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Daily rhythm of insecticide susceptibility in the silkworm *Bombyx mori*

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The present study investigated daily changes in susceptibility to insecticides in the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). The daily rhythm of permethrin susceptibility in fifth instar larvae of the p63 silkworm strain was significant, with the lowest mortality rate during the late photophase under a 12 h light – 12 h dark cycle. The rhythms were similar whether permethrin was intraperitoneally injected into hemolymph or topically applied to the dorsal abdomen. Drug intake through the cuticle probably did not significantly contribute to the organization of this susceptibility rhythm. We also found that the daily susceptibility profiles depended on insecticide type and silkworm strain. Temporal susceptibility variations might be attributable to the drug metabolic system. To our knowledge, this is the first description of an insecticide susceptibility rhythm in the larval stage of lepidopteran insects. Specifically timed delivery of insecticides might enable more efficient control of pest caterpillars that cause serious crop damage.

Keywords: *Bombyx mori*; insecticide susceptibility rhythm; lepidopteran larvae

1. Introduction

The behavioural and physiological processes of most organisms including insects, exhibit daily rhythms (Dunlap et al. 2004; Saunders 2002) that synchronize to environmental fluctuations and consequently increase the fitness of organisms. Several studies have found daily susceptibility rhythms to insecticides in insects: for example, in the pine weevil, *Hylobius abietis* (Pszczolkowski and Dobrowolski 1999) and the mosquito, *Aedes aegypti* (Yang et al. 2010) to pyrethroids, as well as in the fruit fly, *Drosophila melanogaster* (Hooven et al. 2009) to carbamate, phenylpyrazoles and organophosphates. Thus, time-specific delivery of insecticides to target insects when they are most susceptible would allow more efficient control of insect pests.

Lepidopteran larvae cause serious crop damage; however, although Ware and McComb (1970) reported the daily susceptibility rhythm to the acetylcholinesterase inhibitor, azinphosmethyl (organophosphate), in adult moths of the pink bollworm *Pectinophora gossypiella*, daily variations in susceptibility to insecticides have not been determined in the larval stage of lepidopteran insects as far as we can ascertain.

The silkworm *Bombyx mori* (L.) has served as a lepidopteran model insect for studies of drug toxicity (Hamamoto et al. 2009). This species is suitable for physiological and biochemical studies, since it can be easily reared to generate large populations with a genetically uniform background and it has a large enough body to facilitate surgical procedures. Furthermore, a recent study of the silkworm genome has showed that there is extensive synteny conservation between *Bombyx mori* and wild noctuid moths (d'Alençon et al. 2010). Therefore, the present study investigated daily changes in the susceptibility of *Bombyx mori* to insecticides.

2. Materials and methods

2.1. Animals

The p63 (Chinese improved) strain with sex-limited larval markings was the subject of all experiments including a comparison with the p20 (Japanese native) and p50 (Chinese native) strains. Silkworm eggs provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Science, Sports and Culture of Japan (http://www.shigen.nig.ac.jp/silkwormbase/index.jsp) were incubated at 25°C under continuous darkness. Hatched larvae were fed with the Silkmate PS artificial diet (Nihonnousan Kogyo Co. Ltd., Yokohama, Japan), and reared at 25°C under daily 12 h light – 12 h dark cycles (lights on: 0:00 – 12:00 h). The Animal Care and Use Committee at Kobe University approved all experiments and procedures.

2.2. *Insecticide administration*

The insecticides, permethrin (pyrethroid, sodium channel modulator; 20% flowable, Sumitomo Chemical Co., Ltd, Tokyo, Japan), acephate (organophosphate, acetylcholinesterase inhibitor; 15% flowable, Sumitomo Chemical Garden Products, Inc., Tokyo, Japan) and emamectin benzoate (avermectin, chloride channel activator; 1% flowable, Syngenta Japan, Tokyo, Japan) were diluted to appropriate working concentrations with either acetone or saline (0.8% NaCl) for topical application (6 μL) to the dorsal abdomen using micropipets or for intraperitoneal injection (6 μL) into hemolymph using Hamilton syringes, respectively.

