Relationship of Larval Phase and Susceptibility of the Armyworm, *Pseudaletia separata* WALKER (Lepidoptera: Noctuidae) to a Nuclear Polyhedrosis Virus and a Granulosis Virus

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Larval susceptibility in solitary and gregarious phases of *Pseudaletia separata* to nuclear polyhedrosis virus (PsNPV) and granulosis virus (GVH) was examined under laboratory condition. As the rearing density was decreased, larvae became more susceptible at the 5th instar and died within a shorter period when perorally inoculated with PsNPV. The larvae reared in isolation were more susceptible than those reared in crowded condition when the 2nd instar larvae were perorally inoculated with GVH. The larvae which had been transferred from isolated to crowded condition or vice versa after viral administration had intermediate susceptibility to PsNPV infection relative to the susceptibility of larvae reared in isolated and in crowded condition throughout the larval stage. The pale larvae produced from crowded rearing were slightly more susceptible than the dark larvae. No differences in susceptibility and time of death were observed between the solitary and the gregarious larvae when PsNPV was injected into the 5th instar larvae.

INTRODUCTION

The armyworm, *Pseudaletia separata* WALKER, shows color polymorphism in the larval stages which occurs depending on population density (IWA0, 1962). At low population density larvae have pale cuticle, while black cuticle occurs as population density increases (IWA0, 1962). The “gregarious” larvae grow faster, consume more food, respond more actively to a physical disturbance, and are more tolerant of unpalatable food or starvation than the “solitary” larvae (IWA0, 1962, 1963; SHIBAZAKI, 1969). There are also differences in respiratory rates between these 2 types of larvae (SHIBAZAKI and ITÔ, 1969). However, little attempt has been made to investigate the effect of larval phase on susceptibility of *P. separata* larvae to entomogeneous viruses.

In this paper, differences in susceptibility of the gregarious and solitary phases of *P. separata* to a nuclear polyhedrosis virus and a granulosis virus are described.

MATERIALS AND METHODS

Insect. The armyworm, *P. separata* was originally supplied by Dr. S. YAGI and reared continuously in our laboratory. Larvae were reared on either maize leaves or on artificial diet (HATTORI and ATSUSAWA, 1980) supplemented with 800 ppm sorbic acid and hydroxybenzoic acid. The rearing chambers used were plastic containers

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(15 cm dia. $\times$ 9 cm in depth) placed in a 25°C incubator under a long photoperiod regime (16L–8D). Color grade of the larvae was determined according to IWA0 (1962).

Virus. The nuclear polyhedrosis virus used in this experiment was obtained from Dr. M. OKADA of Hokkaido National Agricultural Experiment Station. This nuclear polyhedrosis virus (PsNPV) was originally isolated from *P. separata* larvae collected in Hiroshima Prefecture in 1969 (OKADA, 1977). The PsNPV was propagated in laboratory-reared larvae of *P. separata*. Dead larvae were homogenized in an electric blender. The resulting suspension was filtered through 4 layers of gauze. The polyhedral inclusion bodies (PIBs) were purified by 2 cycles of centrifugation (500 rpm for 3 min, 3,000 rpm for 15 min). The resulting pellet was suspended in a small amount of phosphate-buffered saline (PBS). One volume of this suspension was layered on the top of 9 volumes of Percoll™ (Pharmacia) solution previously adjusted to pH 7.0 with 0.1 N HCl, and spun at 35,000 rpm for 30 min. A band containing PIBs was collected by Pasteur pipette. To remove residual Percoll™, PIBs were resuspended in PBS and recentrifuged twice at 5,000 rpm for 15 min. The number of PIBs was determined with Thoma hemocytometer.

Non-occluded virus used for the injection test was prepared by collecting and centrifuging the hemolymph of heavily infected 6th instar larvae at 10,000 rpm for 30 min. The supernatant containing the non-occluded PsNPV (NOPsNPV) was stored at –80°C until use.

The granulosis virus (GVH) used in this experiment was originally isolated from *P. unipuncta* larvae collected in Hawaii (TANADA and HUKUHARA, 1968). The GVH was propagated in laboratory-reared 2nd instar larvae of *P. separata*. Two hundred and fifty 6th instar larvae infected with GVH were homogenized in 100 ml of PBS. The resulting suspension was filtered through 4 layers of gauze. Viral occlusion bodies were purified by differential centrifugation. The final pellet containing granular inclusions was resuspended in 50 ml of PBS. Concentration of this virus suspension was denoted as 10^0 .

