OBSERVATIONS ON MOULTING OF FOURTH-STAGE LARVAE OF
PARATYLENCHUS NANUS

By J. M. Fisher*

[Manuscript received April 4, 1966]

Summary

Moulting of fourth-stage larvae of Paratylenchus nanus was stimulated by apricot root secretion but the activity was lost on boiling. Dormant apricot seedlings did not produce an active secretion. The effect was on the anterior part of the nematode and 1 day in secretion was sufficient to initiate the moult. Five to 10 days after stimulation the larvae became motionless and the moult was complete about 3 days later. Moulting took place most rapidly at about 20°C and was not affected by pH between 4 and 7. Moulting but not stimulation was inhibited at temperatures of 25 and 30°C. Shedding of the cuticle differed from exsheathment of trichostrongylid larvae in that no refractile ring formed in the anterior part of the cuticle.

I. Introduction

Moulting of plant parasitic nematodes has not been examined in detail but shedding of the cuticle has been described (van Gundy 1959; Rhoades and Linford 1961). Understanding of the mechanisms involved in moulting in insects (Wigglesworth 1964) and in parasitic nematodes of animals (Rogers 1962) has developed from those in which an initial stimulus for moulting or exsheathment is known. For most plant parasitic nematodes, the initial stimulus is unknown but Rhoades and Linford (1959) have shown that an external stimulus from some plant roots will initiate moulting of fourth-stage larvae of some species of Paratylenchus. This paper records experiments on moulting of P. nanus.

II. Materials and Methods

All fourth-stage larvae of P. nanus were obtained from an apple orchard at Basket Range, South Australia.

Apricot seedlings (cv. Moorpark), a known host of P. nanus, were used as the source of root secretion. Seeds were surface-sterilized for 2 min in mercuric chloride (1000 p.p.m.), thoroughly washed in sterile distilled water, then immersed in sterile gibberellic acid (100 p.p.m.) for 24 hr at room temperature before being planted in sterilized sand. Seedlings were removed from the sand when at the three- to four-leaf stage, the roots were washed in distilled water and then immersed in 20 ml of glass-distilled water at 20°C in the dark for 14 hr. Root secretion was freshly collected for each experiment and was stored at 5°C.

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Moulting was assessed at low magnification ($\times 50$) and a nematode was determined as moulted when the new stylet in females or spicules in males could first be clearly seen. Of several methods used for testing activity of solutions, the best was to immerse fourth-stage larvae in about 2 ml of solution in small syracuse dishes (B.P.I. type). The dishes were placed in petri dishes to which a little distilled water had been added and incubated under the test conditions.

III. Results

(a) Moulting

Larvae became quiescent prior to moulting and assumed a characteristic curved shape. In potential males, first indications of moulting were clearing or transparency of the tail, initiation of cloacal and spicular formation, and development of the gonad. Similar clearing occurred in females but was more anterior in position and later in development. In both sexes, shedding of the cuticle was first observed at the head; the cuticle became free and elongated (Plate 1, Fig. 1) and the cuticular lining of the amphidial pouches and stylet was shed as well (Plate 1, Fig. 2). Shedding of the cuticle at the tail occurred shortly afterwards, and at this stage formation of vagina and spermatheca could be seen (Plate 1, Fig. 3). A refractile ring did not occur in the cuticle near the excretory pore (Plate 1, Fig. 4) but two groups of clearly defined granules could be seen in the body cavity near the posterior end of the oesophagus. The remainder of the cuticle was closely adpressed to the body of the nematode but the hypodermis appeared thickened. In late stages of moulting, the cuticle became almost completely free and inflated but was still attached at irregular intervals (Plate 1, Fig. 5). At no stage was the anterior portion of the cuticle released from the posterior; in fact throughout the experiments, no nematode ever managed to free itself completely from cast cuticle which remained as a sheath around it.

