Size-related Susceptibility of *Spodoptera litura* (Lepidoptera: Noctuidae) Larvae to Entomogenous Nematode, *Steinernema feltiae* (DD-136)¹

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Laboratory inoculation experiments were conducted to study the infectivity of *Steinernema feltiae* (DD-136) on fed and unfed larvae of common cutworm, *Spodoptera litura*, of different body sizes. All larvae used were killed within 48 hr after inoculation with ca. 1,000 infective juveniles. With increasing body weight, longer time was required to kill the larvae: the average body weights of sluggish, moribund, and dead larvae 24 hr after inoculation were about 390, 280, and 200 mg, respectively. Nematode infectivity on fed and unfed larvae became considerably different with decreasing inoculum size of nematodes from 500 to 5 per insect. Number of invading nematodes in dead larvae ranged from 1 to 6 per mg body weight of insect, while that in living ones was less than 1. Similarly, larvae which fed other larvae under crowded conditions were infected with fewer nematodes. Generally, nematode infection through the anus was less in the fed larvae than in the unfed. Average number of symbiotic bacteria, *Xenorhabdus nematophilus*, isolated from 10 μl hemolymph 24 hr after inoculation was $3 \times 10^4$ for dead larvae and only about 5 for those still living, including the sluggish. The susceptibility of *S. litura* larvae of different sizes was discussed from the standpoint of the nematode infection process.

The entomogenous nematode, *Steinernema feltiae*, has a wide host range and is highly infectious to lepidopterous insects, however, its infectivity is quite different depending on species and developmental stage of insects (PoNAR, 1979). This is also true for the common cutworm, *Spodoptera litura*: the infectivity of this nematode differs considerably with developmental stage of the insect (KONDO and ISHIBASHI, 1984).

Various stages of *S. litura* inhabit fields during its active season. Of all stages, the last instar larva is economically most important because of its severe feeding damage on various crops and its high resistance to pesticides. After the final molt, the larva shows a good appetite resulting in a rapid body size increase, although its feeding is suspended in daytime: it feeds on plants at night and hides in the daytime in soil around the plant base without feeding. For practical application of entomogenous nematodes to control an insect pest having these properties, nematode infectivity on the larvae of different body sizes and feeding habits must be determined. The present inoculation

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experiments were conducted to show how the infectivity of *S. feltiae* (DD-136) differs with body size and feeding condition of *S. litura* larvae.

**MATERIALS AND METHODS**

**Nematode used.** The DD-136 strain of *Steinernema feltiae* was used. The nematode was cultured on media prepared with liver and intestine of chicken (Kondo et al., 1985) and harvested 30–40 days after inoculation. The harvested infective juveniles (hereafter called J₉₅%) were suspended in 0.1% formalin solution to prepare inocula. Unless otherwise particularly mentioned, inoculum size was ca. 1,000 J₉₅% per plastic petri dish.

**Insect used.** The insects used were larvae of the common cutworm, *Spodoptera litura*, reared on an artificial diet (Koyama and Kamano, 1976) at 25°C under long day photoperiod (16L–8D).

**Inoculation experiments.** To study the infectivity of *S. feltiae* (DD-136) on *S. litura* larvae of different sizes, experiments were conducted at 25°C on the following items.

1) **Times required to kill larvae:** Larvae of different body weights were placed individually on a filter paper in a petri dish (5.5 cm in diam.) inoculated with 0.4 ml of 0.1% formalin solution containing ca. 1,000 J₉₅%. A total of 50 insects were used. Insect survival was followed for 48 hr at 2 hr intervals and body weight was measured at the time of insect death.

2) **Nematode infection to larvae:** Twenty-four hr after inoculation, dead and still living larvae of different body weights were washed with 0.1% formalin solution to recover nematodes on the insect body. Then, the surface-washed insect was dissected along a ventral line and placed dissected side down in a 1/2 diluted physiologically balanced saline solution (Kondo and Ishibashi, 1986) in a petri dish for 12 hr. Nematodes swimming out in the solution were counted as those having been in the insect body.

