Walker) and *Plodia interpunctella* (Hubner; T. Miyatake 2007–2009, personal observation). In this community of prey, predators such as jumping spiders may respond more to moving prey than to non-moving prey. If the prey struggles or moves after the first attack by a spider, the spider immediately attacks it again and, usually, the prey is eaten (Miyatake *et al.* 2004). However, if the prey shows thanatosis, or tonic immobility, and remains immobile after the first attack, the spider often loses interest and the prey survives. Spiders appear to rely on prey locomotion in order to catch prey, and in addition may rely on tactile movement signals to initiate the kill behaviour. Potentially the latter informs the spider that its food is still alive. This expression of the predator's character and prey's actions probably caused the significant difference in predation frequency between immobilized and moving individuals, and it may cause the evolution of tonic immobility behaviour. These results may explain the prevalence of tonic immobility or death-feigning in nature, especially in group-living organisms. Thus, a selfish prey immobilized tonically can survive by sacrificing its neighbours in the group or community in a manner akin to the idea of the selfish herd (Hamilton 1971; Beauchamp 2007). This study is the first to use Hamilton's selfish herd hypothesis to explain the evolution of death-feigning behaviour in group-living prey. However, the selfish hypothesis is not sufficient to explain our results as tonic immobility reduces predation even in the absence of neighbours (Miyatake *et al.* 2004).

Ruxton (2006) suggests that, rather than feigning death *per se*, tonic immobility could be a way of signalling a chemical defence; therefore beetles selected for increased duration of tonic immobility might also be expected to have greater chemical defences than control lines. However, the present study suggests that this is not the case, at least for this prey–predator system. We found no differences in the quantity of MBQ released into the air when either short or long strain beetles were caught by a spider, suggesting that in *T. castaneum* there is no genetic linkage between chemical defence and the death-feigning posture. The present results suggest there is an adaptive significance to immobility in itself, unrelated to the release of defensive chemicals. Such a system may however occur in other prey–predator systems, for example where prey exhibit an alarm colour and show immobility; the newt *Cynops pyrrhogaster*, which has a red-coloured abdomen and poisonous chemicals within its body, turns over on its back and is immobilized when appropriate stimulus is provided (Brodie 1977). In this case, predators might recognize the colour and the immobilized posture may play a role in alarm signalling.

In conclusion, we found that tonically immobilized selfish prey have increased survival, at the cost of their neighbours; survival rates of feigners significantly increased when with conspecific non-feigners, and when with prey of a different species, compared to when alone. It would be intriguing to examine the prevalence of tonic immobility or death-feigning behaviour in other prey–predator systems in community-living animals.

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