2.3. *Measurement of temporal fluctuations in insecticide susceptibility*

Insecticide susceptibility was investigated in male larvae on days 2 and 3, or only on day 2 of the fifth instar (where day 0 was defined as the day of the fourth larval molt). Mortality was assessed six hours after drug treatment and moribund larvae, namely those that fell from a paper towel placed at a 60° incline were taken as being dead. We initially determined the median lethal dose (LD_{50}) for each agent at the midpoint (6:00 h) of the photophase on each experimental day in 200 larvae using Probit analysis (Finney 1971) (Table 1). Then, we applied the LD_{50} of each insecticide at 3:00, 9:00, 15:00 and 21:00 h on each experimental day and compared larval mortality rates to determine temporal fluctuations in susceptibility. Eight or nine groups (n = 10 larvae each) were assessed at each time point. Mean values were statistically analyzed using one-way ANOVA followed by the Tukey HSD test. $P < 0.05$ was considered significant. We observed daily susceptibility variations in larvae of a different batch from that we used for the determination of LD_{50}. Therefore, the temporal mortality curves did not exactly pass the 50% point at 6:00 h in some experiments.

3. Results and discussion

3.1. Daily susceptibility rhythm to permethrin

The daily rhythm of susceptibility to the sodium channel modulator, permethrin, was significant in the silkworm larvae (Fig. 1). The susceptibility oscillated with the lowest mortality at 9:00 h during the late photophase on days 2 and 3 of the fifth instar. The finding of rhythmic insecticide susceptibility in domestic silkworms indicated that wild lepidopteran larvae also have such rhythms. In addition, the rhythms were similar when permethrin was either intraperitoneally injected (Fig. 1a) or topically applied (Fig. 1b). Drug intake through the cuticle probably did not significantly contribute to the organization of this susceptibility rhythm.

3.2. Temporal susceptibility to different insecticides

We also found significant daily changes in the susceptibility of silkworms to the acetylcholinesterase inhibitor, acephate, and the chloride channel activator, emamectin benzoate (Fig. 2). However, the susceptibility rhythms to both of these insecticides differed from that to permethrin. Susceptibility to acephate peaked at 9:00 h during the late photophase and that to emamectin benzoate was lowest at 21:00 h during the late scotophase. The daily susceptibility of *Bombyx mori* depended on the nature of the tested insecticides, as it does in other insect species such as the malarial mosquito *Anopheles gambiae* (Balmert et al. 2014). The *Drosophila* enzymes that are involved in insecticide detoxification appear to have daily fluctuations in susceptibility (Hamby et al. 2013). Temporal susceptibility variations might be attributable to the metabolic systems involved in detoxifying each insecticide in *Bombyx mori*.

3.3. Susceptibility rhythm in different strains

We found that daily insecticide susceptibility profiles differed among silkworm strains (Fig. 3). Permethrin susceptibility was lowest at 21:00 h during the late scotophase in the Japanese native p20 strain, but at 9:00 h during the late photophase in the Chinese native p50 and Chinese improved p63 strains (*cf*. Fig. 1). Differences in susceptibility rhythms between local populations of target species should be taken into account when considering pest control.

3.4. Conclusions

The present study found that the larvae of a lepidopteran species (*Bombyx mori*) have daily susceptibility rhythms to insecticides. The temporal susceptibility profiles depended on insecticide type and silkworm strain. Caterpillar pests could be more efficiently controlled if the rhythmic properties of susceptibility to insecticides are considered.

Acknowledgements

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Disclosure

We do not have any potential conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

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Table 1. The median lethal dose (LD_{50}) of insecticides to larvae of the silkworm *Bombyx mori*.

Insecticide	Administration method	Silkworm strain	Experimental day (in fifth instar)	LD_{50} (μg/each larva)
Permethrin	Topical application	p63	Day 2	0.060
	Topical application	p63	Day 3	0.065
	Injection	p63	Day 2	0.140
	Injection	p63	Day 3	0.224
	Topical application	p20	Day 2	0.043
	Topical application	p50	Day 2	0.045
Acephate	Topical application	p63	Day 2	33.208
Emamectin benzoate	Injection	p63	Day 2	9.775

