A STUDY OF SOME FREE LIVING MARINE NEMATODES AND OF *MERMIS NIGRESCENS* WITH REGARD TO THE DEVELOPMENT, CHEMICAL NATURE AND FUNCTION OF THEIR ANTERIOR PIGMENT SPOTS.

by

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A Study of Some Free Living Marine Nematodes and of Mermis nigrescens with Regard to the Development, Chemical Nature and Function of Their Anterior Pigment Spots.

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ABSTRACT

In order to elucidate the possible visual function of the anterior pigment spots found in certain nematodes, studies were made of the eyespots of several species of free-living marine nematodes of the Burrard Inlet area of British Columbia and of the chromatrope of the insect parasite, Mermis nigrescens.

During laboratory rearing of the nematodes, it was observed that embryogenesis in Oncholaimus vesicarius, O. skawensis, Enoplus anisospiculus, Enoplus sp., Seuratiella sp. and Symplocostoma sp. followed the same general pattern as in other free-living nematodes. The process of blastopore closure differed from that of parasitic nematodes reported in the literature. Development of the eyespots occurred just prior to hatching in all species except Symplocostoma sp. The specificity of the time of development and the discrete location of the eyespots suggested that they perform a definite function in the nematode and are not simply storage or excretory products. Post parasitic M. nigrescens females were reared to maturity and the eggs produced parthenogenetically yielded viable larvae when fed to locusts. The chromatrope pigment first appeared just prior to the post parasitic moult.

The chemical characteristics of the pigment spots were determined through solubility tests, microspectrophotometry, histochemistry and examination for fluorescence. In E. anisospiculus, two pigments were found, a melanin confined to the eyespot area and a hemosiderin deposited along the eso-

phageal muscle. The eyespots of O. vesicarius consisted mainly of a melanin, but also had a minor, possibly carotenoid component, while O. skawensis, Chromadorina germanica and Symplocostoma sp. eyespots contained only a carotenoid-like pigment. Seuratiella sp. eyespots contained a melanin similar to that of O. vesicarius. The presence of an oxyhemoglobin in the M. nigrescens chromatrope was confirmed by microspectrophotometry. The spectrum was similar to that found for other parasitic nematode hemoglobins in that the beta peak absorption was much higher than the alpha peak absorption indicating that the hemoglobin has a high affinity for oxygen.

The probability that the eyespot pigments of the marine nematodes do not function directly in photoreceptive processes, but act rather as shading pigment for adjacent photoreceptors is discussed.

ACKNOWLEDGEMENTS

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CHAPTER 1

GENERAL INTRODUCTION

What is the function of the anterior pigment spots in nematodes? They are commonly called eyespots and it has long been conjectured that they might be involved in photoreception. An early nematologist, Eberth (1863), in a discussion of nematode sense organs referred to them as "eyes" and his contemporary, Bastian (1865) called them ocelli. There was however no real evidence to support this terminology then, nor was there at the time this work was begun.

Anterior pigment spots are found in many species of free-living aquatic nematodes that might be exposed to light and are not found in terrestrial or parasitic nematodes except in the free-living adult female of the insect parasite Mermis nigrescens. Among the aquatic nematodes, the pigment spots are typically paired and embedded in the anterior esophageal musculature. The pigment is granular and ranges in colour from orange through red, violet, brown and black. The pigment spots have varying degrees of structural complexity in different species. Among the more specialized are those of Parasymplocostoma formosum (Schultz, 1931) and Deontostoma californicum (Siddiqui and Viglerchio, 1970a) in which the eyespot is cup shaped and is associated with a refractile body and nucleated cell. In some species such as Enoplus communis, the pigmentation is not confined to the paired anterior spots, but also extends posteriorly along the esophagus (Schultz,

1931). In adult Mermis females, a red non-granular pigment identified as hemoglobin occurs as a band in hypodermal chord material anterior to the trophosome (Ellenby and Smith, 1966b).

Behavioural responses to light have been demonstrated in only three nematodes with eyespots. In Diplolaimella schneideri a rapid negative phototaxis was observed upon exposure of the nematodes to either daylight or fluorescent light (Chitwood and Murphy, 1964). Chemical identity of the pigment in this species is not known. In Chromadorina viridis, a positive phototaxis was demonstrated at most wavelengths of visible light (Croll, 1966a). The action spectrum of this response was at first thought to correlate with the absorption spectrum of hemoglobin contained in the eyespots. More recently, it has been found that these pigment spots do not contain hemoglobin (Croll et al., 1972). In M. nigrescens both phototaxis (Croll, 1966b) and an increase in the rate of oviposition (Cobb, 1926; Croll, 1966b) have been shown to have an action spectrum similar to the absorption spectrum of hemoglobin.

Hemoglobin has never before been implicated as a visual pigment and it would be very interesting if it should function in this capacity since all the known visual pigments are retinal-protein complexes. The anterior pigment of E. communis has been identified as melanin (Croll, 1966c) which is also not known to be a visual pigment. These considerations raise the following questions: (1) Are the anterior pigment spots of nematodes involved in any way in phototaxis? (2) If so, are

they directly involved in the photoreceptive process? and (3) What is their chemical composition?

The present work was done in order to answer these questions by examining the pigment spots of several local species of free-living marine nematodes and of adult M. nigrescens females. The chemical composition was determined with solubility tests, histochemistry and microspectrophotometry. Observations on the time of development and the specialized location and morphology of the eyespots suggest that they may well be involved in phototaxis. However, the variable composition of the eyespot pigment together with recent ultrastructural evidence suggest the the pigment spots are unlikely to be directly involved.

CHAPTER II

OBSERVATIONS ON NEMATODE PIGMENT SPOTS AND EMBRYOGENESIS

A. Materials and Methods

1. Sites and Methods of Nematode Collection

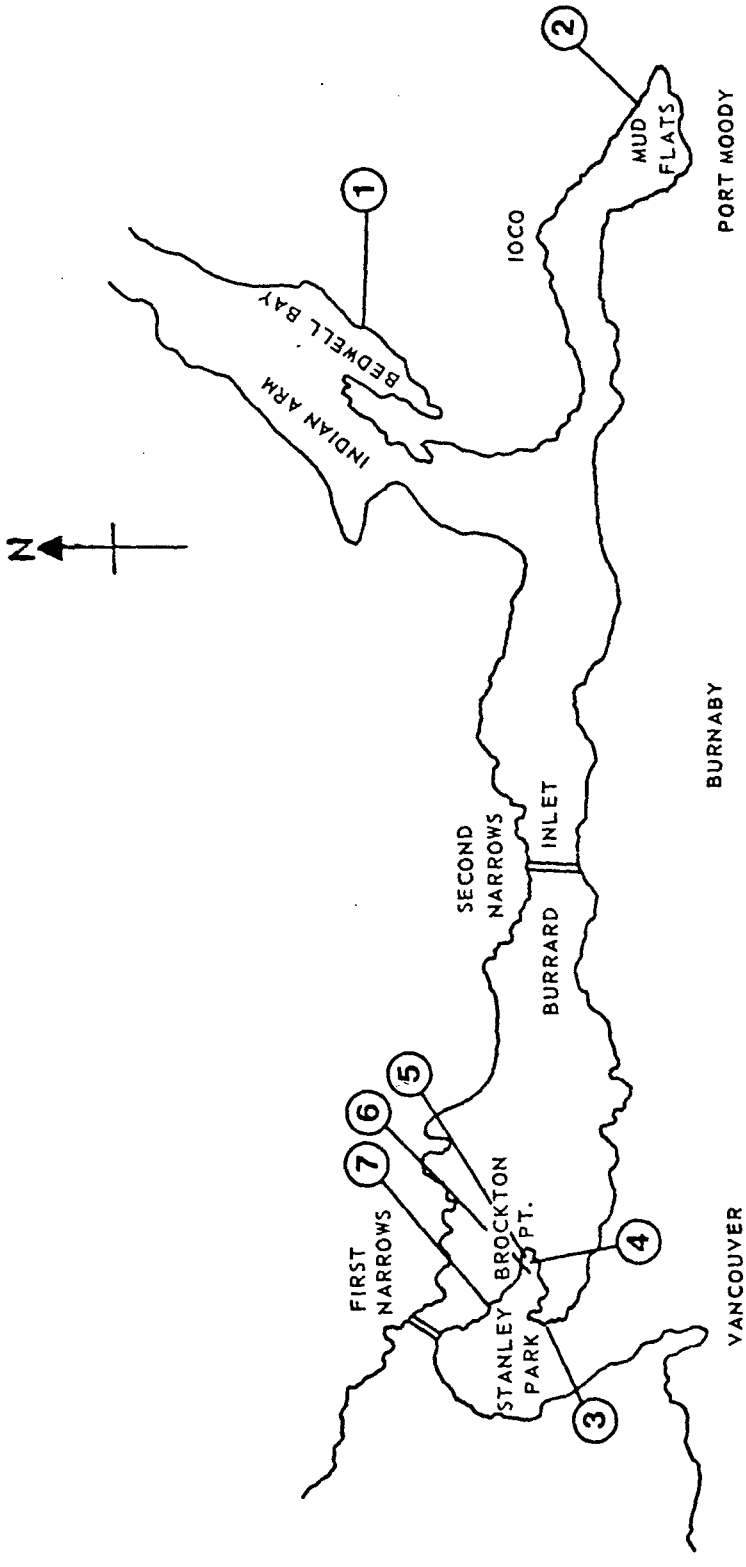
The marine nematodes were collected from the intertidal zone at seven sites on Burrard Inlet, British Columbia. Of these, five were at Brockton Point near the mouth of the Inlet, one near Port Moody at the upper end of the Inlet and one at Bedwell Bay on Indian Arm (Figure 1). The methods used for collecting samples varied with the nature of the site.

