The Fine Structure of the Cuticle of Tylenchorhynchus Martini and Meloidogyne Hapla.

Ibrahim Khayry atris Ibrahim
Louisiana State University and Agricultural & Mechanical College

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THE FINE STRUCTURE OF THE CUTICLE OF
TYLENCHORHYNCHUS MARTINI AND MELOIDOGYNE
HAPLA.

Louisiana State University and Agricultural and
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Botany

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THE FINE STRUCTURE OF THE CUTICLE OF
TYLENCHORHYNCHUS MARTINI AND MELOIDOGYNE HAPIA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Botany and Plant Pathology

by

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B.Sc., Alexandria University, 1960
M.S., University of California, 1964
January, 1967
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>Phytoparasitic Nematodes</td>
<td>4</td>
</tr>
<tr>
<td>Zooparasitic Nematodes</td>
<td>7</td>
</tr>
<tr>
<td>Free-Living Nematodes</td>
<td>11</td>
</tr>
<tr>
<td>Swarming Phenomenon</td>
<td>13</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>16</td>
</tr>
<tr>
<td>RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>ABBREVIATIONS FOR FIGURES</td>
<td>25</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>45</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>51</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>53</td>
</tr>
<tr>
<td>VITA</td>
<td>57</td>
</tr>
</tbody>
</table>

iii
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cuticular structure of adult females of some phytoparasitic nematodes</td>
<td>8</td>
</tr>
<tr>
<td>2. Cuticular structure of some zooparasitic nematodes</td>
<td>12</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cross section through the cuticle of Tylenchorhynchus martini, nonswarmer (X 22,000)</td>
</tr>
<tr>
<td>2.</td>
<td>Cross section through the cuticle of T. martini, swarmer, showing the lateral fields (X 23,000)</td>
</tr>
<tr>
<td>3.</td>
<td>Longitudinal section through the anal opening of T. martini, swarmer, showing internal cuticle (X 15,600)</td>
</tr>
<tr>
<td>4.</td>
<td>Cross section through the esophageal region of T. martini, swarmer, showing the cuticle, hypodermis, muscle cells and the esophageal lumen (X 13,500)</td>
</tr>
<tr>
<td>5.</td>
<td>Oblique cross section through the cuticle of T. martini, swarmer, showing changes at the edges of the interstitial regions (arrows), early stage in change from nonswarming to swarming condition (X 26,500)</td>
</tr>
<tr>
<td>6.</td>
<td>Oblique longitudinal section of the cuticle of T. martini, swarmer, showing morphological changes in the outer layers, late stage in change from nonswarming to swarming condition (X 23,500)</td>
</tr>
<tr>
<td>7.</td>
<td>Cross section through the cuticle of T. martini, swarmer, showing bright colored-spots in the matrix and the fiber layers (arrows), late stage in change from nonswarming to swarming condition (X 25,500)</td>
</tr>
<tr>
<td>8.</td>
<td>Oblique longitudinal section through the cuticle of T. martini, swarmer, showing a cuticular canal (X 28,800)</td>
</tr>
<tr>
<td>9.</td>
<td>Longitudinal section through the body wall of Meloidogyne hapla, adult female, showing 4 cuticular layers and the hypodermis (X 9,100)</td>
</tr>
<tr>
<td>10.</td>
<td>Longitudinal section through the cuticle of M. hapla, adult female, showing 3 fiberal zones (X 7,500)</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>11.</td>
<td>Three cortical layers (arrows) in the cuticle of <em>M. hapla</em>, adult female.</td>
</tr>
<tr>
<td></td>
<td>Note the regular-type annulation.</td>
</tr>
<tr>
<td></td>
<td>A. Oblique cross section (X 18,000). B. Longitudinal section (X 15,000)</td>
</tr>
<tr>
<td>12.</td>
<td>Longitudinal section through the cuticle of <em>M. hapla</em>, adult female,</td>
</tr>
<tr>
<td></td>
<td>showing rising-type annulation (X 14,000)</td>
</tr>
<tr>
<td>13.</td>
<td>Longitudinal section through the cuticle of <em>M. hapla</em>, adult female,</td>
</tr>
<tr>
<td></td>
<td>showing serrate-type annulation (X 10,500)</td>
</tr>
<tr>
<td>14.</td>
<td>The body wall of <em>M. hapla</em>, second stage larva.</td>
</tr>
<tr>
<td></td>
<td>A. Longitudinal section (X 10,500). B. Oblique cross section (X 11,000)</td>
</tr>
<tr>
<td>15.</td>
<td>The body wall of <em>M. hapla</em>, second stage larva.</td>
</tr>
<tr>
<td></td>
<td>A. Oblique cross section (X 6,500). B. Longitudinal section (X 17,000)</td>
</tr>
<tr>
<td>16.</td>
<td>Cross section through the esophageal lumen of <em>M. hapla</em>, second stage larva</td>
</tr>
<tr>
<td></td>
<td>A. Anteriorly (X 20,000). B. Posteriorly (X 15,500)</td>
</tr>
<tr>
<td>17.</td>
<td>Cross section through the esophageal region of <em>M. hapla</em>, second stage larva</td>
</tr>
<tr>
<td></td>
<td>(X 3,600)</td>
</tr>
<tr>
<td>18.</td>
<td>Enlarged portion of the esophageal lumen radius of <em>M. hapla</em>, second stage larva</td>
</tr>
<tr>
<td></td>
<td>A. Showing parallel shape cuticularization and a cavity in the radius wall (X 27,100). B. Showing scissor shape cuticularization (X 28,500)</td>
</tr>
<tr>
<td>19.</td>
<td>Dorsal radius of the esophageal lumen of <em>M. hapla</em>, second stage larva, showing a branching tip of the radius near the intestinal tissues (X 18,000)</td>
</tr>
</tbody>
</table>
ABSTRACT

Electron microscopy of Tylenchorhynchus martini, swarming and nonswarming specimens, and Meloidogyne hapla, adult females and second stage larvae, revealed that the cuticle of these nematodes is noncellular and multilayered; consisting of three main layers: cortex, matrix and a fiber layer.

Seven cuticular layers observed in T. martini were: external cortex, internal cortex, first boundary layer, matrix, second boundary layer, fiber and third boundary layer. The cuticle ranged in thickness from 0.75 μ to 1.0 μ, except in the lateral fields where it was about two-fold thicker. Four incisures appeared in each of the two lateral fields, except in the anterior and posterior regions where there were two or three incisures.