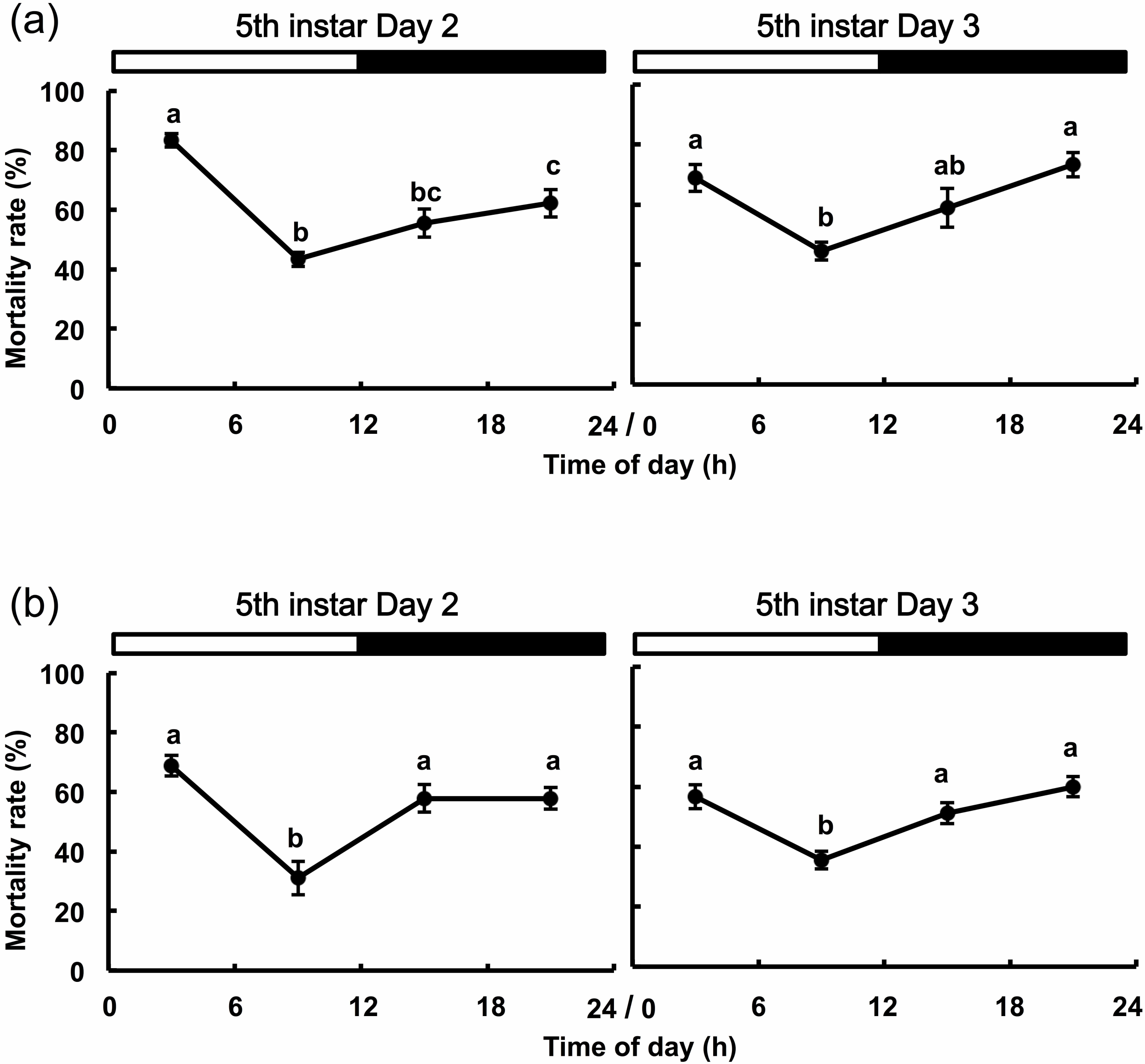
We determined the median lethal dose (LD_{50}) for each insecticide at the midpoint (6:00 h) of the photophase on each experimental day in 200 male larvae using Probit analysis (Finney 1971). Insecticides were diluted to appropriate working concentrations with either acetone or saline for topical application to the dorsal abdomen using micropipets or for intraperitoneal injection into hemolymph using Hamilton syringes, respectively.