(b) Effect of Heat on Apricot Root Secretion

Secretion was thoroughly mixed and divided into two equal parts, one of which was boiled for 5 min. After cooling, approximately 20 fourth-stage larvae were placed

<table>
<thead>
<tr>
<th>Treatment of Apricot Root Secretion</th>
<th>Av. No. of Males</th>
<th>Av. No. of Females</th>
<th>Av. No. Larvae Unmoulted</th>
<th>Percentage Moult*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unboiled</td>
<td>5·8</td>
<td>10·4</td>
<td>3·0</td>
<td>84</td>
</tr>
<tr>
<td>Boiled for 5 min</td>
<td>3·6</td>
<td>5·3</td>
<td>10·4</td>
<td>47</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4·6</td>
<td>5·6</td>
<td>7·0</td>
<td>59</td>
</tr>
</tbody>
</table>

* Least significant difference at 5% level = 14.
in each of five replicates of boiled secretion, untreated secretion, and distilled water. Significantly more larvae moulted in unboiled than in boiled secretion or distilled water (Table 1).

(c) Effect of Temperature on Moulting

Five replicates of approximately 20 fourth-stage larvae were incubated in secretion at 15, 20, 25, and 30°C. Moulting was assessed after 13 and 15 days (Table 2). Moulting proceeded very slowly at 15°C and was still continuing at 15 days. At 20°C, almost all larvae had moulted after 15 days. At 25 and 30°C, a few larvae moulted initially but these temperatures were apparently too high to allow further moulting. This experiment suggests that the optimum temperature for moulting is about 20°C.

Table 2

**Effect of Temperature on Moulting of Fourth-Stage Larvae of P. nanus in Apricot Root Secretion**

20 fourth-stage larvae incubated at four temperatures for 13 and 15 days. Values are averages for five replications.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Average Numbers after 13 Days</th>
<th>Percentage Moulte after 13 Days*</th>
<th>Average Numbers after 15 Days</th>
<th>Percentage Moulte after 15 Days*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Larvae Unmoulter</td>
<td>Males</td>
</tr>
<tr>
<td>15</td>
<td>2·0</td>
<td>2·6</td>
<td>14·0</td>
<td>26·0</td>
</tr>
<tr>
<td>20</td>
<td>5·0</td>
<td>11·8</td>
<td>5·4</td>
<td>75·0</td>
</tr>
<tr>
<td>25</td>
<td>1·8</td>
<td>2·4</td>
<td>14·2</td>
<td>23·0</td>
</tr>
<tr>
<td>30</td>
<td>0·25</td>
<td>0·0</td>
<td>18·5</td>
<td>1·25</td>
</tr>
</tbody>
</table>

* Least significant difference at 5% level = 10.

After 15 days the larvae which had not moulted at 25 and 30°C were removed, washed thoroughly in distilled water, and incubated in distilled water at 20°C. Larvae from both temperatures commenced to moult at 20°C without further stimulation.

(d) Effect of pH on Moulting

To examine the effect of pH on moulting, roots of apricot seedlings were immersed in 20 ml of phosphate–citrate buffer (Hale 1958) at approximate pH’s of 4·0 (0·13M), 6·0 (0·09M), and 8·0 (0·04M). When the seedlings were removed, the pH’s of the buffer solutions which now contained secretion were 4·0, 6·0, and 7·0; collecting the secretion had lowered the pH of the most alkaline buffer. Five replicates of approximately 18 fourth-stage larvae were incubated at 23°C at each pH and in distilled water. The numbers of larvae which had moulted after 13 days (Table 3) were the same at all pH’s, but significantly fewer moulted in distilled water.
To test whether a stimulant could be obtained from a dormant seedling, secretion was collected in the usual way from the roots of a 2-year-old dormant apricot seedling.

**Table 3**

**Effect of pH on Moult ing of Fourth-stage Larvae of P. Nanus**

In each experiment approximately 18 fourth-stage larvae were incubated for 13 days at 23°C in buffers containing apricot root secretion at various pH's and in distilled water. Values given are averages for five replications.

<table>
<thead>
<tr>
<th>pH of Buffer Solution</th>
<th>Av. No. of Males</th>
<th>Av. No. of Females</th>
<th>Av. No. of Larvae Unmoulted</th>
<th>Percentage Moult*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4·6</td>
<td>5·2</td>
<td>8·4</td>
<td>54</td>
</tr>
<tr>
<td>4·0</td>
<td>5·3</td>
<td>10·4</td>
<td>2·6</td>
<td>86</td>
</tr>
<tr>
<td>6·0</td>
<td>5·4</td>
<td>10·6</td>
<td>2·0</td>
<td>89</td>
</tr>
<tr>
<td>7·0</td>
<td>5·3</td>
<td>10·6</td>
<td>2·4</td>
<td>87</td>
</tr>
</tbody>
</table>

* Least significant difference at 5% level = 14.