Morphological differences between the cuticle of swarming and nonswarming specimens of T. martini appeared in swarming specimens as modifications in some layers of the cuticle. These changes probably started at the edges of the interstitial regions as swellings in the external cortex and as a separation of the two cortical layers from each other because in later stages they extended throughout the interstitial regions. Some specimens exhibited abnormally bright light-colored spots in the matrix and fiber layers in addition to the cortical
changes. Sections of nonswarming specimens of *T. martini* showed no changes in the cuticular layers and the cuticle appeared intact.

Electron micrographs of *M. hapla* adult females showed the cuticle to consist of four main layers: cortex, matrix, fiber and a basal layer. Three cortical layers distinguished, particularly in the anterior striated portion of the body were: dense external layer, light median layer, and dense inner layer. Each cortical layer was about 30 μ thick and together they appeared as a triple-layered unit membrane. The fiber layer was the thickest layer of the cuticle and was not separated from other layers by boundaries. It consisted of three fiber zones, especially in the posterior region of the body. The cuticle ranged in thickness from 2.2 μ anteriorly to 3.4 μ in the posterior regions.

The cuticle of the second stage larva of *M. hapla* was about 1.6 μ thick and consisted of three main layers: the cortex, matrix and a fiber layer. The cortex had an electron-dense structure and was deeply striated. Beneath the cortex was an electron-transparent layer, the matrix, which was dissected by the striae of the cortex. The fiber layer beneath the matrix showed two zones: an outer zone which appeared in longitudinal sections as a palisade-like array of dense structures and an inner zone containing six dark bands separated from each other by light-colored areas. Cuticular thickening of the esophageal lumen wall was evident and in addition there was a pair
of tooth-like cuticular thickenings along both sides of each lumen radius. The cuticular thickening was composed of individual platelets arranged in longitudinal rows, which appeared in some sections parallel to each other and in other sections crossing each other forming scissor-shaped structures.
INTRODUCTION

Nematodes are a sharply differentiated group of invertebrates. They constitute one of the most important groups of organisms which inhabit the soil. An important part of the nematode body is the cuticle, which is tough, flexible, and noncellular.

The nematode cuticle acts not only as the boundary between the animal and its environment but also as a part of the hydrostatic skeletal system on which many of the characters considered diagnostic of the Nematoda are functionally dependent (12). The cuticle of nematodes plays an important part in the physiology and immunology of these animals (27). It is important also as the major barrier to the entry of nematocides. It is significant that millions of dollars have been spent on the development of nematocides and antihelminthics while only shreds of information are available on the nature of permeability and structure of the nematode cuticle (40).

Knowledge of cuticular structure is important to an understanding of several nematode manifestations. An interesting cuticular phenomenon is the swarming (aggregation in masses) of Tylenchorhynchus martini Fielding, 1956 and other nematodes. Preliminary investigations of the swarming phenomenon in T. martini indicated that it results from an apparent sticky condition of the nematode cuticle (17).
The principle objective of this work was to illustrate and compare the fine structure of the body wall of both swarming and nonswarming specimens of *T. martini* and nonswarming specimens of *Meloidogyne hapla* Chitwood, 1949. Electron microscope techniques were used to define structural details of the body wall of these nematodes.

It is the purpose of this thesis to illustrate and evaluate some fundamental differences which were discovered in the cuticle between swarming and nonswarming specimens of *T. martini* and to relate these to the general morphology of the surface layers in *T. martini* and *M. hapla*. 
REVIEW OF LITERATURE

Little is known about the fine structure of plant parasitic nematodes because much of their structural detail lies beyond the resolution of the light microscope and electron microscopy has been used only during the past ten years. Much has been published about the structure of the nematode cuticle, but most of the reports describe the cuticle as a simple structure consisting of a few layers (11, 39).

Von Siebold in 1848 (38) first called attention to the many-layered structure of the cuticle of a phasmidian nematode, *Ascaris lumbricoides* Linnaeus, 1758, and his observations were confirmed and extended by Czermak, Bastian, Von Bommel, and Goldschmidt (7). According to Chitwood and Chitwood, 1950 (7), all nematodes have a layered cuticle which is fundamentally similar in the various groups.

Goodey, 1963 (11), described the cuticle of nematodes as follows: It is composed of two or three layers; it may be quite smooth on the surface but very often is marked by a regular series of transverse striations. These striations are often interrupted by the lateral fields which are quite prominent in some forms. These lateral fields bear longitudinal lines which, together with the edges, are termed incisures (involution). Longitudinal striations sometimes occur also. Beneath the cuticle is the hypodermis, which has the form of a protoplasmic tube, inwardly bulging as four chords, one each located dorsally and
ventrally and two located laterally. Inside the hypodermis is the body musculature composed of a single layer of cells, whose outer fibrillated regions are attached to the hypodermis and whose protoplasmic inner sides abut on the body cavity. The body muscles are arranged in four principal groups which lie between the four chief hypodermal chords.

**Phytoparasitic nematodes**

Although the histological anatomy of nematodes representative of numerous groups have been investigated, a review of literature did not reveal much information about the fine anatomy of phytoparasitic species, rather emphasis has been placed upon their general morphology and life cycle.

Elsea, 1951 (8), studying cross sections of the root-knot nematode *Meloidogyne hapla* Chitwood, 1949, with the light microscope, reported the cuticle of adult females to consist of at least three layers: an external cortical layer, a middle matrix layer, and an innermost fiber layer. He found that the hypodermis lay beneath the cuticle and that it was syncytial and completely encircled the nematode body. Maggenti and Allen, 1960 (30), reported that the hypodermis of adult *Meloidogyne* females is thick and syncytial in structure. The hypodermal nuclei were found to be small and numerous. Discrete somatic muscles were not observed.