Bioassay procedures

1) Effect of rearing density on oral susceptibility to PsNPV: In all bioassays, larvae were continuously reared on artificial diet in a 25°C incubator with a 16L–8D photoperiod unless otherwise specified.

Neonate larvae from an eggmass were reared on maize leaves. At the end of the 1st instar, larvae were transferred to rearing densities of one, 2, 5, and 20 larvae per 50-, 100-, 200- and 430-ml volume plastic cups, respectively. Immediately after the 4th molt, the larvae were allowed to feed on PsNPV-treated maize leaf discs (1 cm dia.). Forty larvae were used for each dilution of PsNPV. Two days later, the larvae were fed uncontaminated leaves of maize until pupation.

2) Oral susceptibility of larvae to PsNPV when rearing density was shifted after administration: At the end of the 1st instar, larvae were divided into isolated or crowded rearing condition (20 individuals per container). Four experimental plots were constructed: (1) rearing in isolation from 2nd to final instar (S-S larvae), (2) rearing in isolation from 2nd to 4th instar and in crowded condition afterwards (S-G larvae), (3) crowded rearing from 2nd to final instar (G-G larvae), and (4) crowded rearing from 2nd to 4th instar and in isolation afterwards (G-S larvae). Larvae were transferred to these rearing conditions at the end of the 1st instar. Newly molted 5th

instar larvae were fed one gram of artificial diet containing 100 μ l of viral suspension. In all experimental plots 7 dilutions of PsNPV suspension were used for each group. Forty larvae used for each PsNPV dilution. Two days after administration of PsNPV, larvae were transferred to uncontaminated diet.

3) Oral susceptibility of solitary and gregarious larvae to GVH: Newly molted 2nd instar larvae were allowed to feed for 2 days on GVH-treated artificial diet (1 ml GVH suspension/10 g diet). After administration of GVH, the larvae were fed uncontaminated diet and reared in crowded condition (20 larvae per container) or in isolation for 36 days. The isolated and crowded larvae were reared in 50 and 430 ml plastic containers, respectively.

4) Oral susceptibility of pale and dark larvae to PsNPV: Crowded rearing (20 larvae/430-ml container) was used to produce color polymorphism in *P. separata*. The pale (color grade I, II) and dark (color grade III, IV) larvae were chosen from these cohorts at the beginning of the 5th instar. Larvae were fed PsNPV-treated diet containing 1 ml viral suspension per 10 g artificial diet. Fifteen larvae of each color type were used for each of the 5 dilutions of PsNPV. The larvae were transferred to uncontaminated diet 2 days after administration and placed in plastic containers (15 cm dia. $\times$ 9 cm ht.).

5) Percutaneous susceptibility of solitary and gregarious larvae to PsNPV: Newly molted 6th instar larvae which had been reared in crowded condition or in isolation from the 2nd instar were used for this experiment. Each larva was etherized and then injected with 10 μ l of each dilution of NOPsNPV using a hand microapplicator with 50 μ l microsyringe. Twenty larvae were used at each dilution. After injection, larvae were reared individually in 50 ml plastic containers on artificial diet for 10 days.

6) Calculation of LC_{50} and LT_{50} values: In all bioassays, larval mortality was monitored daily. Tissue smears prepared from dead larvae were examined for virus occlusions using a light microscope. Probit analysis was used to calculate the LC_{50} and LT_{50} values as described by FINNEY (1952).

RESULTS

Effect of rearing density on oral susceptibility to PsNPV

Frequency distribution patterns of color grade of 5th instar larvae were significantly different among larvae reared at different densities. With increase of rearing density,

Fig. 1. Frequency distribution of color types in the final instar larvae of *Pseudaletia separata* reared on fresh leaves of maize at different densities.

Fig. 2. Cumulative mortality for *Pseudaletia separata* larvae infected with a nuclear polyhedrosis virus under each rearing density.

Table 1. Summary of analysis of dose-mortality lines for *Pseudaletia separata* larvae infected with PsNPV under each rearing density

Rearing density	log LC ₅₀ (PIBs/ml)	95% fiducial limits		Slope ¹⁾ (b)	Intercept ¹⁾ (a)	Correlation coefficient
		Lower	Upper			
1	7.2	7.0	7.3	1.7	-7.2	0.97
2	7.5	7.3	7.7	1.4	-5.5	0.96
5	8.1	7.9	8.3	1.5	-7.1	0.94
20	8.2	7.9	8.4	1.2	-4.8	0.98

¹⁾ Regression model is $Y=a+b\log X$, where Y is mortality probit and X is numbers of polyhedra per one ml of suspension.

the larval coloration became darker (Fig. 1). Mean color grades (Iwao, 1962) of the 5th instar larvae reared at the densities of 1, 2, 5, and 20 individuals per container were estimated as 1.7, 2.6, 3.5, and 3.9, respectively.