Invasion and development of nematodes were also investigated in sluggish, moribund, and dead larvae 24 hr after inoculation. Living larvae which hardly moved and those which moved only when picked up with a glass rod were respectively referred to as moribund and sluggish. After surface-wash to recover nematodes from their bodies, these classified larvae were individually transferred to a petri dish without nematodes and incubated for an additional 24 hr. Forty-eight hr after inoculation, nematodes in insect bodies were recovered and counted. Percentage of adults of the recovered nematodes was also calculated.

3) **Isolation of symbiotic bacteria:** Bacterial cells of *Xenorhabdus nematophilus* were isolated 24 hr after inoculation from hemolymph of *S. litura* larvae of different body sizes. The insect was immersed in 70% ethanol solution for 1 min with agitation, rinsed three times with distilled water, and blotted dry on a filter paper. Hemolymph was bled by cutting the 1st abdominal leg of the surface-sterilized larvae. To reduce the chance of contamination, the 1st drop of hemolymph was discarded and a 2nd one was withdrawn into a 10 μl microcap (Drummond Scientific Co., U.S.A.). During bleeding, the head and tail parts of insects were wrapped with cotton to avoid contamination by discharged gut juice and feces. The hemolymph sample thus obtained was suspended in 1 ml of saline solution in a petri dish and a pour plate was prepared by adding about 20 ml of nutrient agar containing 0.0045% (w/v) triphenyltetrazolium
chloride and 0.025% (w/v) bromotymol blue (Akhurst, 1980). To make cell counting easy, hemolymph samples diluted to 1/10, 1/100, and 1/1,000 were also used to prepare pour plates. All procedures described above were conducted in a laminar flow chamber using sterilized implements and solutions. Number of cell colonies was counted 5 days after incubation at 25°C. As a control, hemolymph of non-inoculated larvae was processed as above. Diagnosis of bacteria was primarily based on shape and color characteristics of colonies on the media, response to Gram staining, and morphology.

4) Effect of insect density on nematode infection: Selective nematode infection to larger and smaller larvae was studied by placing 1, 2, 5, 10, and 20 insect individuals in a petri dish (9 cm in diam.) inoculated with ca. 1,000 J15S. In this experiment, the head slippage stage which develops up to the last instar was used to decrease the chance of devouring other larvae under a crowded condition. Forty-eight hr after inoculation, nematodes were recovered from the body of larvae which were not devoured. Bigger larvae having material in their alimentary tract were considered to have devoured others.

5) Infectivity on fed and unfed larvae: Effects of feeding conditions of S. litura larvae on the infectivity of nematodes were studied. Newly molted last instar larvae were individually placed in a petri dish (5.5 cm in diam.) inoculated with ca. 1,000 J15S and allowed to starve or feed on insect artificial diet. As a control, larvae were either starved or fed in a petri dish without nematodes. At 24 hr intervals, body weight of these insects was measured for 5 days and survival was followed until all insects had died or developed to pupae.

Infection via mouth and anus of fed and unfed 5th instar larvae was examined under scanning electron microscope (SEM). Roughly 18 hr after inoculation with ca. 20,000 J15S per larva, the insect was fixed at 5°C for 2 days in 5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2). After dehydration through a graded ethanol series, the ventral side of the insect was cut with a razor blade in two pieces through a dorso-ventral plane to expose nematodes in the alimentary tract, critical-point dried, ion-sputter coated with gold, and observed under a SEM (JSM F-15) operated at 15 kV.

RESULTS

Time required to kill larvae

Although the time required to kill insects varied, smaller larvae tended to die earlier than larger ones: the average body weight of larvae dying before 24 hr, between 24 and 36 hr, and after 36 hr were 154, 277, and 345 mg, respectively (Fig. 1). Generally, this tendency was more clearly recognized in insects dying within 32 hr after inoculation than those dying thereafter.