50 fourth-stage larvae were then placed in secretion from a growing seedling, from a dormant seedling, or in distilled water and incubated at 23°C. Secretion from the dormant seedling did not stimulate moulting (Table 4). The experiment was not replicated and needed repeating but attempts to induce dormancy in young seedlings were unsuccessful.

**Table 4**

**Effect of Apricot Root Secretion from Dormant Seedlings on Moult ing of Fourth-stage Larvae of P. Nanus**

50 fourth-stage larvae incubated at 23°C for 15 days in distilled water and in secretion from dormant and growing seedlings.

<table>
<thead>
<tr>
<th>Source of Secretion</th>
<th>Av. No. of Males</th>
<th>Av. No. of Females</th>
<th>Av. No. of Larvae Unmoulted</th>
<th>Percentage Moult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growing seedlings</td>
<td>14</td>
<td>22</td>
<td>14</td>
<td>72</td>
</tr>
<tr>
<td>Dormant seedlings</td>
<td>4</td>
<td>13</td>
<td>32</td>
<td>35</td>
</tr>
<tr>
<td>Distilled water (control)</td>
<td>4</td>
<td>10</td>
<td>32</td>
<td>30</td>
</tr>
</tbody>
</table>
MOLTING OF PARATYLENCHUS NANUS

(f) Mode of Action of Stimulus

In all these experiments, the time necessary for moulting was 8–13 days. When larvae were placed in root secretions they usually remained active for 5–10 days, after which they became motionless and, in the next day or two, would commence to moult. From the time the larvae became motionless to completion of the moult took approximately 3 days. Thus a considerable time elapsed before moulting commenced and, as concentration of the secretion might affect this time, attempts were made to collect a more concentrated solution from larger seedlings growing in pots. Distilled water was added to a 6-month-old apricot seedling and the first 20 ml of solution which percolated through the pot was collected. This solution failed to stimulate moulting of fourth-stage larvae.

**Table 5**

<table>
<thead>
<tr>
<th>Treatment of Larvae</th>
<th>Time of Immersion (days)</th>
<th>Av. No. of Males</th>
<th>Av. No. of Females</th>
<th>Av. No. of Larvae Unmoulted</th>
<th>Percentage Moulting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immersed in root secretion</td>
<td>1</td>
<td>1.5</td>
<td>2.5</td>
<td>14.0</td>
<td>22.5*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.0</td>
<td>4.5</td>
<td>14.0</td>
<td>31.5*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.5</td>
<td>1.0</td>
<td>15.0</td>
<td>19.0*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.0</td>
<td>2.5</td>
<td>14.5</td>
<td>27.8*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.2</td>
<td>4.8</td>
<td>13.6</td>
<td>34.7*†</td>
</tr>
<tr>
<td>Immersed in distilled water</td>
<td>14</td>
<td>0.8</td>
<td>0.8</td>
<td>19.6</td>
<td>7.6†</td>
</tr>
</tbody>
</table>

* Standard error of difference between means of percentage moult = 8.5.
† Standard error of difference between means of percentage moult = 6.2.

In attempts to find a more active secretion, various other plants, e.g. peas (*Pisum sativum*), beans (*Phaseolus vulgaris*), corn (*Zea mays*), and lucerne (*Medicago sativa*) were tested but all failed to stimulate moulting.

The time interval before larvae commenced to shed their cuticle needed further examination. It was not known whether stimulation occurred in a short time or not. Two replicates of approximately 20 larvae were placed in root secretion for each of 1, 2, 3, and 6 days. The root secretion was removed, the larvae were thoroughly washed in distilled water, and then left in distilled water. Controls consisted of five replicates of approximately 20 larvae in distilled water and in root secretion; all treatments were incubated at 20°C and moulting was assessed after 14 days. Increasing length of time in root secretion did not influence the number of larvae which moulted (Table 5). Fewer larvae moulted in distilled water than in root secretion.
(g) Site of Action of Stimulus