Hirschmann, 1959 (13), made histological studies on males of *Heterodera glycines* Ichinohe, 1952, and adults of *Hoplolaimus*
tylenchiformis Daday, 1905. She found that the external cuticle of these nematodes consists of three distinct layers: a hyaline coarsely-annulated outermost layer, a middle layer which is homogeneous in structure, and a third or innermost layer. She reported that the third layer appears in *Heterodera* males as a double annulation or as a row of dots; 2 annules or dots correspond to 1 annule of the outer layer. In *Hoplolaimus*, this layer was visualized as a dense refractive material with a slightly fibrous appearance in cross-section, and double annulation. She concluded that due to the relatively small size of these nematodes, it was difficult to compare their cuticular layering with that of *Ascaris lumbricoides*. It seems likely, however, that the outermost layer would correspond to the cortical layers, the middle layer to the matrix, and the innermost layer to the fiber layer (14).

Bird, 1958 (4), studying the cuticle of adult females of *M. hapla* and *M. javanica* Chitwood, 1949, with the light microscope, reported that there was considerable variation in the thickness of the female cuticle both in adults of different ages and in different regions of the same cuticle. The cuticle of these two species consists of a thin, darkly-staining surface layer covering a homogeneous substance which is divided into three layers by two darkly-staining bands. He also reported vertical structures running from the hypodermis to the outermost layer.
In an electron microscope study on \textit{M. javanica}, Bird and Rogers (5) reported that the cuticle consisted of a clearly-defined osmiophilic external cortical layer, and an internal cortical layer morphologically distinct from a thick fiber layer. In adult specimens this fiber layer appeared to consist of two layers particularly in the posterior region. They stated that high magnifications revealed vertical striae in the external cortical layer which appeared to connect with structures in the internal cortical layer. Externally, the striae were covered by a distinct membrane which probably corresponds to the osmiophilic surface membrane of the \textit{Ascaris} cuticle. The fiber layer merged with the hypodermis and was not separated from it by a basal lamella.

Wright, 1965 (45), studying the histology of the esophageal region of \textit{Xiphinema index} Thorne and Allen, 1950 reported that the external body cuticle appeared similar in structure throughout the esophageal region. He found four cuticular zones: an outer thin zone of high density, a thick granular zone, a fiber zone, and an inner lamellate zone. The outermost zone was approximately 30 \textmu m thick. The granular zone contained basally a palisade-like array of dense structures. The fiber zone contained two layers of obliquely-oriented fibers, and each layer was approximately 0.5 \textmu m thick. The innermost zone of the cuticle consisted of a variable number of layers of material that did not appear discretely fibrous, but resembled the texture of the fiber layer in its reaction with permanganate.
A summary and comparison of the cuticular structure of some phytoparasitic nematodes, which have been studied with the electron microscope, are shown in Table 1.

**Zooparasitic Nematodes**

Chitwood and Chitwood, 1950 (7), reported that the cuticle of *Ascaris lumbricoides* Linnaeus, 1758 is divisible into 9 distinct layers: (1) an external cortical layer; (2) an internal cortical layer; (3) a fibrillar layer; (4) a matrix layer (homogenous layer); (5) a boundary layer (bandschichte of Van Bommel); (6, 7 and 8) external, middle and internal fiber layers; and (9) a basal lamella. The above description in substance the same as that given by Van Bommel, 1894 (7). According to Chitwood and Chitwood, the cuticle of *Parascaris equorum* Goeze, 1782, *Toxocara canis* Werner, 1782, *Toxascaris leonina* Linstow, 1902, and other ascarids has the same fundamental structure.

Watson, 1965 (42), studying the fine structure of the body wall in adults of *A. lumbricoides*, reported that during growth of adult worms the cuticle increased in volume due to growth of all layers of the cuticle. The matrix layer of the cuticle increased in thickness more rapidly than the fiber layer, and both of these layers grew faster than the cortex. Ribonucleic acid was detected in the epidermis of the growing adult worms and its presence was correlated with the development of the endoplasmic reticulum and ribosomes. She reasoned that
Table 1. Cuticular structure of adult females of some phytoparasitic nematodes.

<table>
<thead>
<tr>
<th>Species</th>
<th>External Cortex</th>
<th>Internal Cortex</th>
<th>First Boundary</th>
<th>Matrix</th>
<th>Second Boundary</th>
<th>Fiber</th>
<th>Basal</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meloidogyne javanica</td>
<td>+(^b)</td>
<td>+</td>
<td>0(^c)</td>
<td>0</td>
<td>0</td>
<td>double-layered</td>
<td>0</td>
<td>Bird and Rogers, 1965 (5)</td>
</tr>
<tr>
<td>M. hapla</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>triple-layered</td>
<td>+</td>
<td>Ibrahim, Hollis and Birchfield, 1966 (21)</td>
</tr>
<tr>
<td>Tylenchorhynchus martini</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>single-layered</td>
<td>+</td>
<td>Ibrahim, 1965 (20)</td>
</tr>
<tr>
<td>Xiphinema index</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>double-layered</td>
<td>+</td>
<td>Wright 1965 (45)</td>
</tr>
</tbody>
</table>

\(^a\)Listed in order from outside to inside.

\(^b\) + present

\(^c\) 0 absent.
cuticular canals which she observed were probably involved in the
transport of the cuticular precursors from the hypodermis to the cuticular
layers.

Lee, 1965 (27), studying the cuticle of adult *Nippostrongylus*
brasiliensis Travassos, 1914, reported that the cuticle had the follow­
ing layers: an outer triple-layered membrane; a single cortical layer; a
fluid-filled layer which was traversed by numerous collagen fibrils and
struts which were suspended by collagen fibrils in the fluid-filled layer;
two fiber layers, each apparently containing 3 layers of fibers; and a
basement lamella. The fluid-filled layer contained haemoglobin and
esterase. The muscles of the body wall were attached to either the
basement lamella or to the fiber layers of the cuticle.

Lee, 1966 (28), studying the third stage larva of *N. brasiliensis*,
reported that the cuticle consists of 7 layers, an outer triple-layered
membrane, a double-layered outer cortex, an inner cortex, a matrix
layer, a striated layer and two fibril layers. There was no basement
lamella and the hypodermis was seen as a thin layer between the muscles
and the cuticle which expanded to form the dorsal, ventral and lateral
chords.