Nematode infection to larvae

Total number of nematodes recovered from the surface and within the body of an S. litura larva fluctuated with body weight and activity of the insect (Fig. 2 A). Nematodes recovered 24 hr after inoculation with ca. 1,000 J15S were most numerous in dead larvae weighing about 250 mg, and larger living larvae had fewer nematodes on average. The ratio of nematodes remaining on the insect body surface was generally higher in living larvae with greater body weight (Fig. 2 B). Consequently, the number of
invading nematodes ranged from 1 to 6 for dead larvae, while it was less than 1 per mg body weight for living insects (Fig. 2 C).

Table 1 shows the infection and development of nematodes in the last instar larvae which showed different activity 24 hr after inoculation. The insect activity decreased more quickly in smaller larvae: the average body weight of sluggish, moribund, and dead larvae was 390, 279, and 196 mg, respectively. Fewer nematodes were recovered from sluggish larvae than from moribund and dead ones. The rate of nematodes in the insect body was highest for dead larvae followed by moribund and sluggish ones. The same tendency was observed for the number of invading nematodes on an insect weight basis. The development of invading nematodes was earlier in smaller larvae as was indicated by the percent of adult nematodes in the infected larvae.

**Isolation of symbiotic bacteria**

Bacteria were not isolated from the hemolymph of non-inoculated larvae. Prop-
Table 1. Comparison of numbers and development of invading *Steinernema feltiae* (DD-136) in the last instar larvae of common cutworm, *Spodoptera litura*, of different body sizes\(^a\)

<table>
<thead>
<tr>
<th>Insect activity(^b)</th>
<th>No. of insects examined</th>
<th>Fresh body weight of insect (mg)</th>
<th>Number of nematodes per</th>
<th>% Nematode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Insect</td>
<td>mg body weight</td>
</tr>
<tr>
<td>Sluggish</td>
<td>19</td>
<td>390 ± 78</td>
<td>368</td>
<td>0.79</td>
</tr>
<tr>
<td>Moribund</td>
<td>7</td>
<td>279 ± 36</td>
<td>435</td>
<td>1.33</td>
</tr>
<tr>
<td>Dead</td>
<td>14</td>
<td>196 ± 53</td>
<td>421</td>
<td>2.06</td>
</tr>
</tbody>
</table>

\(^a\) Insects were surface-sterilized with 0.1% formalin solution 24 hr after inoculation with ca. 1,000 infective juveniles, incubated at 25°C for an additional 24 hr, and dissected to investigate number and development of invading nematodes.

\(^b\) Insect activity at the time of surface-rinsing (24 hr after inoculation). All inoculated larvae died within 48 hr after inoculation.

\(^c\) Ratio of nematodes recovered from the insect body to those recovered from the surface and within the body of a larva.

\(^d\) % adults of all nematodes recovered from the insect body.

Table 2. Isolation of symbiotic bacterium, *X. nematopilia*, from hemolymph of *S. litura* larvae 24 hr after inoculation with ca. 1,000 infective juveniles of *S. feltiae* (DD-136)

<table>
<thead>
<tr>
<th>Condition of insect at the time of isolation</th>
<th>Fresh body weight (mg) of insect</th>
<th>Average number of bacteria per 10 (\mu)l hemolymph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead</td>
<td>131 ± 46</td>
<td>3.0 (\times ) 10(^4)</td>
</tr>
<tr>
<td>Living(^a)</td>
<td>349 ± 153</td>
<td>5.4 (\times ) 10(^6)</td>
</tr>
</tbody>
</table>

\(^a\) Sluggish and moribund larvae were included in living larvae.

Agitation of symbiotic bacteria in hemolymph differed considerably with larval body size. The average body weight of dead and living larvae 24 hr after inoculation was 131 and 349 mg, respectively (Table 2). In 10 \(\mu\)l hemolymph of these larvae, an average of 3 \(\times \) 10\(^4\) bacterial cells were isolated from dead larvae and only about 5 from still living ones including the sluggish. The hemolymph of smaller dead larvae tended to contain more cells than that of larger ones on unit volume basis: ranging from 10\(^5\) to 10\(^4\) cells per 10 \(\mu\)l hemolymph (Fig. 3). Most of the living larvae had bacteria fewer than 10 cells per 10 \(\mu\)l hemolymph.