Site 1, at Bedwell Bay. A small rocky bay on which numerous logs were beached at low tide. Nematodes were obtained from samples of green algae scraped off the logs and adjacent rocks. Few nematodes were found in samples of sand deposited between the logs and rocks.

Site 2, near Port Moody. A large mud flat exposed by tides of less than 2.4 m. The thick, viscous surface layer was composed predominantly of mud. Several fresh water streams drained into the inlet over the mud flats. At most locations on the flats large numbers of nematodes were found in the surface centimeter of mud and very few below this depth. Samples of the surface mud were scraped up with a trowel and sieved at the site by washing with sea water through a 100 mesh screen. The fine mud was discarded and the remaining coarse debris consisting largely of wood chips and fibres together

Figure 1. Collection sites for marine nematodes on Burrard Inlet, British Columbia.

Scale 1 cm = 1.3 km


N
1
2
3
4
5
6
7
BEDWELL BAY
INDIAN ARM
IOCO
MUD FLATS
PORT MOODY
BURNABY
SECOND NARROWS
INLET
BURRARD
BROCKTON PT.
FIRST NARROWS
STANLEY PARK
VANCOUVER

with entrapped nematodes was collected in plastic bags.

Site 3, Brockton Point, at the Yacht Club Basin. A smaller mud flat exposed by tides of less than 0.9 m. Several sewage outfalls drained onto the flats. The surface centimeter of mud had a rich and varied nematode fauna with few found below this depth. Samples of surface mud were scooped up with a trowel and transferred to plastic bags. Initially an attempt was made to sieve the samples at the site by washing with sea water but large masses of dead and decaying filamentous algae clogged the screen and trapped most of the nematodes. Subsequent disentanglement of the nematodes from the algae proved very difficult and so samples were usually collected without screening.

Site 4, Brockton Point at the lighthouse, and Site 5, about 0.8 km west of the lighthouse. The sites were characterized by sand and large boulders interspersed with small muddy pools exposed by tides of less than 0.9 m. There were very few nematodes in the sand, many in the half centimeter of mud sediment removed from the pools and in green algae on the rocks.

Site 6, on a small bay between Brockton Point and Lumberman's Arch. This site was composed of mixed sand and mud. Samples of the surface centimeter were collected.

Site 7, Brockton Point at Lumberman's Arch. The site consisted predominantly of sand, the surface centimeter of which occasionally contained a large number of nematodes.

In the laboratory, the samples of mud and sand together with sea water were transferred to clear plexiglass trays, aerated using aquarium pumps and covered to minimize evaporation. The trays were kept in an incubator at 16 - 19° C under an alternating 12 hour light and dark cycle. After settling for 24 to 48 hours during which time the nematodes migrated to the surface, aliquots were scooped from the surface of the mud or sand and the nematodes picked out with a fine wire needle under a dissecting microscope. The nematodes were placed in petri dishes of aerated sea water and stored in either the incubator or a refrigerator at 4° C until required. The field samples of algae were kept in covered glass jars in aerated sea water in the incubator.

Nematodes in samples of mud from sites 3 to 7 remained active for up to a month if kept well aerated. In the sieved samples from site 2, the nematodes remained active for 2 to 3 months. Nematodes suspended in non-aerated sea water survived for 1 to 4 weeks in the incubator depending on the species. Stored in the refrigerator, they remained active for longer periods.

Adult females of Mermis nigrescens (Dujardin, 1842) were collected during late May and early June from a cultivated field near Chilliwack, B. C. as they emerged from the soil to lay their eggs early in the morning following overnight rain. They were placed in plastic bags containing moist soil and stored in the refrigerator at 4° C until required.

2. Culture of Marine Nematodes

In order to obtain a constant supply of the marine nematodes, attempts were made to culture them on unfiltered sea water agar plates to which small amounts of mud and algae were added. Adult males and gravid females were placed together on the agar and incubated at 18 - 20° C under an alternating 12 hour light and dark cycle. Initial survival of the nematodes was best on 1% agar where they could move freely both through and on the surface of the medium.

3. Rearing of Free-Living Stage of M. nigrescens

M. nigrescens larvae were removed from the haemocoel of a locust (Schistocerca gregaria) infected three weeks previously with 50 eggs from an adult Mermis female collected in the field. The larvae were sexed by examination of the genital primordia and placed on the surface of a 20 cm column of loosely packed soil in a 3000 ml beaker. To simulate natural conditions, the sides and bottom of the beaker were darkened with aluminum foil and kept at 18 - 20° C under an alternating 12 hour light and dark cycle. Distilled water was added as required to keep the soil slightly moist and the top of the beaker was covered with perforated clear plastic to minimize evaporation but permit oxygen exchange. The nematodes were removed from the soil at intervals and the progress of pigment development noted.

4. Embryogenesis and Pigment Development of Marine Nematodes

Studies of embryogenesis and pigment development in six

of the marine nematode species were done on eggs obtained from the 1% sea water agar cultures described above. The rate and sequence of embryonic development was observed and photographed in eggs suspended in sea water on microscope slides. The sea water was replaced daily with fresh aerated sea water. During observations a heat filter was used to minimize the heating effects of the microscope lamp. Observations were also made on the time and position of pigment development in the embryos. Progress of pigment development in later larval stages was observed in larvae obtained from field samples.

B. Results and Discussion

1. Site Occurrence of the Marine Nematodes

The eleven species of free-living marine nematodes with eyespots which were found in the Burrard Inlet area, the sites at which they were found, their relative numbers and the months during which adults were most numerous are shown in Table I. Fewer adults and larvae were found at other times of the year.

2. Description of Nematode Pigment Spots

The anterior pigment spots of the nematodes exhibited a wide range of colour and morphology and will be described by species.

Chromadorina germanica (Butschli, 1894) (Fig. 2A)

Adults of both sexes possessed paired orange-red eyespots 8 μ in length and 5 μ in width. The spots were located dorso-laterally in swellings of the esophageal muscle 25 μ from the anterior end of the nematode. The pigment was granular and

Table I

Site Occurrence of the Marine Nematodes

Species	Site	Occurrence of Adults		Habitat
Chromadorina germanica	1	May-Dec.	numerous	filamentous green algae
	3	May-Dec.	occasional	filamentous green algae
Oncholaimus skawensis	3	June-Nov.	numerous	mud
	4	June-Nov.	moderate	mud
	5	June-Nov.	moderate	mud
Oncholaimus vesicarius	3	Mar.-Aug.	numerous	mud
	4	Mar.-Aug.	numerous	mud
	7	Mar.-Aug.	occasional	sand
Enoplus anisospiculus	2	Oct.-Apr.	numerous	mud
Enoplus sp.	5	Mar.-Sept.	numerous	mud
Seuratiella sp.	3	July-Aug.	numerous	mud
	4	July-Aug.	numerous	mud
Symplocostoma sp.	3	Apr.-Aug.	numerous	mud
	4	Apr.-Aug.	numerous	mud
Euchromadora sp.	6	July-Sept.	numerous	filamentous green algae
Halichoanolaimus sp.	4	Aug.	rare	mud
Araeolaimus sp.	3	July	rare	mud
Unidentified sp.	5	July	rare	mud

closely packed. In some specimens, a third smaller spot was located ventrally in the mid line of the esophageal muscle. The pigment faded in either light or dark within a few hours of nematode death or after formalin fixation.