Jamuar, 1966 (24), studying third stage larva and adult specimens
of *N. brasiliensis*, reported that the cuticle apparently was noncellular
in nature since none of the cellular organelles could be found in this
region. In adult specimens, the cuticle consisted mainly of three
layers: cortex, matrix, and fiber layers. Externally the cortex was covered by an osmiophilic membrane about 7 mu thick, outside of which was a less regular layer about 17 mu thick; the two being separated by a lighter zone approximately 10 mu in width. The cortex was composed of homogeneous, faintly-granular material. Transverse bands were embedded in the matrix and were spaced at intervals varying between 1 and 2 u. Below the matrix there were two fibrous, collagen-like layers arranged obliquely to the body axis. The fibers ranged in width from 45 to 75 mu. The cuticle of the third stage larva appeared to be less complicated. The cortex itself was composed of dense homogeneous material and was bordered on the outer side by a membrane. It was divided into numerous annuli by transverse depressions in the external surface of the cuticle. In the matrix between annules there were two dense bodies. These were thought to be precursors of the transverse bands in the adult cuticle. The fiber layer was apparently absent. The innermost layer of the matrix was composed of a crystalloid structure showing dense parallel lines in longitudinal section.

Anya, 1966 (2), investigating the cuticular structure of Aspiculuris tetraperta Nitzsch, 1821, reported that there were 3 basic layers in the cuticle of this nematode: the cortex, which consisted of an outer and an inner layer, the matrix layer, and the complex fiber layer consisting of three layers. There was, in addition, a thin osmiophilic superficial membrane on the surface of the cuticle. This membrane was seen only
with the electron microscope. It was triple-layered and consisted of
two electron-dense layers separated by a less electron-dense layer.

A summary and comparison of the cuticular structure of some
zooparasitic nematodes, which have been studied with the electron
microscope, are shown in Table 2.

Free-Living Nematodes

Inglis, 1964 (23), studying the cuticle of a Chromadorida, reported
that the nematode cuticle consisted grossly of 3 layers: an outer layer -
the cortical layer; an intermediate layer - the matrix layer; and an inner
layer - the basal layer. With these layers was associated a punctation
channel component. The cortical layer carried externally a wholly
sclerotized layer, the epi-cortex. The inner part of the basal layer was
modified as a basal lamella for the attachment of the hypodermis, from
which it was not sharply delimited. He suggested that the treatment of
the cuticle as basically three layers with a punctation channel system can
be expanded to cover conditions in all the Nematoda. No fiber layers
were present in the Chromadorida and this condition can be applied also
to the Monhysterida and the Axonolaimida.

According to Chitwood and Chitwood, 1950 (7), the cuticle of the
Chromadorida usually appears as two dense layers, a cortical layer
and a basal layer separated by a more or less distinct matrix layer. In
the genus Aphanolaimus, the matrix layer was scarcely distinguishable
from the basal layer. In Halichoanolaimus and similar forms, the
Table 2. Cuticular structure of some zooparasitic nematodes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Triple-layered membrane</th>
<th>Outer layer</th>
<th>Middle layer</th>
<th>Fiber</th>
<th>Basal lamella</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspiculuris tetraptera^d</td>
<td>+</td>
<td>outer and inner cortex</td>
<td>matrix</td>
<td>triple-layered</td>
<td>0^c</td>
<td>Anyia, 1966 (2)</td>
</tr>
<tr>
<td>Nippostrongylus brasiliensis^e</td>
<td>+</td>
<td>single-layered cortex</td>
<td>matrix, dense bodies and crystalloid bodies</td>
<td>0</td>
<td>0</td>
<td>Jamuar, 1966 (24)</td>
</tr>
<tr>
<td>N. brasiliensis^a</td>
<td>+</td>
<td>double-layered outer cortex and inner cortex</td>
<td>matrix and striated layer</td>
<td>double-layered</td>
<td>0</td>
<td>Lee, 1966 (28)</td>
</tr>
<tr>
<td>N. brasiliensis^d</td>
<td>+</td>
<td>single-layered cortex</td>
<td>matrix and transverse bands</td>
<td>double-layered</td>
<td>0</td>
<td>Jamuar, 1966 (24)</td>
</tr>
<tr>
<td>N. brasiliensis^d</td>
<td>+</td>
<td>single-layered cortex</td>
<td>fluid-filled and struts</td>
<td>double-layered</td>
<td>+</td>
<td>Lee, 1965 (27)</td>
</tr>
</tbody>
</table>

^a Listed in order from outside to inside.
^b + present.
^c 0 absent.
^d Adult female.
^e Third stage larva.
matrix layer appeared in cross-section as a cavity traversed by the fibril layer and the "tubes" of the fibril layer caused the appearance of punctuation when viewed in toto. In Monoposthia, the cortical and basal layers were separated by a distinct cavity which contained a pair of lateral cuticular ridges. The cuticle of such forms as the chromadorids probably contains oblique fibers; presumably the fiber layers are represented by the basal layer and punctuation is a manifestation of the fibril layer.

Swarming Phenomenon

In 1955, Meyl (33) reported a phenomenon, which resembles swarming, in the nematode species Hemicyclophora typica de Man, 1921. He called it Nesterbildung and attributed it to a sexual function in the life cycle of this nematode.

Hollis, 1958 (15) discovered and named the swarming phenomenon in Tylenchorhynchus martini Fielding, 1956 and reported such stimulating factors as a high proportion of active nematodes and a high suspension density. He found that a range of room temperature and of pH 3.5 - 11.0 did not influence swarming intensity. In 1960 (16), he found that the swarming reaction in T. martini resulted from an apparent sticky condition of the cuticle and that it was nonsexual and species specific. He suggested a hypothesis that this phenomenon may be conditioned by the state of polysaccharide, lipid, or protein substances of the cuticle. He also found that swarming occurred in a species of Hemicyclophora.
In another study, Hollis, 1962 (17), reported that the state of swarming was induced in *T. martini* by abundant and rapid host-plant growth. It was characterized by: an innate morphological modification of the cuticle, participation of all stages of worm development, and higher egg-laying rates in swarming specimens. Swarming was inhibited by enzymes such as papain, ficin, and trypsin. This inhibition was removed by washing to eliminate the enzymes and this was believed to provide evidence of the proteinaceous nature of the cuticle surface layer. He hypothesized that the capacity for centering the swarming state is under genic control, and that its induction is determined by nutritional factors.