Lethal time for the larvae administered with PsNPV was longer when larvae were reared at higher densities (Fig. 2). The relative susceptibility of the larvae reared at different densities was determined by comparing their LC₅₀ values (Table 1). The LC₅₀ values for larvae reared at the densities of 1, 2, 5, and 20 per container were estimated as 1.3×10^7 , 3.2×10^7 , 1.0×10^6 , and 1.3×10^8 PIBs/ml, respectively. With increase of rearing density, the larvae became less susceptible to oral infection with PsNPV. Individually reared larvae were ca. 2, 8 and 10 times more susceptible to PsNPV than the larvae reared at densities of 2, 5 and 20 per container, respectively. The slope of the dose-mortality regression line (b) obtained from density 20 plots was smaller than those from other density plots.

Oral susceptibility of larvae to PsNPV when rearing density was shifted after administration

As concentration of administered PsNPV was increased, LT₅₀ values decreased in

Table 2. Summary of analysis of dose-mortality lines for *Pseudaletia separata* larvae infected with PsNPV when rearing density was shifted after administration

Rearing density		log LC ₅₀ (PIBs/ml)	95% fiducial limits		Slope ¹⁾ (b)	Intercept ¹⁾ (a)	Correlation coefficient
1st-4th instar	5th-6th instar		Lower	Upper			
1	1	6.4	6.2	6.6	1.8	-6.8	0.9396
1	15	6.9	6.8	7.0	1.4	-4.9	0.9997
20	1	6.8	6.6	7.0	1.1	-2.3	0.9938
20	15	7.2	7.1	7.3	1.4	-5.1	0.9968

¹⁾ Regression model is $Y=a+b\log X$, where Y is mortality probit and X is numbers of polyhedra per one ml of suspension.

Fig. 3. LT₅₀ values for *Pseudaletia separata* larvae infected with a nuclear polyhedrosis virus when rearing density was shifted after administration. ○: S-S larvae, ●: S-G larvae, □: G-S larvae, ■: G-G larvae.

Fig. 4. Dosage-mortality regressions for solitary (□) and gregarious (■) larvae of *Pseudaletia separata* inoculated with a granulosis virus percutaneously.

all the experimental plots. Among the experimental plots, LT₅₀ values of G-G larvae receiving PsNPV were the longest, those of S-S larvae were the shortest and those of S-G and G-S larvae were intermediate (Fig. 3). At lower concentrations of administered PsNPV, the differences in LT₅₀ values among experimental plots increased.

LC₅₀ values of S-S, S-G, G-S, and G-G larvae were estimated as 2.5×10^6 , 7.9×10^6 , 6.3×10^6 and 1.6×10^7 PIBs/ml, respectively (Table 2). The S-S larvae were ca. 6 times more susceptible than the G-G larvae. Though LC₅₀ values were not significantly different between S-G and G-S larvae, LC₅₀ values of S-G and G-S larvae were significantly different from those of G-G and S-S larvae ($p < 0.05$). The susceptibilities of S-G and G-S larvae to PsNPV were intermediate between the most susceptible S-S larvae and the least susceptible G-G larvae.

Oral susceptibility of solitary and gregarious larvae to GVH

The relative susceptibilities of the solitary and gregarious larvae to GVH were

Fig. 5. Dosage-mortality regressions for pale (□) and dark (■) larvae of *Pseudaletia separata* inoculated with a nuclear polyhedrosis virus percutaneously. Crowded rearing was used to produce color polymorphism. Pale (color grade I, II) and dark (color grade III, IV) larvae were chosen from these cohorts at the beginning of the 5th instar.