Oncholaimus skawensis (Ditlevson, 1921) (Fig. 2B)

Adults of both sexes had a pair of pigment spots located laterally in the esophageal muscle at the base of the buccal cavity. A third smaller spot sometimes occurred dorsally in the esophageal muscle. The granular pigment in the spots was red and rather diffusely distributed. Posterior to the pigment spots was a clear unpigmented area and posterior to this a second granular pigment, pale green in colour was distributed along the esophagus. The anterior red pigment will be called the eyespot pigment and the posterior green pigment, the esophageal pigment. The eyespot pigment faded in either light or dark within a few hours of nematode death or after formalin fixation.

Oncholaimus vesicarius (Wieser, 1959) (Fig. 2C)

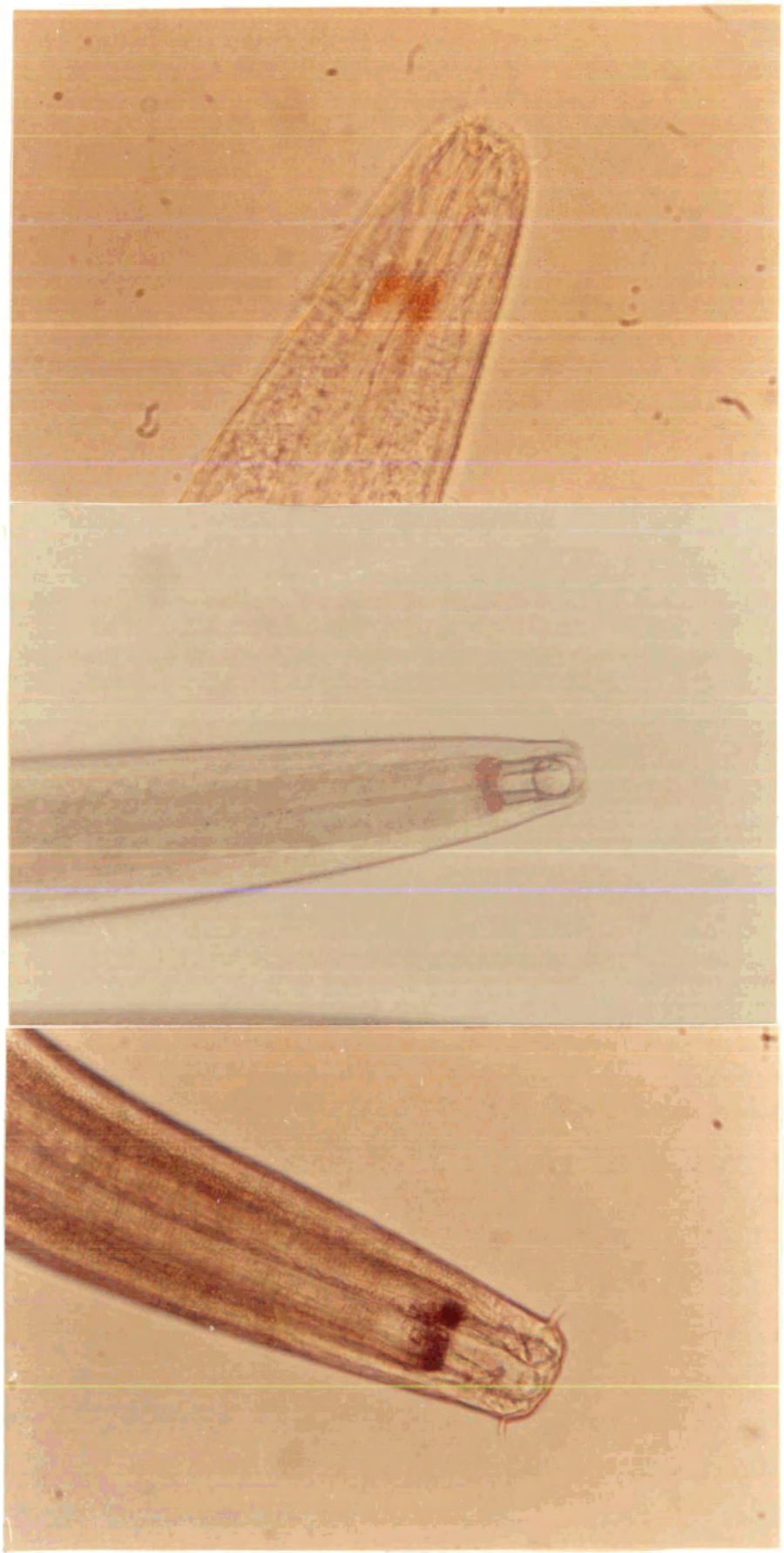
Adults of both sexes possessed pigment spots identical in morphology to those of O. skawensis. The eyespot pigment in this species was, however, reddish-brown to purplish-black in colour and the esophageal pigment was more pronounced and of a darker colour than in O. skawensis. Within a few days of nematode death or exposure to formalin, some of the eyespot

Figure 2. Pigment spots of *C. germanica*, *O. skawensis* and *O. vesicarius*

A. (top) *C. germanica* adult male dorsal view X1000

B. (center) *O. skawensis* adult male dorsolateral view X300

C. (bottom) *O. vesicarius* adult male dorsolateral view X450



pigment, particularly in the posterior portion of the spots, faded. In specimens with reddish-brown pigment, this fading was accompanied by a change in colour of the remaining pigment to purple. The purple pigment was unaffected by several weeks exposure to formalin.

Enoplus anisospiculus (Nelson et al., 1972) (Fig. 3A) and Enoplus sp. (Fig. 3B)

Since the colour and distribution of the pigment in both species of Enoplus was identical, only one description will be given.

Adults of both sexes possessed paired dorsolateral pigment spots embedded in slight swellings of the esophageal muscle approximately 60 from the anterior end of the nematode. The spots consisted of closely packed reddish to dark brown pigment granules about 1 in diameter. The spots, when viewed dorsally appeared oval in shape and laterally almost triangular with a broad base in the ventral position. This pigment will will be referred to as the eyespot pigment.