Swarming was noted by Whitehead (42) in Kenya in species of *Hemicyclyiophora*, *Rotylenchulus*, and *Scutellonema*. Steiner (31) reported what appears to be swarming in an *Oncholaimus* species in Puerto Rico. Greenhouse populations of swarming *T. claytoni* Steiner, 1937 were observed by Chapman (31) in Kentucky. A *Mononchus* sp. and a *Hemicyclyiophora* sp., from several different grasses in Kenya, were observed in a swarming condition by Hollis (18). Swarming has been reported also in *Dorylaimus pusillus* Cobb, 1893 in Louisiana (17) and in *Helicotylenchus nannus* Steiner, 1945 in Florida (31, 32).

Hollis and McBride, 1962 (19) studying the swarming phenomenon in *T. martini*, reported that swarming, unlike anabiosis, is induced by favorable conditions of rapid and abundant plant growth. Changes from
a nonswarming to a swarming condition apparently depends on specific nutritional factors or a host-parasite relation conducive to rapid buildup of the nematode population.

McBride, 1964 (31), and McBride and Hollis (32), after extensive studies on the induction of swarming in *T. martini*, found that complete nutrition of the host with all essential nutrient elements was necessary to the induction of swarming. Organic supplements to nutrient solutions had no effect on the induction of swarming. They reported that *T. martini* and *T. claytoni* swarmed together. *T. martini* and unidentified species of *Tylenchorhynchus* swarmed separately in mixtures, indicating that both species and group specificity of the swarming reaction may be common in the genus *Tylenchorhynchus*. The activity of swarming nematodes is best characterized as continual and rapid, jerky movements which result apparently from attempts of individuals to break free from swarms. Aggregation due to swarming was differentiated from that due to anabiosis by the mode of separation of specimens. Swarmers separate only at right angles to the plane of contact whereas anabiosis separate by gliding motions parallel to contact planes.
MATERIALS AND METHODS

*Tylenchorhynchus martini*

Swarming and nonswarming specimens of *T. martini*, grown on rice plants in the greenhouse, were sieved from soil with tap water and placed in 1% unbuffered osmium tetroxide solutions in small vials for 2 hours at 5°C. Specimens were then washed in distilled water and dehydrated gradually in a series of 4 aqueous ethanol solutions, then in absolute ethanol as outlined below:

1. 25% ethanol for 1 hour.
2. 50% ethanol for 1 hour.
3. 70% ethanol for 1 hour.
4. 90% ethanol for 1 hour.
5. Absolute ethanol for 6 hours.
6. Absolute ethanol for 8 hours.

The solvent was decanted or replaced in the vials between each step with a hypodermic needle. The specimens were then washed twice in propylene oxide for 1 hour each time because the embedding resin, maraglas (Polysciences Inc., Rydal, Pennsylvania), was soluble in propylene oxide and insoluble in alcohol. Specimens then were incubated in a mixture of propylene oxide plus maraglas mixture 1:1 v/v and maraglas mixture for about 4 hours each in a vacuum desiccator to remove any air bubbles from the tissues and to increase penetration by
the maraglas mixture. The maraglas mixture used for embedding consisted of ml proportions of maraglas 34, cardolite 10, dibutyl phthalate 5, and benzyl dimethylamine 1 (10).

A drop of fresh maraglas mixture was added to the bottom of a Beem plastic capsule; nematode specimens were introduced and oriented, and maraglas mixture added to fill the capsule. The capsules were incubated at 60 C for 48 hours, then blocks were trimmed and sections were cut to give a bright gold to silver interference color with a glass knife in a Sorvall MT-2 Porter-Blum Ultra-Microtome. Sections were stained in 1% aqueous lead oxide for 10 minutes (25) and then viewed under a HU-11A Hitachi electron microscope at 50 KV.

**Meloidogyne hapla**

Adult females and second stage larvae of *M. hapla* were obtained from infected roots of tomato plants grown in the greenhouse. Females were separated by placing a galled piece of root in water and dissecting away the root tissues from the body of the animals. Small galls on fine roots were found to be the most satisfactory, because the females were easily exposed by dissection. Second stage larvae were collected from around roots and from hatching eggs. Several cuts were made in the body of adult females just before killing and fixing. This was necessary because otherwise the presence of a thick cuticle hindered proper fixation of internal structures.
Specimens of *M. hapla* were fixed at room temperature in small vials for 2 hours in 6% gluteraldehyde in 0.05 M phosphate buffer at pH 6.8. Specimens were then exposed for 2 hours in 2% osmium tetroxide in 0.05 M phosphate buffer at pH 6.8 for final fixation. The fixed specimens were then washed thoroughly with distilled water and dehydrated in a graded series of ethanol aqueous solutions as in the case of *T. martini*.

*M. hapla* specimens were washed twice with propylene oxide then soaked in 1:1 v/v mixture of propylene oxide and Epon mixture for 3 hours in a vacuum desiccator. The Epon mixture was prepared as follows (29, 34):

- (Epon 812) 15 ml (The Ring Chem. Co., 1112 Rosine Street, Houston, Texas).
- (Araldite 506) 20 ml (Ciba Products Co., Fair Lawn, New Jersey).
- Dibutyl phthalate 1.5 ml.
- Benzyl dimethyl amine 1.0 ml.

Specimens were treated in the same manner as those of *T. martini*, except sections of *M. hapla* were stained with 2% aqueous lead oxide for 15 minutes and then with 2% aqueous urinyl acetate for 30 minutes.
RESULTS

*Tylenchorhynchus martini*

Sections of mature females of swarming and nonswarming *T. martini* viewed and photographed under the electron microscope show that the cuticle is about 1.0 μm thick and consists of seven layers (20). These cuticular layers are (listed from outside to inside): external cortex, internal cortex, first boundary layer, matrix layer, second boundary layer, fiber layer, and third boundary layer (Fig. 1, 4).

The external cortex is the outermost layer of the cuticle, it appears as an electron-dense layer about 40 μm thick. The internal cortex is an electron-transparent structure and has the same thickness as the outer layer. The first boundary layer, which separates the internal cortex from the matrix layer, is an electron-dense layer about 50 μm thick. These three outer layers appear as a thick unit-membrane covering the entire body of the nematode in both the striae and interstrial regions. In some regions of the cuticle, these three layers appear undifferentiated as a thick line.