**Effect of insect density on nematode infection**

With increasing insect density from 1 to 20 per petri dish, number of invading nematodes per insect decreased: 254, 169, 80, 34, and 19 nematodes at the densities of 1, 2, 5, 10, and 20, respectively (Table 3). At the two lower densities, all larvae were killed by nematodes before devouring others. At densities of 2 and 5, the number of invading nematodes per larva showed a nearly normal distribution (Fig. 4). At such high densities as 10 and 20, however, the frequency distribution broadened: insects infected with considerably larger or smaller numbers of nematodes increased. Those insects which increased in size by devouring others were usually infected with the fewest number of nematodes among all insects in a dish.
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**Fig. 3.** Densities of symbiotic bacteria (*Xenorhabdus nematophilus*) in hemolymph of *S. littura* larvae of different body weights 24 hr after inoculation with ca. 1,000 infective juveniles of *S. feltiae* (DD-136). Closed and open circles indicate dead and living insects, respectively.

**Fig. 4.** Effect of density of *S. littura* larvae on invasion of *S. feltiae* (DD-136). Nematodes were recovered 48 hr after inoculation with ca. 1,000 infective juveniles. Numerals in squares indicate number of insects per petri dish (9 cm in diam.). Black parts of columns indicate larvae which devoured other larvae during inoculation time at 25°C.

<table>
<thead>
<tr>
<th>Insect density</th>
<th>Hours after inoculation</th>
<th>% insects</th>
<th>No. of invading nematodes per insect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>survived</td>
<td>devoured</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>92</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>9.5</td>
</tr>
</tbody>
</table>

- **a** Number of *S. littura* larvae placed per petri dish (9 cm in diam.). In order to decrease the chance to devour other larvae, the larvae inoculated were in the head slippage stage which develops up to the last instar.
- **b** Larvae devoured by other larvae and those killed due to nematode infection, respectively.
- **c** Nematode invasion was investigated 48 hr after inoculation with ca. 1,000 infective juveniles. Values are the mean of 10 replicates.
Infectivity on fed and unfed larvae

The body weight of non-inoculated unfed larvae decreased to about 1/2 of the weight before inoculation within 5 days (Fig. 5 B) and all insects died within 6 days after onset of an experiment (Fig. 6 B). With increasing of nematode inoculum size, body weight of unfed larvae decreased more rapidly. However, weight loss stopped earlier in the larvae administered higher inocula: for instance, all larvae inoculated with ca. 500 or 100 \( J_{118} \) died within 2 days after inoculation (Fig. 6 B) and the larval weight was almost stationary thereafter (Fig. 5 B). The degree of weight loss and mortality of larvae inoculated with twenty nematodes were between those of control and of insects administered higher inocula. The body weight of all non-inoculated fed larvae increased linearly during the 3 days after the final molt and then decreased during
Fig. 8. Light and scanning electron micrographs of anterior (A, C, E) and posterior (B, D, F) parts of S. litura larvae fed or unfed on insect artificial diet after inoculation with ca. 20,000 infective juveniles of S. feltiae (DD-136). Bars in all pictures indicate 1 cm in length. A and B: Longitudinal sections of non-inoculated insects. b, brain; p, pharynx; s, subesophageal ganglion; m, midgut, f, feces in rectum. C and D: Internal view of fed insects inoculated with nematodes (n). d, diet; f, feces in rectum. E and F: Internal view of unfed larvae inoculated with nematodes. Note many nematodes (n) in pharynx, midgut, hindgut, and rectum.

the development to wandering stage larvae and prepupae.

Compared to unfed larvae, the fed ones showed slower body weight loss and longer survival after inoculation (Figs. 5 A and 6 A). For the fed larvae inoculated with 500 J_N, body weight did not exceed the original level and the survival curve was similar to that of unfed larvae. A difference in susceptibility between the fed and unfed larvae became obvious with decreasing nematode inoculum size from 500 to 5 per insect. At inoculum sizes of 5 and 20, more than half of the larvae died after developing to wandering stage larvae, prepupae or pupae. At all inoculum sizes examined, the infec-
tive juveniles emerged earlier and more frequently from the cadavers of unfed larvae than from those of fed ones (Fig. 7).