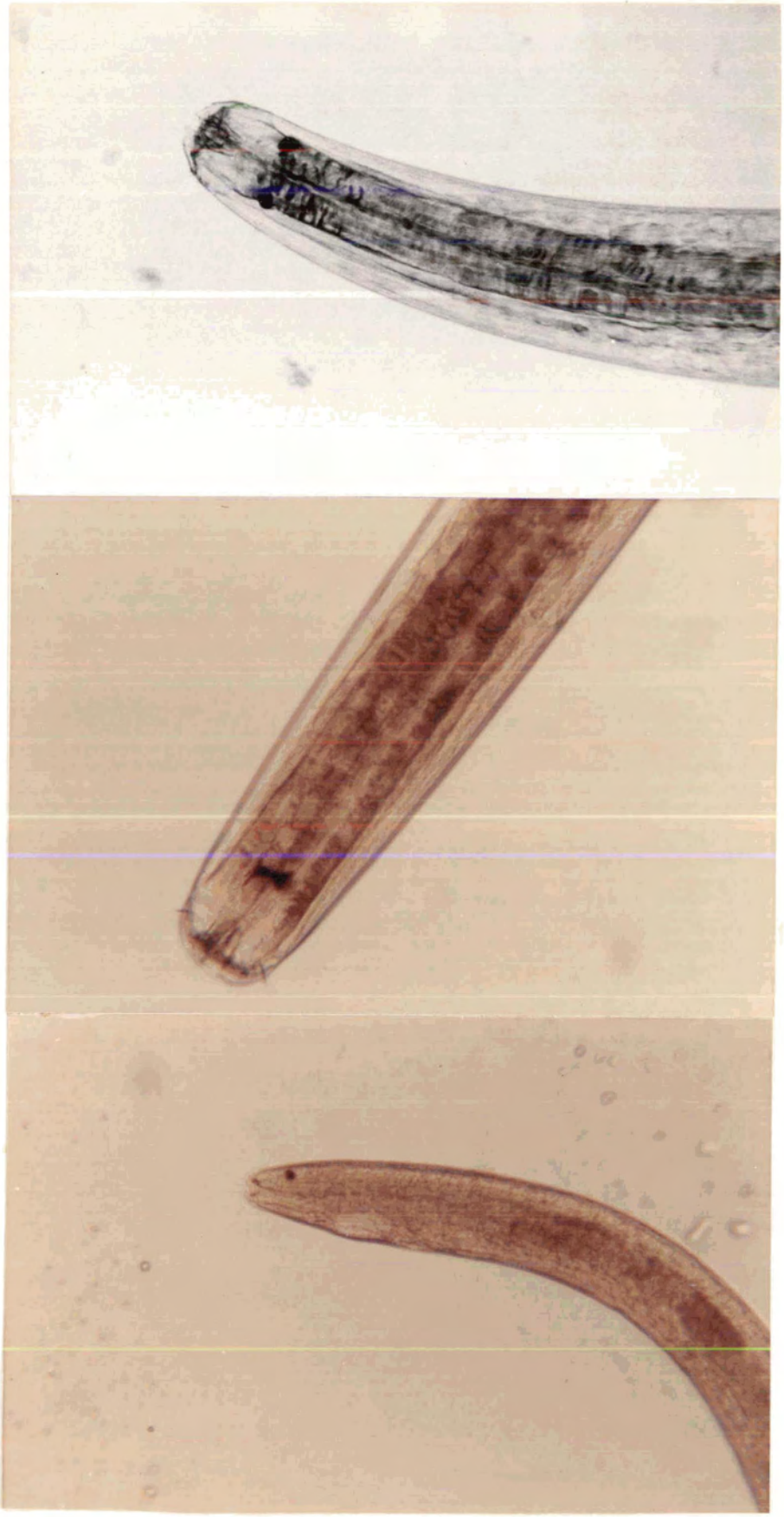
A second granular pigment, slightly lighter in colour than the eyespot pigment was distributed along the length of the esophageal muscle posterior to the eyespots. This pigment, which will be called the esophageal pigment, appeared to line three channels in the muscle and also filled numerous irregularly shaped vesicles projecting radially from the channels. The eyespots were located at the anterior ends of the the dorsolateral channels, but there was no corresponding

Figure 3. Pigment spots of E. anisospiculus, Enoplus sp. and Seuratiella sp.

A. (top) E. anisospiculus adult male ventral view X400

B. (center) Enoplus sp. adult male lateral view X450

C. (bottom) Seuratiella sp. adult male lateral view X450



pigment spot at the anterior end of the ventral channel. Both pigments were very stable and remained unfaded after several months exposure to formalin. The pigment of these two Enoplus species had the same colour and distribution as that of E. communis (Schultz, 1931).

Seuratiella sp. (Fig. 3C)

Adults of both sexes possessed paired, round, purple to black spots 3 to 4 μ in diameter located 13 μ from the anterior end of the nematode. The spots were dorsolateral in location, closely spaced and apparently not embedded in the esophageal muscle. The pigment was granular and relatively stable after death of the nematode. In formalin, it faded after 3 to 4 days to a reddish colour which remained unchanged for several weeks. The shape and location of the pigment spots was the same as that of Acanthoncus rostratus (Murphy, 1963) but unlike this species, Seuratiella sp. did not appear to have a refractile body anterior to the pigment.

Symplocostoma sp. (Fig. 4)

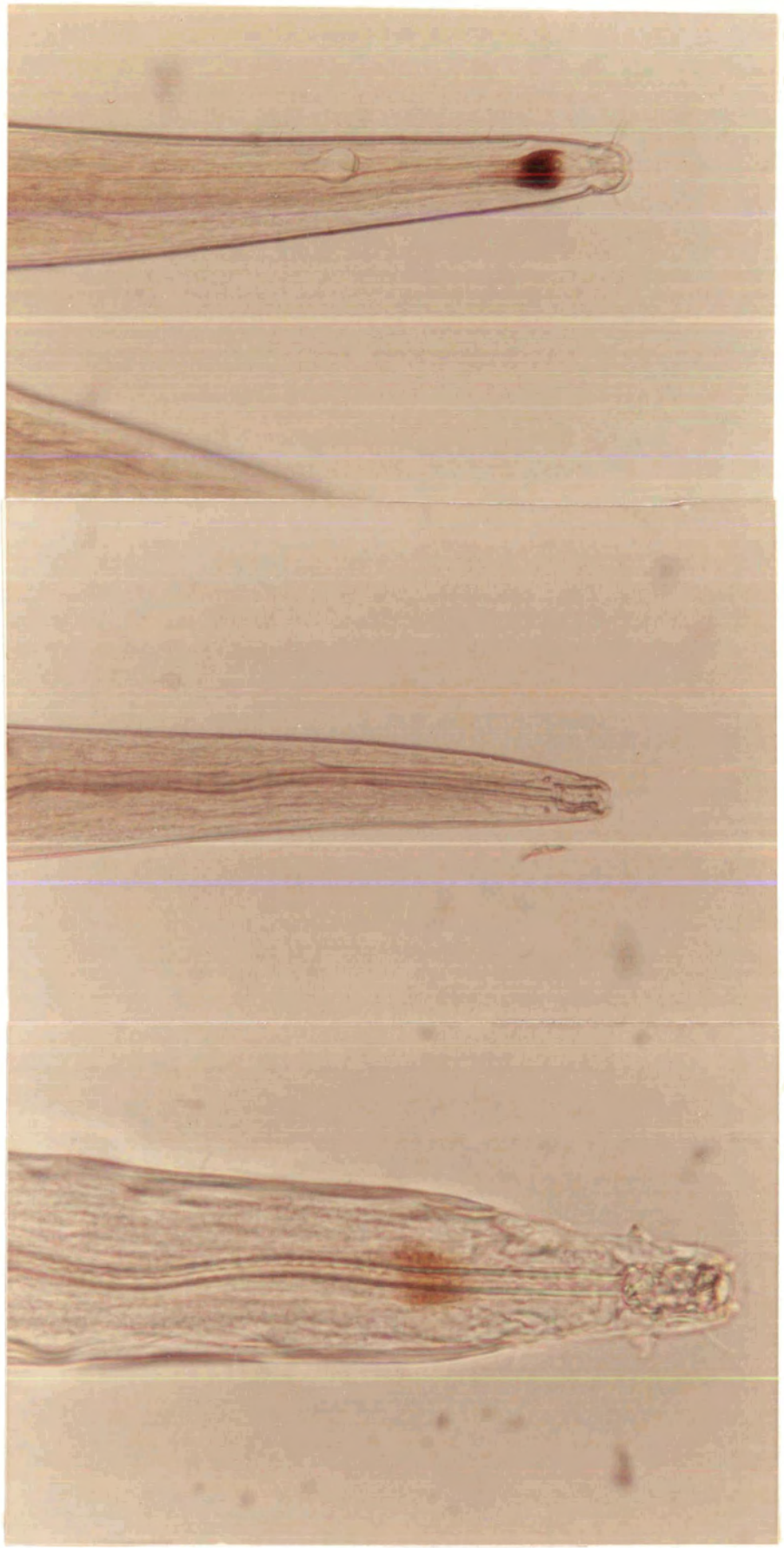
This species exhibited the sexual dimorphism that is typical of the Enchelidiidae (Wieser, 1953). The males had a greatly reduced buccal cavity and two prominent cup shaped pigment spots 24 μ in length and containing reddish-brown, finely granular pigment embedded laterally in swellings of the esophageal muscle 30 μ from the anterior end of the nematode. Just anterior to the pigment spots was a pair of colourless, refractile bodies also embedded laterally in the esophageal

Figure 4. Pigment spots of Symplocostoma sp.