The matrix layer is light-colored, electron-transparent and about 120 μm in thickness. Separating the matrix layer from the fiber layer is the second boundary layer. It is a dark discontinuous layer and probably serves to connect the layers above and below it with each other. The second boundary layer is about 60 μm thick. The fiber
layer is 0.4-0.5 μ thick and has a low optical density similar to the matrix. The third boundary layer, which separates the fiber layer from the hypodermis, has a high density and excepting the occurrence of some light-colored areas appears similar in structure to the first boundary layer. It has a thickness of 60-80 μ.

The cuticle in the lateral fields is about two-fold thicker than in other areas and the depth of the longitudinal striae (grooves) in these regions is 0.7-1.0 μ. Each lateral field consists of four longitudinal striae, except at the anterior and posterior ends where they may be reduced to three or two striae. In the lateral fields, the cuticular layers are not clearly differentiated and the layers, especially the matrix and the fiber layers, are much thicker and more irregular than in the other areas of the cuticle (Fig. 2).

Striation occurs longitudinally and transversely in the cuticle of *T. martini* (Fig. 1, 3). This pattern of striation suggests that aerolation of the cuticle may occur. The average length of the interstrial regions is about 1.0 μ.

The internal cuticle, which lines all the natural openings of the nematode body, is similar in structure to the external cuticle (Fig. 3). Longitudinal sections through the anal opening show that the lower side is lined with a cuticle similar to the external cuticle. The three outer layers (on the upper side of the anal opening) appear normal but the lower layers are irregular in shape.
The hypodermis is a thin tubular layer about 80 μm thick lying beneath the cuticle. This layer bulges into the body cavity at four places to form the chords; the two lateral chords are large and the dorsal and ventral chords are small (Fig. 4, 5).

The Cuticle of Swarming and Nonswarming T. martini

There are morphological differences between the cuticle of swarming and nonswarming populations of T. martini. Such differences appear in swarming specimens as modifications in some cuticular layers, especially the cortex and the fiber layers. In many sections of swarming specimens, changes in the outer layers of the cuticle were detected. These changes probably started at the edges of the interstitial regions (Fig. 5) as a swelling in the external cortex and separation of the two cortical layers from each other. In later stages, these changes extended throughout the interstitial regions (Fig. 6). In some specimens, abnormal bright light-colored spots occurred in the matrix and fiber layers in addition to the changes in the cortex (Fig. 7). Sections of nonswarming specimens of T. martini showed no changes in the cuticular layers and the cuticle appeared intact (Fig. 1).

Meloidogyne hapla, Adult Female

Electron micrographs of M. hapla adult females show the cuticle as a multilayered structure ranging in thickness from 2.2 μm anteriorly
to 3.4 μm in the posterior regions. The cuticle consists of four main layers: cortex, matrix, fiber and basal layer (Fig. 9).

The cortex appears as a dark line covering the entire cuticle and is about 80 μm thick. Three cortical layers can be distinguished, particularly in the anterior striated portion of the body: dense external layer, light median layer, and dense inner layer (Fig. 11). Each cortical layer is about 30 μm thick and together they appear as a triple-layered unit membrane. These layers are undifferentiated in the posterior regions of the body (Fig. 9).

The matrix is a dense interrupted layer lying beneath the cortex and ranging in thickness from 0.3 μm posteriorly to 1.0 μm anteriorly.

The fiber layer is 1.4-2.5 μm thick, is less dense than the matrix, and is not separated from other layers by boundaries. Three fiberal zones can be seen (Fig. 10). They are: outer fiber zone, about 0.5 μm thick; middle fiber zone, about 1.0 μm thick, and inner fiber zone, about 0.4 μm thick. The basal layer is very light-colored, discontinuous layer about 0.5 μm thick. Either the inner fiber layer or the basal layer is attached to the hypodermis.

Striation occurs both longitudinally and transversely in the cuticle of adult females of *M. hapla* (Fig. 11, 13). Three types of annulations have been found; these are the regular-type (Fig. 11), the rising-type (Fig. 12), and the serrate-type (Fig. 13). Striation occurs in the anterior portion of the body and in the perineal region. The cuticle
appears stretched in the middle region of the nematode body because it is without annulation (Fig. 9).

The hypodermis is 2.5-3.0 \( \mu \text{m} \) thick and contains many protoplasmic structures in a syncytial layer (uninterrupted cellular structure) (Fig. 9). Their diameters in microns are: nuclei 1.2-2.8, mitochondria 0.5-1.2, lipid bodies 0.4-0.6, and protein bodies averaging 0.7.

**Meloidogyne hapla, Second Stage Larva**

The cuticle of the second stage larva of *M. hapla* is about 1.6 \( \mu \text{m} \) thick. It is multilayered and deeply striated. The average length of the interstitial regions is about 0.4 \( \mu \text{m} \) and depth of the striae is about 0.3 \( \mu \text{m} \) (Fig. 14, 15).

The outermost layer of the cuticle, the cortex, is an electron-dense layer about 0.05 \( \mu \text{m} \) in thickness. Lying beneath the cortex is an electron-transparent layer, the matrix, which is dissected by the striae of the cortex. The matrix has a thickness of 0.2 \( \mu \text{m} \). Beneath the matrix is the fiber layer, in which two zones can be distinguished: an outer zone and an inner zone. The outer fiber zone is about 0.3 \( \mu \text{m} \) thick; in oblique longitudinal sections, it appears as a lamellate or palisade-like array of dense structures (Fig. 15). The inner fiber zone is about 0.8 \( \mu \text{m} \) thick; it contains 6 dark bands which are separated from each other by light-colored areas (Fig. 14).

The hypodermis lies between the cuticle and the muscle regions. It appears as a tubular protoplasmic layer and has dark particles, about
0.2 μ in diameter, scattered throughout its volume. The hypodermis bulges in four regions to form two large lateral chords and a small dorsal and ventral chord. Its thickness is about 0.4 μ (Fig. 14, 17).

The muscle region consists of elliptical-shaped cells, filling the areas between the hypodermal chords. The thickness of this layer is about 2.0 μ (Fig. 14, 15, 17).

Cross-sections in the anterior part of the body of second stage larva, show that the esophagus has an elliptical shape with a diameter of 14-30 μ. The esophageal lumen is triradiate in form with two sub-ventral radii and one dorsal radius; average diameter of each radius is about 5.0 μ (Fig. 16, 17). Along both sides of each lumen radius is a pair of tooth-like cuticular thickenings, and in addition there is a cuticular thickening of the wall of the esophageal lumen (Fig. 16, 17). This cuticular thickening is composed of individual platelets arranged in longitudinal rows, which appear in some sections parallel to each other and in other sections crossing each other to form scissor-shaped structures (Fig. 18).