Table 3. Mortalities of solitary and gregarious larvae of *Pseudaletia separata* inoculated subcutaneously with non-occluded NPV

Types of larvae	Virus concentration	No. of larvae used	Larval mortality rate (%)
Solitary	Control	20	0
	$10^{-3.0}$	20	0
	$10^{-2.5}$	20	10.0
	$10^{-2.0}$	20	25.0
	$10^{-1.5}$	20	25.0
	$10^{-1.0}$	20	30.0
	$10^{-0.5}$	20	75.0
	10^0	20	100.0
Gregarious	Control	20	0
	$10^{-3.0}$	20	5.0
	$10^{-2.5}$	20	10.0
	$10^{-2.0}$	20	15.0
	$10^{-1.5}$	21	14.3
	$10^{-1.0}$	20	50.0
	$10^{-0.5}$	21	90.5
	10^0	21	100.0

Newly molted 6th instar larvae which had been reared in crowded condition or isolation from the 2nd instar were used for this experiment. Each larva was etherized and then injected with 10 μ l of each dilution of non-occluded NPV using a hand microapplicator with a 50 μ l microsyringe. After injection, larvae were reared individually in 50 ml plastic cups on artificial diet for 10 days.

determined by comparing their LC_{50} values (Fig. 4). The LC_{50} values of the solitary and gregarious larvae receiving GVH were estimated as -63 and -16, respectively. The solitary larvae were ca. 4 times more susceptible to GVH than the gregarious larvae.

Oral susceptibility of pale and dark larvae to PsNPV

The LC_{50} values for pale and dark larvae were estimated as 3.2×10^7 and 6.3×10^7 PIBs/ml, respectively (Fig. 5). Although the pale larvae were slightly more susceptible to oral infection than the dark larvae, the difference was not significant ($p > 0.05$).

Percutaneous susceptibility of solitary and gregarious larvae to PsNPV

Non-occluded PsNPV were injected into the 6th instar larvae reared in isolation or in crowded condition (Table 3). The LC_{50} value calculated by the BEHRENS-KÄRBER method (KÄRBER, 1931) for the isolated larvae was almost the same as that for crowded larvae.

DISCUSSION

The solitary larvae of *P. separata* were more susceptible to nuclear polyhedrosis and granulosis viruses than the gregarious larvae when the virus was administered orally. The latter larvae are characterized by relatively darker pigmentation, greater activity and resistance to starvation, and a faster rate of development (Iwao, 1962, 1963). Iwao (1962) suggested that this marked morpho-functional variation of larvae as seen in *P. separata* may have evolved among noctuid species in which the level of population density fluctuates between wide extremes in both space and time. Acquired resistance to entomopathogenic virus under crowded condition can be regarded as adaptive and consistent with the above-mentioned responses to crowding. This point of view is further supported by the fact that viral diseases of wild insects are likely to be transmitted horizontally in crowded populations (ANDERSON and MAY, 1981).

The susceptibilities of S-G and G-S larvae to PsNPV were intermediate between the most susceptible S-S larvae and the least susceptible G-G larvae. This observation suggests that rearing densities both before and after virus administration affect the susceptibility of the larvae to PsNPV. Iwao (1962) studied the sensitivity of different instars to crowding in *P. separata*. He found that when the crowded larvae were separated at middle stage, their dark pigmentation gradually faded and that when the isolated larvae were crowded together, rapid melanization occurred. We observed similar phenomena in S-G and G-S larvae in the present experiment. This indicates that phase-transformation might have occurred after changes in density. Therefore, the susceptibility of *P. separata* larvae to peroral infection of PsNPV seems to vary with phase-transformation. The larval phase of *P. separata* is controlled by the neuro-hormone, MRCH, secreted from both the brain-corpora cardiaca-corpora allata complex and the suboesophageal ganglion (OGURA, 1975; MATSUMOTO et al., 1984). Direct effect of MRCH on susceptibility of *P. separata* larvae to PsNPV has not been studied, and its analysis will be important to further explore the relationship between the larval phase and susceptibility to PsNPV.

No differences in susceptibility to PsNPV between the gregarious and solitary larvae were observed when PsNPV was injected into the hemocoel. WATANABE (1966) tested

the susceptibility of several silkworm strains, which exhibit differences in susceptibility to the peroral infection of a CPV, to subdermal infection. He found that differences in susceptibility among the strains were quite small. These results suggest that resistance of the larvae to peroral infection mainly depends on inhibitory mechanisms in the gut lumen against the invasion of the virus into midgut cells. Several antiviral substances in the silkworm gut juice have been found to inactivate both NPV and CPV (ARUGA and WATANABE, 1964; ARATAKE and UENO, 1973; ARATAKE et al., 1974; HAYASHIYA et al., 1968; FUNAKOSHI and AIZAWA, 1989). However, the presence of these substances in *P. separata* gut juice is as yet unknown. To understand the resistant mechanism of peroral infection, it will be necessary to determine the presence or absence of these substances in the gut juice of gregarious larvae.

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