A. (top) Symplocostoma sp. adult male lateral view X450

B. (center) Symplocostoma sp. adult female dorsal view X450

C. (bottom) Symplocostoma sp. fourth stage male larva moulting to adult stage X700



muscle (Fig. 4A). The females had a well developed heavily chitinized buccal cavity and no pigment spots. They did, however, have a pair of colourless, refractile bodies laterally located at the base of the buccal cavity 25 μ from the anterior end of the nematode. (Fig. 4B). The pigment in the males remained unfaded for several days after nematode death or for several months after exposure to formalin.

Euchromadora sp. (Fig. 5A)

Adults of both sexes possessed paired, round, reddish-brown spots 20 μ in diameter. The spots were located dorso-laterally in the esophageal muscle 40 μ from the anterior end of the nematode. A third smaller spot was located ventrally in the mid-line of the esophageal muscle. The pigment was finely granular and closely packed, and sometimes difficult to see because of the thickness and dark colour of the cuticle. The pigment faded in either light or dark within 1 to 2 days of nematode death or 2 to 3 days after formalin fixation.

Halichoanolaimus sp. (Fig. 5B)

The single adult male of this species had a diffuse band of finely granular brown pigment 45 μ in width and located 60 μ from the anterior end of the nematode. The pigment was distributed fairly uniformly around the esophageal muscle. A second finely granular, grayish pigment was distributed posteriorly along the esophageal muscle. Both pigments were unchanged by several weeks exposure to formalin.

Araeolaimus sp. (Fig. 5C)

The single adult female had a pair of round pigment spots, 8 μ in diameter, located 43 from the anterior end of the nematode. The spots were dorsolateral in location and did not appear to be associated with the esophageal muscle. Just posterior to the pigment spots there was a slight but definite swelling of the esophageal muscle. The pigment had a red colour in the fresh specimen and appeared to consist of either a pair of large single granules or a pair of discrete masses of diffuse pigment. The pigment faded rapidly after formalin fixation, revealing the presence of two refractile bodies of the same size and shape as the original pigment spots.

Unidentified sp. (Fig. 6A)

The single adult female had five red pigment spots embedded in the esophageal muscle. A pair of oval spots, 8 μ in length and 4 μ in width were located laterally 68 μ from the anterior end of the nematode. Twelve μ posterior to these were three round spots 4 μ in diameter, two of which were located dorsolaterally and the third ventrally. There was a slight swelling of the muscle around the spots. The finely granular pigment faded within a few days after formalin fixation to a yellowish colour which thereafter remained unchanged.

M. nigrescens (Fig. 7B)

The pigmented area or chromatrope of the adult female consisted of a band of red pigment 70 μ in width situated 35 μ

Figure 5. Pigment spots of Euchromadora, Haliochoanolaimus and Araeolaimus spp.

A. (top) Euchromadora sp. adult male dorsal view X200

B. (center) Halichoanolaimus sp. adult male dorsolateral view X350

C. (bottom) Araeolaimus sp. adult female dorsolateral view X300

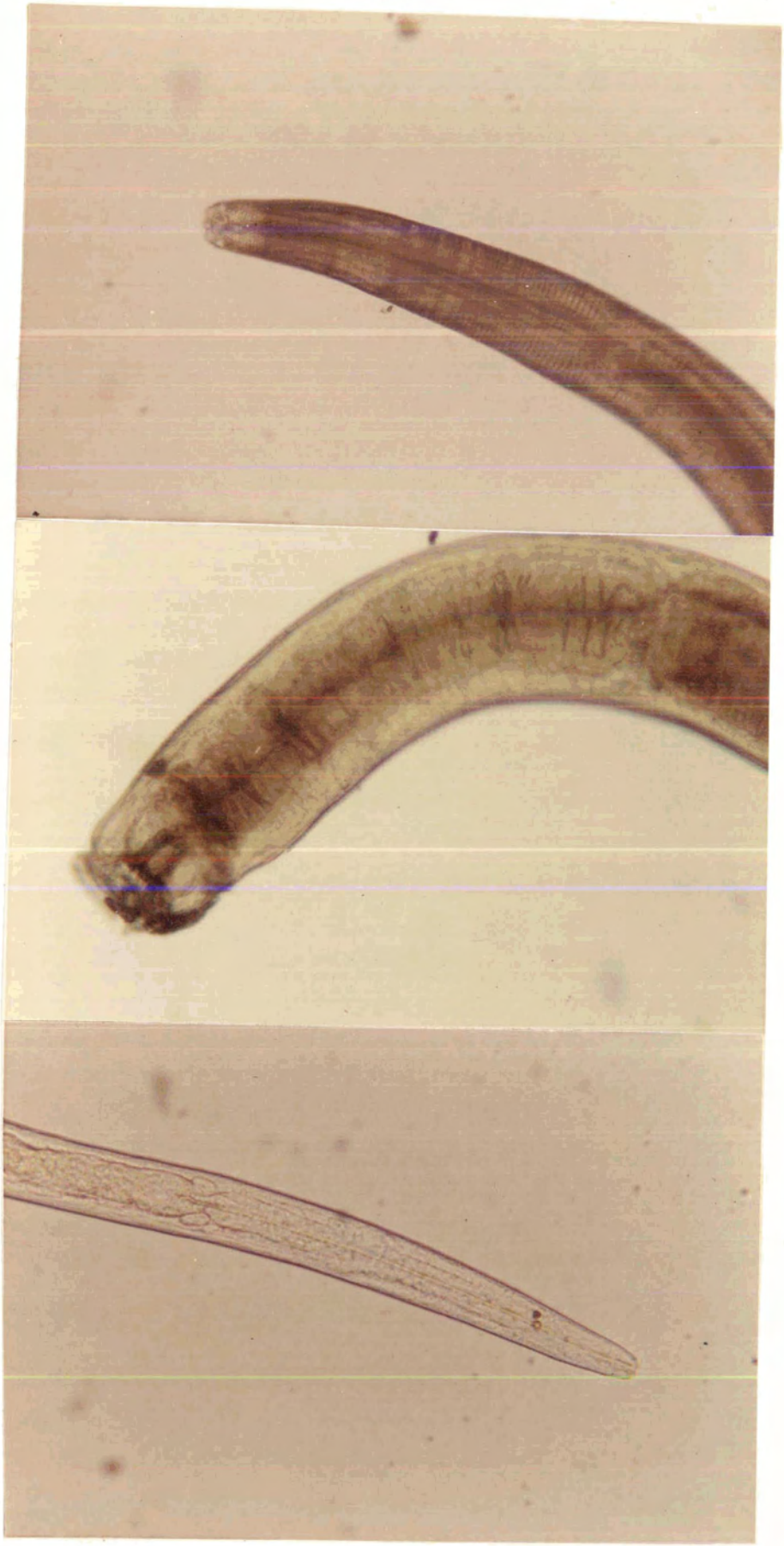


Figure 6. Pigment spots of an unidentified marine nematode and of Oncholaimus spp. larvae; C. germanica gravid female

A. (top) Unidentified marine nematode adult female ventrolateral view X300

B. (center) O. skawensis (upper) and O. vesicarius (lower) third or fourth stage larvae showing difference in pigment colour X150

C (bottom) C. germanica gravid female removed from sea water agar prior to death of culture X100