A cavity in the dorsal radius of the esophageal lumen can be seen in the posterior part of the esophagus (Fig. 18). The dorsal radius, in the region adjacent to the intestine, appears to branch at its tip to form small tubules which open into the intestinal tissue (Fig. 19).
ABBREVIATIONS FOR FIGURES

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Figure 1. Cross section through the cuticle of *Tylenchorhynchus martini*, nonswarmer (X 22,000).
Figure 2. Cross section through the cuticle of *T. martini*, swarmer, showing the lateral fields (X 23,000).
Figure 3. Longitudinal section through the anal opening of *T. martini*, swarmer, showing internal cuticle (X 15,600).
Figure 4. Cross section through the esophageal region of *T. martini*, swarmer, showing the cuticle, hypodermis, muscle cells and the esophageal lumen (X 13,500).
Figure 5. Oblique cross section through the cuticle of *T. martini*, swarmer, showing changes at the edges of the interstitial regions (arrows), early stage in change from nonswarming to swarming condition (X 26,500).
Figure 6. Oblique longitudinal section of the cuticle of *T. martini*, swarmer, showing morphological changes in the outer layers, late stage in change from nonswarming to swarming condition (X 23,500).
Figure 7. Cross section through the cuticle of *T. martini*, swarmer, showing bright colored-spots in the matrix and the fiber layers (arrows), late stage in change from nonswarming to swarming condition (X 25,500).
Figure 8. Oblique longitudinal section through the cuticle of *T. martini*, swarmer, showing a cuticular canal (X 28,800).
Figure 9. Longitudinal section through the body wall of Meloidogyne hapla, adult female, showing 4 cuticular layers and the hypodermis (X 9,100).
Figure 10. Longitudinal section through the cuticle of *M. hapla*, adult female, showing 3 fiberal zones (X 7,500).
Figure 11. Three cortical layers (arrows) in the cuticle of *M. hapla*, adult female. Note the regular-type annulation. A. Oblique cross section (X 18,000). B. Longitudinal section (X 15,000).
Figure 12. Longitudinal section through the cuticle of *M. hapla*, adult female, showing rising-type annulation (X 14,000).
Figure 13. Longitudinal section through the cuticle of *M. hapla*, adult female, showing serrate-type annulation (X 10,500).
Figure 14. The body wall of *M. hapla*, second stage larva.
A. Longitudinal section (X 10,500).
B. Oblique cross section (X 11,000).
Figure 15. The body wall of *M. hapla*, second stage larva.
A. Oblique cross section (X 6,500).
B. Longitudinal section (X 17,000).
Figure 16. Cross section through the esophageal lumen of *M. hapla*, second stage larva. A. Anteriorly (X 20,000). B. Posteriorly (X 15,500).
Figure 17. Cross section through the esophageal region of *M. hapla*, second stage larva (X 3,600).
Figure 18. Enlarged portion of the esophageal lumen radius of *M. hapla*, second stage larva. A. Showing parallel shape cuticularization and a cavity in the radius wall (X 27,100). B. Showing scissor shape cuticularization (X 28,500).
Figure 19. Dorsal radius of the esophageal lumen of *M. hapla*, second stage larva, showing a branching tip of the radius near the intestinal tissues (X 18,000).
DISCUSSION

The value of electron microscopy in the study of nematode structure has been demonstrated recently in a series of publications (2, 3, 24, 26, 27, 42, 44). However, these studies have dealt mainly with the larger animal-parasitic nematodes, especially of the genera Ascaris, Parascaris, and Capillaria, on which standard methods of tissue preparation for the electron microscope have been adequate.

There are at least two difficulties in the preparation of smaller nematodes for electron microscopy which are not encountered in the preparation of larger species: infiltration of the nematode with a suitable embedding medium and orientation of the specimens. These problems were encountered by the writer especially in the case of Tylenchorhynchus martini specimens. Random orientation of these animals in the embedding block did not permit efficient sectioning and an excessive amount of tissue were lost in mounting on the copper grids for viewing under the electron microscope.

The present study shows that the cuticle of T. martini consists of three main layers: cortex, matrix, and fiber layers. These layers are separated from each other by two boundary layers and the fiber layer in turn is separated from the hypodermis by a third boundary layer. The results show that the cuticular morphology of T. martini embraces the general concept of the nematode cuticle as it is understood at the present time.
Three basic cuticular layers have been described in *Heterodera glycines* and *Hoplolaimus tylenchiformis* by Hirschmann, 1960 (14). Electron microscopy was used to demonstrate nine layers in the cuticle of *Ascaris lumbricoides* (3, 42), but these layers are simply derivatives of the three basic layers. An increase in the number of visible layers has been attributed to the large size of ascarid nematodes (14). However the present results and those of Hirschmann demonstrate the cuticle (of small nematodes) to be equally complex.

A striking feature of the cuticle of swarming specimens of *T. martini* is the morphological changes in some cuticular layers. These changes include swelling of the external cortex, separation of the two cortical layers, and bright, light-colored spots in the matrix and the fiber layers. It seems likely that these changes or modifications in the cuticle, especially the rupturing and swelling of the outer layer, are related to the sticky condition of the cuticle of swarming specimens. The cuticle of nonswarming specimens of *T. martini* showed no morphological changes and appeared intact (22).

Rogers (35, 36) suggested that molting and hatching of free-living nematodes are controlled and coordinated by some internal mechanism in the animal. Internal secretion may regulate the time of production of substances which cause molting and hatching. He further suggests that in parasitic species, part of this mechanism might be lost so that the parasite could depend on the host to replace it. The host
might provide a stimulus which induces the nematode to produce the internal secretions or might provide substances which replace the missing internal secretions.

The morphological differences between swarming and nonswarming specimens of *T. martini* indicate that swarming may be initiated by an internal mechanism similar to that described for hatching and molting in nematodes by Rogers (35). Internal secretions may induce the abnormal changes in the cuticle as they reach the outer layers. Special cuticular canals (Fig. 8) in the cuticle may be functioning as avenues for the internal secretions.