Rogers and Sommerville (1960) showed that the area between the excretory pore and the base of the oesophagus was important in exsheathment of larvae of *Trichostrongylus axei*. To test whether the area at the base of the oesophagus was involved in moulting in *P. nanus*, six larvae were ligated (Rogers and Sommerville 1960) with terylene thread, some anterior and some posterior to the base of the oesophagus. The larvae were immersed in root secretion at 20°C. To minimize the effects of bacteria and fungi on the ligated larvae, the solutions were changed every 2 days. Most of the larvae remained active for approximately 10 days then died. One larva remained active longer (Plate 2, Fig. 1), and the portion of the nematode anterior to the ligature moulted completely after about 14 days and was motionless during moulting; the portion posterior to the ligature did not moult and remained motile. The larva would have become a male under normal conditions as no stylet was present and the oesophagus was of the degenerate type normal for males. In further experiments two nematodes survived ligating for more than 16 days. Plate 2, Figure 2, shows a ligated potential-female larva. Moulting and reorganization of the portion of the nematode anterior to the ligature is complete; no change occurred posterior to the ligature. The two larvae which moulted had the ligature tied at about the middle of their bodies. All larvae which had the ligature anterior to the base of the oesophagus died.

IV. Discussion

The experimental procedure used for testing moulting of larvae was unsatisfactory in several ways. Many of the larvae taken from soil moulted in distilled water alone. This was especially true of larvae collected in late summer or autumn. Whether moulting in distilled water occurred as a result of previous stimulation by natural host secretions or whether stimulation was not necessary at certain times of the year is not known. This natural moulting made testing of various factors difficult. Development of fungi and bacteria in the solutions may also have influenced the results; thus a sterile technique would be advantageous.

The area involved in moulting is anterior to the base of the oesophagus but a more exact site could not be determined because all larvae ligated in this region died. Ligation of nematodes for such a long time must have caused considerable physiological upset and the results of such experiments should be treated with caution.

Rogers (1962) suggested that in non-parasitic nematodes the stimulus for moulting might originate internally but that in some parasites this process has been replaced by stimuli from the host. Fourth-stage larvae of *P. nanus* are examples of the initial stimulus being provided by the host. Bird and Rogers (1965) suggested that the reproductive system is involved in moulting of *Meloidogyne* spp. This is most unlikely in *P. nanus* where ligation prevented movement of substances from the developing gonads. Lee (1965) noted a probable relation between size and onset of moulting, and distention of the gut wall has been shown to be responsible for the initiation of moulting in *Rhodinus* sp. (Wigglesworth 1964). Wherever the stimulus is produced internally, it seems likely that it is referred to the oesophageal region from which movement of substances to the cuticle occurs; how many substances are involved is unknown.
Fig. 1.—Release of cuticle at the head.
Fig. 2.—Shedding of lining of amphidial pouches and stylet.
Fig. 3.—Formation of vagina and spermatheca.
Fig. 4.—Cast cuticle in region of excretory pore.
Fig. 5.—Final stage of moulting. Shed cuticle still attached at irregular intervals.

Fig. 1.—Ligatured potential male, showing shed cuticle anterior to ligature.

Fig. 2.—Ligatured potential female, showing reorganization of oesophagus is complete.

The stimulus for moulting of *P. nanus* operates as a “trigger” mechanism and temperatures of 25 and 30°C do not appear to affect receipt of the stimulating substance but do interrupt the processes which are “triggered”. The time necessary for receipt of the stimulating substance is relatively short—1 day or less—but considerable time elapses before moulting can be observed. This time may be necessary for elaboration and release of substances controlling moulting and would constitute a difference between true moulting and exsheathment where the fluids require release only (Rogers and Sommerville 1960).

A least two different methods for shedding the cuticle have been recorded. In *Caenorhabditis briggsae* (Jantunen 1964), the anterior part of the cuticle is shed separately from the posterior part. However, in *P. nanus* and *Seinura oxura* (Hechler 1966) the entire cuticle is shed in one piece. Thus, different processes might be expected in different nematodes. It has already been suggested that exsheathment of *Tripius sciarae* (Poinar and Doncaster 1965) is accomplished in a different way from exsheathment in trichostrongyle larvae (Rogers and Sommerville 1960), so considerable variations in mechanisms of moulting probably occurs.

It has been suggested that *P. nanus* and *P. projectus* may be synonyms (Fisher 1965) but maximum temperature at which moulting would occur in *P. projectus* was above 26°C (Rhoades and Linford 1959) and moulting seemed to be completed more rapidly than in my population. These differences are not valid for species differentiation but do suggest the possibility of races.

V. ACKNOWLEDGMENT

I wish to thank Professor W. P. Rogers for helpful advice and criticism.

VI. REFERENCES


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