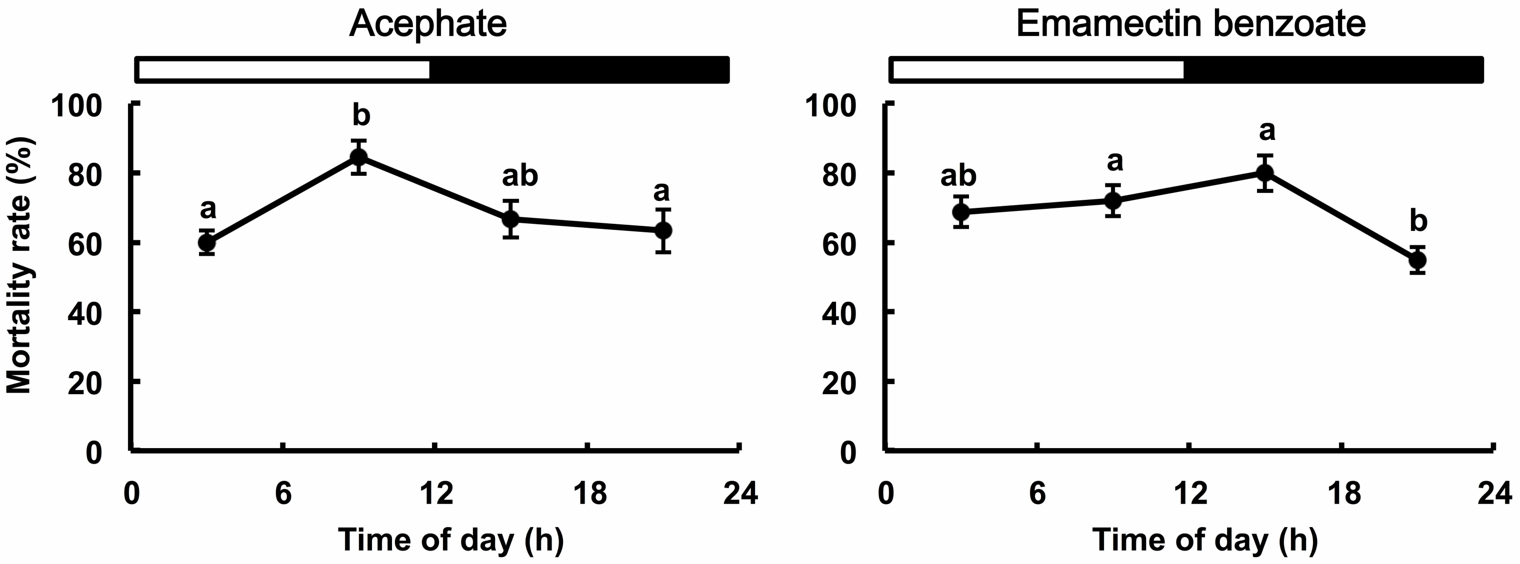
Figure captions

Figure 1. Daily changes in susceptibility of silkworm larvae of the p63 strain to permethrin under 12 h light – 12 h dark cycles (lights on 0:00-12:00 h). Permethrin at LD_{50} doses determined at 6:00 h on each experimental day was administered (a) topically to the dorsal abdomen or by (b) intraperitoneal injection to hemolymph. Unfilled bars, lights on; filled bars, lights off. Data are shown as means ± SEM ($n = 9$ groups of 10 larvae each). Superscript letters represent significant differences in mortality among time points (ANOVA and Tukey HSD, $P < 0.05$).