Rupturing or disintegration of the external cortex of swarming specimens of *T. martini* probably is due to the effect of the internal secretion on this layer. As a result of this, more material of the external cortex will be exposed to the surrounding environment. Association of water molecules with the exposed cuticular material may result in a swollen gel-like structure. This gelation and swelling could account for the sticky condition manifested in the movement of swarmers (17, 31, 32).

The induction of swarming in *T. martini* by rapidly growing plants and by complete nutrition of the host in greenhouse tests (17, 31), gives support to the above idea, that swarming is initiated by an internal mechanism or stimulus. The induction of swarming also showed that the phenomenon is the result of nematode population responses to a superabundant food level.
Wallace (41) stated that in some species an external stimulus from the host is necessary to initiate molting and that the excretory system may be connected in some way with this process. On the other hand, the stimulus to molt may be related to nutrition and growth.

Changes in the morphology of the cuticle have been reported in some nematodes. Allen, 1957 (1), reported that one of the most dramatic changes in the cuticle of the genus *Trichodorus* is the increase in thickness with death. Van Gundy (40) observed the swelling of the cuticle of *Trichodorus* when death occurred in distilled water without any heat or other fixation of the nematodes. Superficially, this resembles a change in the chemical structure whereby water is rapidly imbibed by the cuticle, causing it to swell. This demonstrates that living processes in the nematode may have some control and regulation over the biochemistry of the cuticle.

It has been shown by Anya (2) that the inner cortex in *Aspiculuris*, *Syphacia* and *Ascaris* contained ribonucleic acid (RNA), and the major structural protein of the cuticle is a collagen. He suggested that the inner cortex is involved in protein synthesis.

The cuticle of nematodes grows after the final molt but the synthesis of proteins of the cuticle has always been assumed to be a function of the hypodermis (7, 42). Such a view finds support in the high concentration of enzymes, reserved food materials, such as glycogen and lipids, and RNA in the hypodermis of many nematodes (9, 26, 42).
Watson (42) believes that in *Ascaris* the precursors of the cuticular proteins are secreted from the hypodermis through a system of canals described by her. The system of canals may be necessary if they were needed for the transport of macromolecules from the hypodermis to the outer layers of the cuticle. If, on the other hand, synthesis of the macromolecules which constitute the outer layers takes place in a location in the outer layers, as suggested by Anya (2), then only the transport of smaller molecules would be involved.

The clarity with which the tissues of the esophagus of *M. hapla*, second stage larva, have been resolved in this study provides more precise information on the structure and function of this important part of the nematode than could be obtained with light microscopy. The most striking feature is the cuticular thickening of the lumen radii in the esophagus and the formation of 3 pairs of tooth-like thickenings, around the lumen radii. These cuticular thickenings appear in cross-sections as light-colored structures striped by dark cuticular lines similar to those of the external cortex. The dark cuticular lines are organized in both parallel and scissor shapes. This suggests that not only the cuticular thickenings provide rigidity for the esophageal lumen but also they provide flexibility to the esophagus through their lamellate construction.

This study shows for the first time that posteriorly, the dorsal radius of the esophageal lumen branches at its tip to form small tubules. The end of these tubules open in the nearby tissue of the intestine.
The cuticle of adult females of *Meloidogyne hapla* is a thick multilayered noncellular structure. Four main layers were detected: cortex, matrix, fiber, and basal layer. The cortex appeared to consist of 3 layers: external, internal, and boundary layer. These layers bear a great similarity to the outer 3 layers of the cuticle of *T. martini*. The matrix has a dense structure and is not separated from the fiber layer by a boundary layer as in *T. martini*. The fiber layer consists of 3 fiber zones which can be differentiated particularly in the posterior region, because of the overall thickness of the fiber layer. The basal layer is very light in color and is similar to the fluid-filled layer of adult *Nippostrongylus brasiliensis* (27).

This study shows that the cuticle of both *T. martini* and *M. hapla*, as well as of the other nematodes so far studied, is noncellular, as opposed to the cellular cuticle or integument of cestodes (37) and trematodes (6).
SUMMARY

1. Electron microscopy of swarming and nonswarming populations of *Tylenchorhynchus martini* revealed that the cuticle consisted of seven layers. These cuticular layers were (listed from outside to inside): external cortex, internal cortex, first boundary layer, matrix, second boundary layer, fiber layer, and third boundary layer.

2. The cuticle in the lateral fields was about two-fold thicker than in other areas. Each lateral field consisted of four longitudinal striae, except at the anterior and posterior ends where there were two or three striae.

3. Morphological differences between the cuticle of swarming and nonswarming specimens of *T. martini* appeared in swarming specimens as changes or modifications in some cuticular layers. These changes probably started at the edges of the interstitial regions as swellings in the external cortex and as a separation of the two cortical layers from each other. In later stages these changes extended throughout the interstitial regions. Some specimens exhibited abnormally bright, light-colored spots in the matrix and in the fiber layers in addition to the cortical changes.

4. The cuticle of adult females of *Meloidogyne hapla* consisted of four main layers: cortex, matrix and fiber layers and a discontinuous
basal layer. Three cortical layers were observed in the anterior striated portion of the body; these were: a dense external layer, a light median layer, and a dense inner layer. The fiber layer was the thickest layer and was not separated from other layers by boundaries. It consisted of three fiber zones, especially in the posterior region of the body.

5. Three types of striations were observed in the cuticle of adult females of *M. hapla*. They were: regular-type, rising-type, and serrate-type striation.

6. The cuticle of *M. hapla*, a second-stage larva showed three main layers from outside to inside: cortex, matrix, and fiber layer. The fiber layer showed two zones: an outer zone, which appeared in longitudinal sections as a palisade-like array of dense structures, and an inner zone containing six dark bands separated from each other by light-colored areas.

7. Cuticular thickening of the esophageal lumen wall of *M. hapla*, second stage larva, was evident and in addition there was a pair of tooth-like cuticular thickenings along both sides of each lumen radius. Each cuticular thickening was composed of individual platelets arranged in longitudinal rows.
LITERATURE CITED


53


VITA

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EXAMINATION AND THESIS REPORT

Candidate: Ibrahim Khayry Atris Ibrahim

Major Field: Plant Pathology

Title of Thesis: The Fine Structure of the Cuticle of *Tylenchorhynchus martini* and *Meloidogyne hapla*

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

November 15, 1966