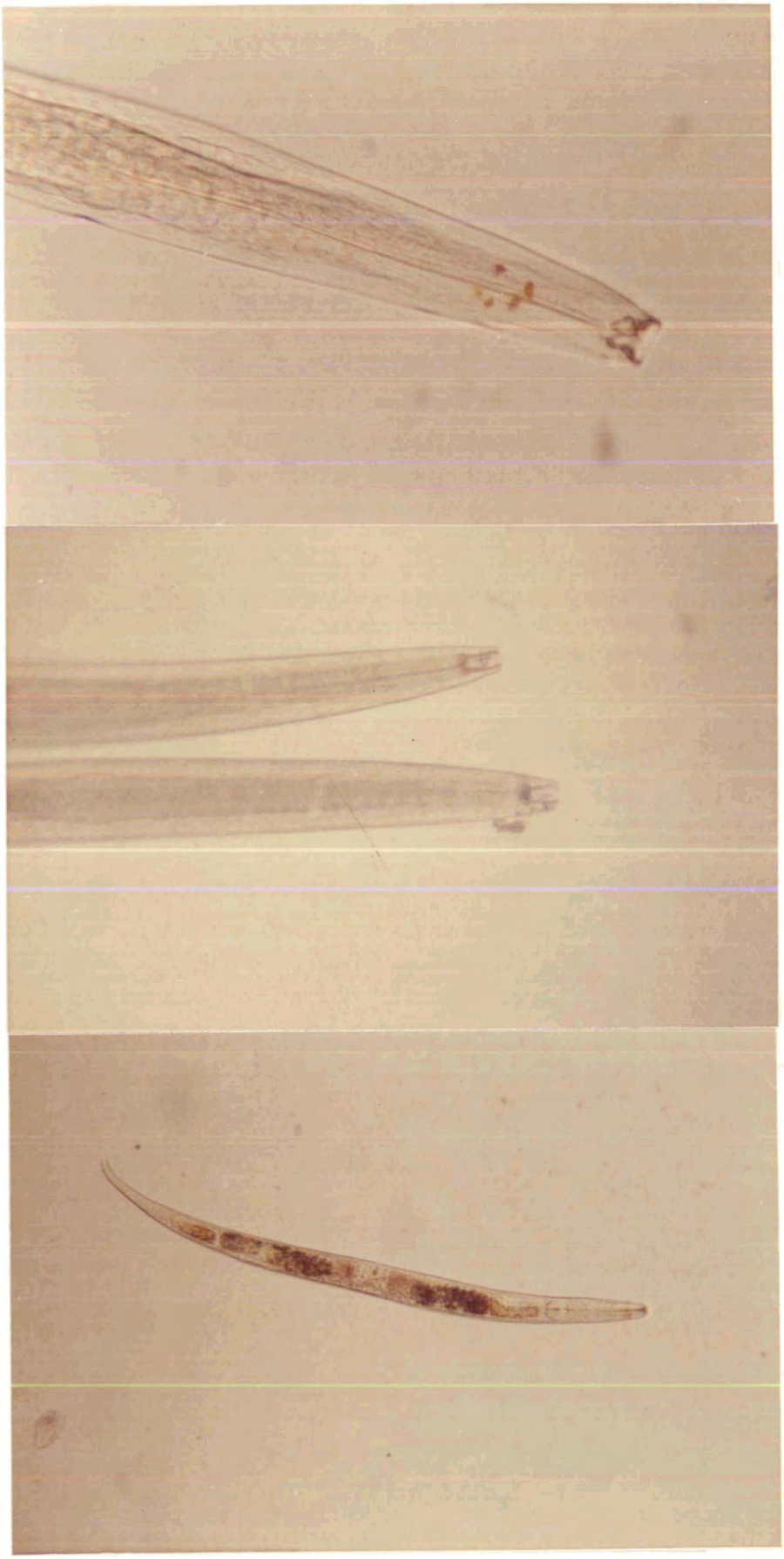
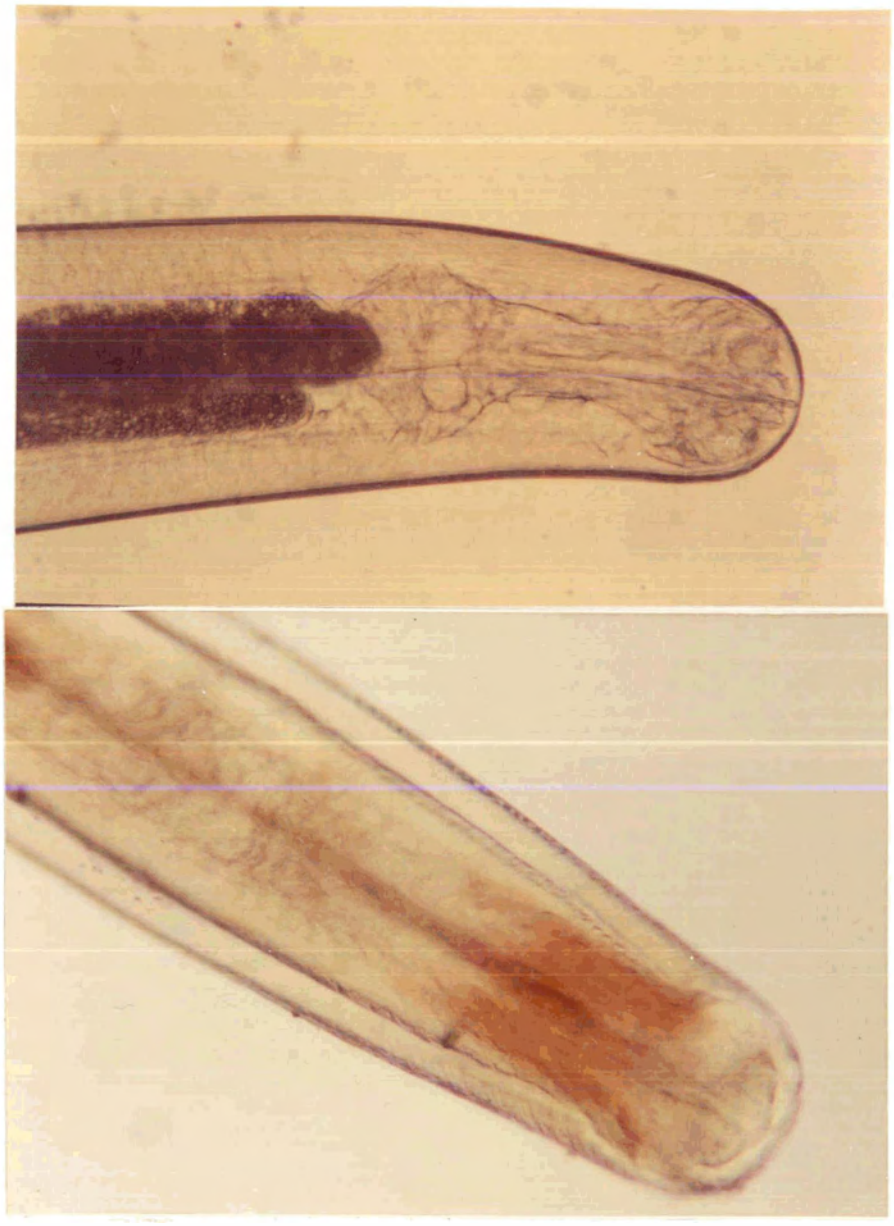


Figure 7. Anterior ends of larval and adult female M. nigrescens

A. (top) Post parasitic female larva prior to pigment development and moulting X450

B. (bottom) Adult female with chromatrope X450



from the anterior end of the nematode. Unlike the marine nematode eyespots, the chromatrope pigment was translucent and non-granular. It was distributed diffusely in the hypodermis and decreased gradually in amount posteriorly from the main pigment spot (Ellenby and Smith, 1964). The pigment was unaffected by formalin fixation, but faded gradually after death of the intact nematode. Fading was very rapid when an amputated head was suspended in water.

3. Culture of Marine Nematodes

On 1% sea water agar C. germanica and Seuratiella sp. adults survived for 1 week, while adults of E. anisospiculus, Enoplus sp., O. vesicarius, O. skawensis and Symplocostoma sp. survived for 2 - 4 weeks. During this time most of the females deposited their eggs. Embryonic development proceeded in the eggs of all species, but in all except C. germanica, the larvae died within 2 - 3 weeks of hatching. C. germanica larvae after hatching fed on a filamentous green algae which had become established in the culture while they were developing. Diatoms and bacteria reproduced in cultures of the other species but were apparently insufficient or unsuitable for supporting larval growth. The C. germanica larvae matured after 4 months and the females began egg laying. At this time all the nematodes died, the eggs disintegrated and one month later the algae also died. The density of the gut contents indicated that the nematodes were feeding actively prior to death (Fig. 6C). Death of the nematodes and eggs followed by

death of the algae suggests that there may have been accumulation of a toxic product in the culture or perhaps development of a nutrient or oxygen deficiency. Sub-culturing of the algae and nematodes at intervals to fresh agar plates might have prevented this.