SEM observations showed many nematodes in the anterior and posterior parts of the alimentary tracts of unfed larvae (Fig. 8). In fed larvae the number of nematodes in alimentary tracts, especially in the rectum was fewer than in fed larvae.

DISCUSSION

To kill an insect, the applied nematode should survive for a desired time in an environment, actively move to the target pest, invade the host, and release its associated bacterium, _Xenorhabdus nematophilus_, causing septicemia. Any variation in this infection process may affect insect mortality. In the case of the _S. litura_ larvae- _S. feltiae_ (DD-136) combination, the mortality differed considerably depending on body size of the insect; larger larvae died more slowly than smaller ones, mainly because of decreased infection, as shown in the present experiments.

In the first step of nematode infection, infective juveniles seemed to move onto various parts of actively moving _S. litura_ larvae, because a similar number of nematodes was recovered from the anterior, middle, and posterior portions of the insect body surface (unpublished data). This was thought to partly explain why the nematode invasion decreased in larger larvae, because nematodes reaching a larger insect have to travel a longer distance to infect through the mouth or anus, the main known infection route for various insects. Although infective juveniles of heterorhabditid and steinernematid nematodes have the ability to infect through the thin cuticular membrane of insects (Bedding and Molyneux, 1982; Kondo and Ishibashi, 1983), infection may become difficult in larger larvae having thicker membrane.

The infective juveniles of steinernematid nematodes are attracted by CO₂ (Gaugler et al., 1980) and by such excretory products of insects as uric acid, xanthine, allantoin, ammonia, and agrine in feces (Schmid and All., 1979). These attractive substances are emitted in more quantity from active larvae. On the other hand, dead or inactive insects emit a smaller amount of these substances, although _S. feltiae_ infected and propagated in _Spodoptera exigua_ larvae killed by insecticides (Hara and Kaya, 1983). Similarly, _S. litura_ larvae killed by such treatments as freezing, immersion in hot water, and exposure to formalin were infected; however, invading nematodes in these killed larvae were fewer than in nontreated larvae having similar body size (unpublished data). Besides the reduced attraction by the moribund insect itself, the discharged gut juice and feces may arrest nematodes and keep them from invading a host. All of these results explain, at least partly, why the number of invading nematodes is generally fewer in smaller larvae which died earlier than larger ones.

Feeding conditions of _S. litura_ larvae also affected the nematode infection and insect survivorship: DD-136 showed lower infectivity to the fed larvae than to the unfed ones. Similarly, larger larvae which had devoured other larvae were infected with fewer nematodes than those which died without devouring others. Nematode infection in fed larvae was considered to decrease partly because of the lower rate of infection through the anus; nematodes are not only arrested by the discharged feces but also their invasion through the anus may be disturbed by the closely packed feces in the rectum. Even if nematodes succeed in advancing to the rectum, some may be forced out during defecation. Also, the thicker wall of the rectum surely acts as a mechanical barrier
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against nematodes. In addition, nematode infection through the mouth seemed lower in the fed than the unfed larvae. Nematodes in the midgut of unfed larvae can easily contact and promptly penetrate the wall of the empty midgut, while in fed larvae, diet taken in the midgut seems to disturb to some extent nematodes from contacting the midgut wall, resulting in a delayed invasion into the hemocoel. Based on these results, it was assumed that the nematode effectively invades the larvae with lowered activity owing to starvation and other physiological stress induced by chemical and physical treatments.

In order to induce septicemia, the nematode-associated bacterium, X. nematophilus, has to break the host defense in the hemocoel. In the present study, the symbiotic bacteria rapidly propagated after the insect death. Considering the fact that lowered insect activity was noticed even when the bacterial density was less than 5 per 10 µl hemolymph, the initial phase of septicemia induction should be studied in detail to reveal the resistance level of an S. litura larva against a nematode and/or bacteria.

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REFERENCES


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