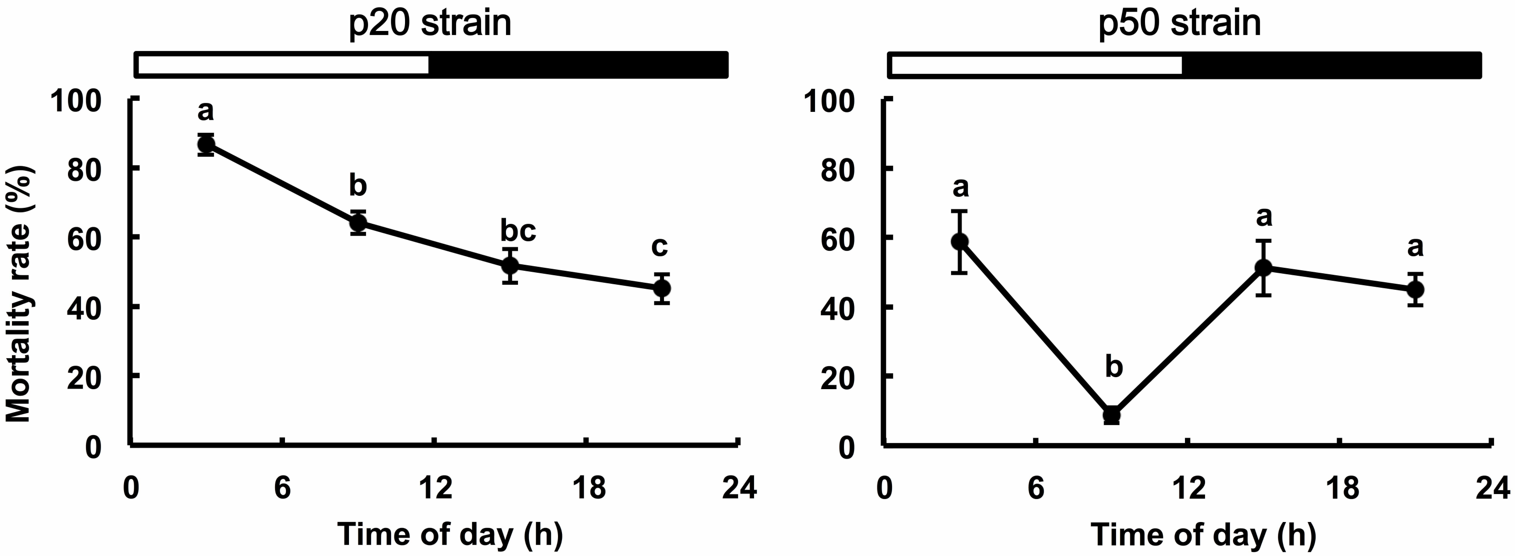
Figure 2. Daily changes in susceptibility of p63-strain silkworm larvae to acephate and emamectin benzoate on day 2 of the fifth instar under 12 h light – 12 h dark cycles (lights on 0:00-12:00 h). Acephate or emamectin benzoate at LD_{50} doses determined at 6:00 h on each experimental day was administered topically to the dorsal abdomen or by intraperitoneal injection into hemolymph, respectively. This is because emamectin benzoate was ineffective when topically administered. Unfilled bars, lights on; filled bars, lights off. Data are shown as means ± SEM ($n = 9$ groups of 10 larvae each). Superscript letters represent significant differences in mortality among time points (ANOVA and Tukey HSD, $P < 0.05$).

Figure 3. Daily changes in susceptibility of p20- and p50-strain silkworm larvae to permethrin on day 2 of the fifth instar under 12 h light – 12 h dark cycles (lights on 0:00-12:00 h). Permethrin at LD_{50} doses determined at 6:00 h on each experimental day was administered topically to dorsal abdomen. Unfilled bars, lights on; filled bars, lights off. Data are shown as means ± SEM ($n = 8$ groups of 10 larvae each). Superscript letters represent significant differences in mortality among time points (ANOVA and Tukey HSD, $P < 0.05$).


(a)
5th instar Day 2
5th instar Day 3
Mortality rate (%)
0
20
40
60
80
100
a
b
bc
c
a
b
ab
a
0
6
12
18
24 / 0
6
12
18
24
Time of day (h)
(b)
5th instar Day 2
5th instar Day 3
Mortality rate (%)
0
20
40
60
80
100
a
b
a
a
a
b
a
a
0
6
12
18
24 / 0
6
12
18
24
Time of day (h)



Acephate
Emamectin benzoate
Mortality rate (%)
100
80
60
40
20
0
a
b
ab
a
ab
a
a
b
0
6
12
18
24
Time of day (h)


p20 strain
p50 strain
Mortality rate (%)
0
20
40
60
80
100
a
b
bc
c
a
b
a
a
0
6
12
18
24
Time of day (h)
Time of day (h)