4. Development of M. nigrescens and its Chromatrope

Within 3 days of being placed on the soil in the incubator all the post parasitic larvae had migrated beneath the surface except three which had died. After 2 weeks the larvae were randomly distributed through the soil 2 to 15 cm beneath the surface and 4 weeks later most of the larvae had accumulated close to the bottom of the beaker where they remained tightly coiled until mature. Three to four months after removal from the host, a faint pigment began to appear in the anterior of the larvae and a moult followed. During the succeeding 5 months the red pigment increased in amount until it was similar in density and distribution to that of the adult female chromatrope. The anterior ends of an unpigmented post-parasitic larva and of a pigmented adult female are shown in Figure 7. Ten months after removal from the host, developing eggs became apparent in the ovaries and in the following 6 months gradually acquired a brown pigmentation. After 20 months the nematodes were apparently mature since they began egg-laying spontaneously when exposed to the heat and light of the microscope lamp. When the eggs were fed to locusts, 25% developed into viable larvae, all of which were females.

Since no males were present post parasitically, this result confirms the report of parthenogenesis in M. subnigrescens (Christie, 1929). It is unlikely that contact with males during the parasitic phase could result in fertilization because at the time of emergence from the host, the male reproductive organs are not yet mature and the developing female organs are still covered by a thick cuticle.

5. Embryogenesis and Pigment Development of Marine Nematodes

a) Results

E. anisospiculus and Enoplus sp.

Females of these species usually laid a cluster of 15 - 30 round to oval eggs 120 - 140 μ in diameter embedded in a tough gelatinous matrix. The eggs were laid uncleaved (Fig. 8A) or in various stages of cleavage within a single cluster (Fig. 10A). Prior to egg laying the females sometimes died, but embryogenesis proceeded normally and eventually the larvae hatched in the decomposing female body (Fig. 9A).

Uncleaved eggs usually commenced cleavage within 24 hours after they were laid. The first cleavage was complete and two equal sized blastomeres were formed (Fig. 8B). The second cleavage, oblique to the first, occurred simultaneously in both blastomeres and four equal cells arranged in a rhomboid were formed (Fig. 8C). During the early stages of embryogenesis, the cell nuclei were visible as round clear areas in the dense cytoplasm (Fig. 8D).

Figure 8. Embryogenesis of E. anisospiculus.

n, nucleus

A. uncleaved eggs X140

B. 2 celled stage X230

C. 4 celled stage X230

D. 8 and 16 celled stages X200

E. 16 and 32 celled stages X260

F. coeloblastula X230

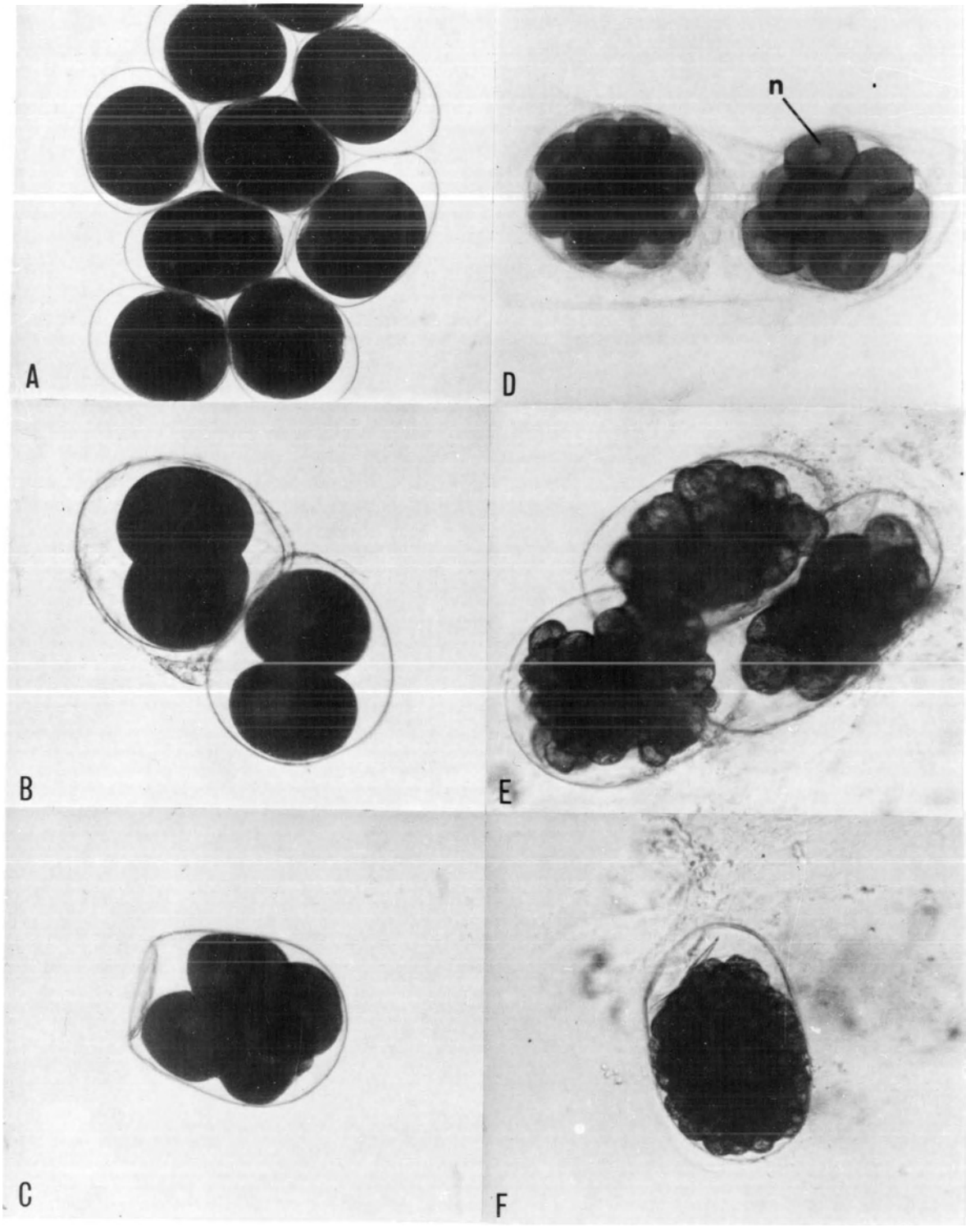

A
B
C
D
n
E
F

Figure 8. Embryogenesis of E. anisospiculus (cont'd.)

ep, eyespot pigment

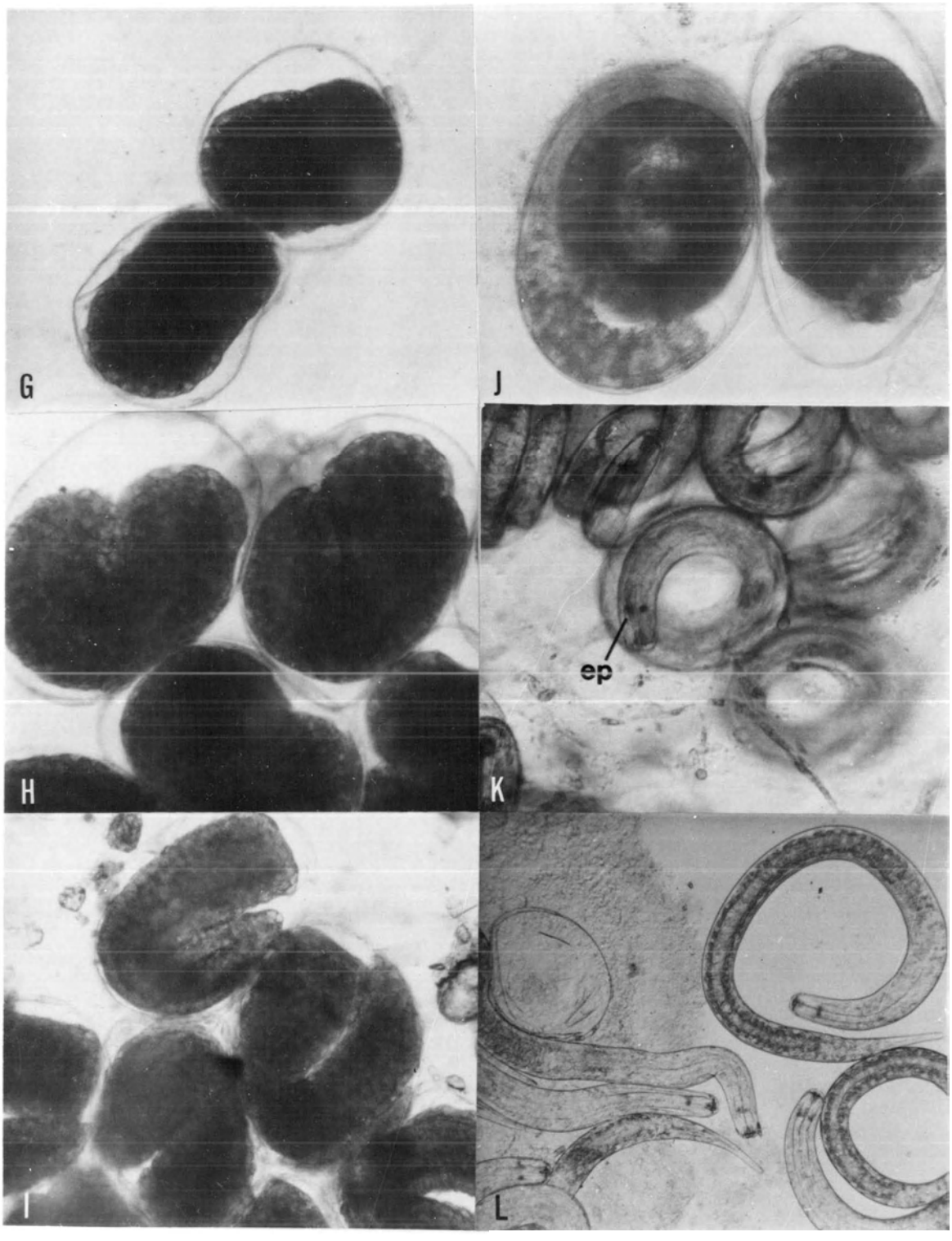
G, H. early larval formation G, X230; H, X300

I tadpole stage X230

J larva prior to pigment development X300

K larvae just prior to hatching X200

L first stage larvae hatching X160


G
J
H
ep
K
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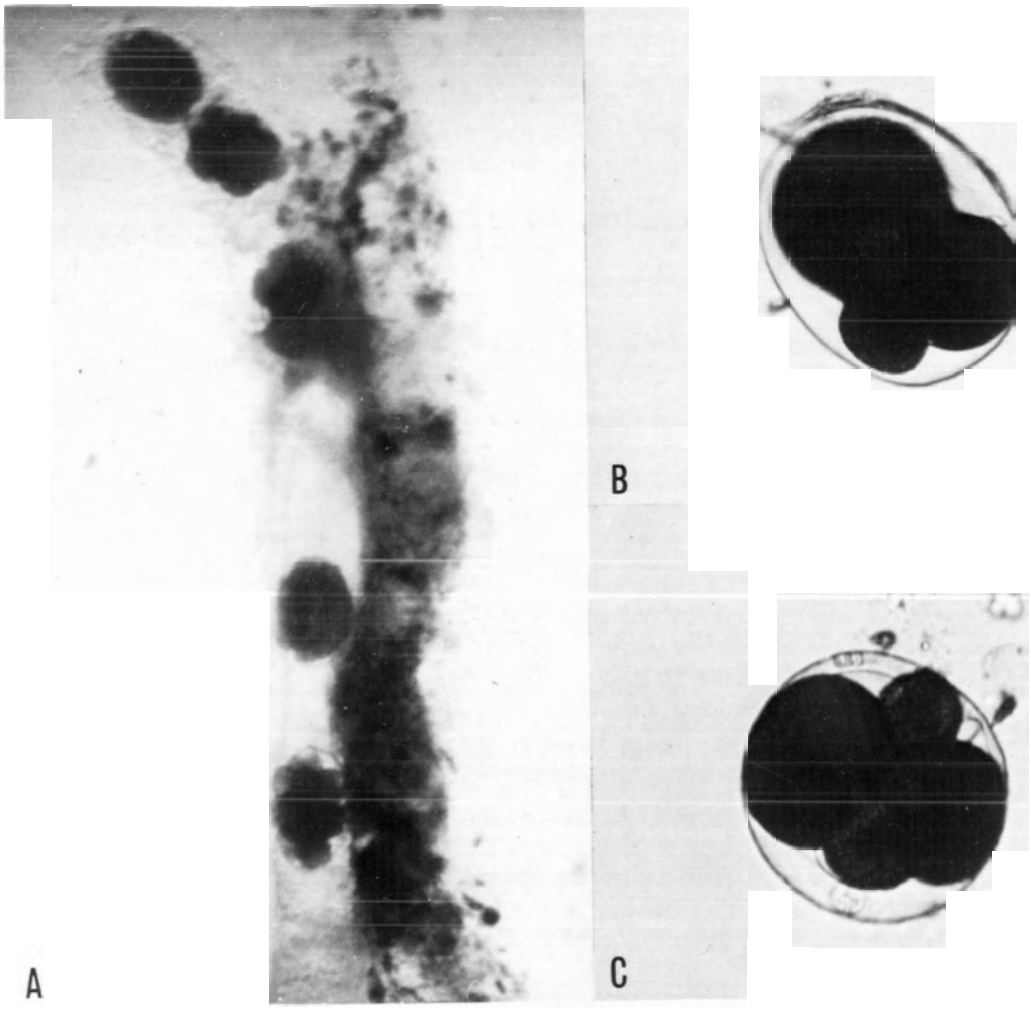
L

Figure 9. Embryogenesis of E. anisospiculus

A. eggs developing in remains of mother's body X100

B. abnormal three celled embryo X250

C. abnormal four celled embryo X250


A
B
C

Eggs that commenced cleavage did not always continue to develop (Fig. 10 A-D). If the eggs were left in unaerated sea water, embryonic development was usually arrested at the two cell stage, but in a few embryos development was arrested after an abnormal second cleavage (Fig. 9 B and C).

In a normally developing egg, the second cleavage was usually completed within 12 hours of initiation of the first cleavage. Rapid successive cleavages followed, the sequence of events being difficult to follow since groups of cells divided independently of one another (Fig. 8 D and E). The coeloblastula (Fig. 8F) was usually formed within 48 to 72 hours of the initiation of cleavage. During the next 24 hours gastrulation occurred. The blastopore appeared as a longtudinal groove on the surface of the embryo which closed first in the middle and then progressively towards each end (Fig. 10 E-G). At completion of gastrulation, the embryo was bilaterally symmetrical and slightly flattened on the ventral surface.

The beginning of larval formation was indicated by a rapid growth of the embryo and bending over of the ends to form first a kidney shape (Figs. 8G, 10 D) and then a U-shape (Fig. 8H). The anterior end then broadened and rounded an the posterior end became narrow and pointed (Fig. 8L). Shortly after formation of this tadpole stage, the first slight movements of the embryo were seen.

By 14 days after the initiation of cleavage, the larva was worm-like and active (Fig. 8J). In the following two to four

days, the buccal cavity developed and eyespot pigment appeared in the anterior esophageal muscle (Fig. 8K). Three to five days later the larva hatched (Fig. 8L). The time from initiation of cleavage to hatching was 19 to 23 days. The larvae did not appear to moult prior to hatching, the first moult occurring about two weeks after hatching.

The newly emerged larvae had two well defined eyespots in the same position and configuration as in the adult, but smaller in size (Fig. 12A). A third patch of pigment was located in the mid-line of the ventral esophageal muscle (Fig. 12B). The granular reddish-brown pigment appeared identical in all three locations. As the larvae matured, the eyespots increased in size and density and esophageal pigment was gradually deposited along the muscle progressively from the anterior end just behind the eyespot pigment.

b) O. vesicarius and O. skawensis

Embryogensis was identical in these two species and similar to that of Enoplus spp. The round to oval eggs, 80 - 100 μ in diameter, were deposited in strings or, more often, in clusters of 10 - 40 eggs. The eggs were laid before or after the beginning of cleavage, but in contrast to Enoplus a single cluster usually contained eggs at the same developmental stage (Fig. 11C). As in Enoplus, the females sometimes died prior to egg laying and the larvae developed in the decomposing female body.

Uncleaved eggs usually began cleaving within 1 to 3 days

Figure 10. Embryogenesis of Enoplus sp.

a, b, c, d, and e: eggs selected from clusters for presentation in E, F, G

A. egg cluster 24 hrs after deposition X100

B. 48 hrs after deposition X100

C. 3 days after deposition X100

D. 6 days after deposition X100

E, F, G. progressive stages in closure of the blastopore X200

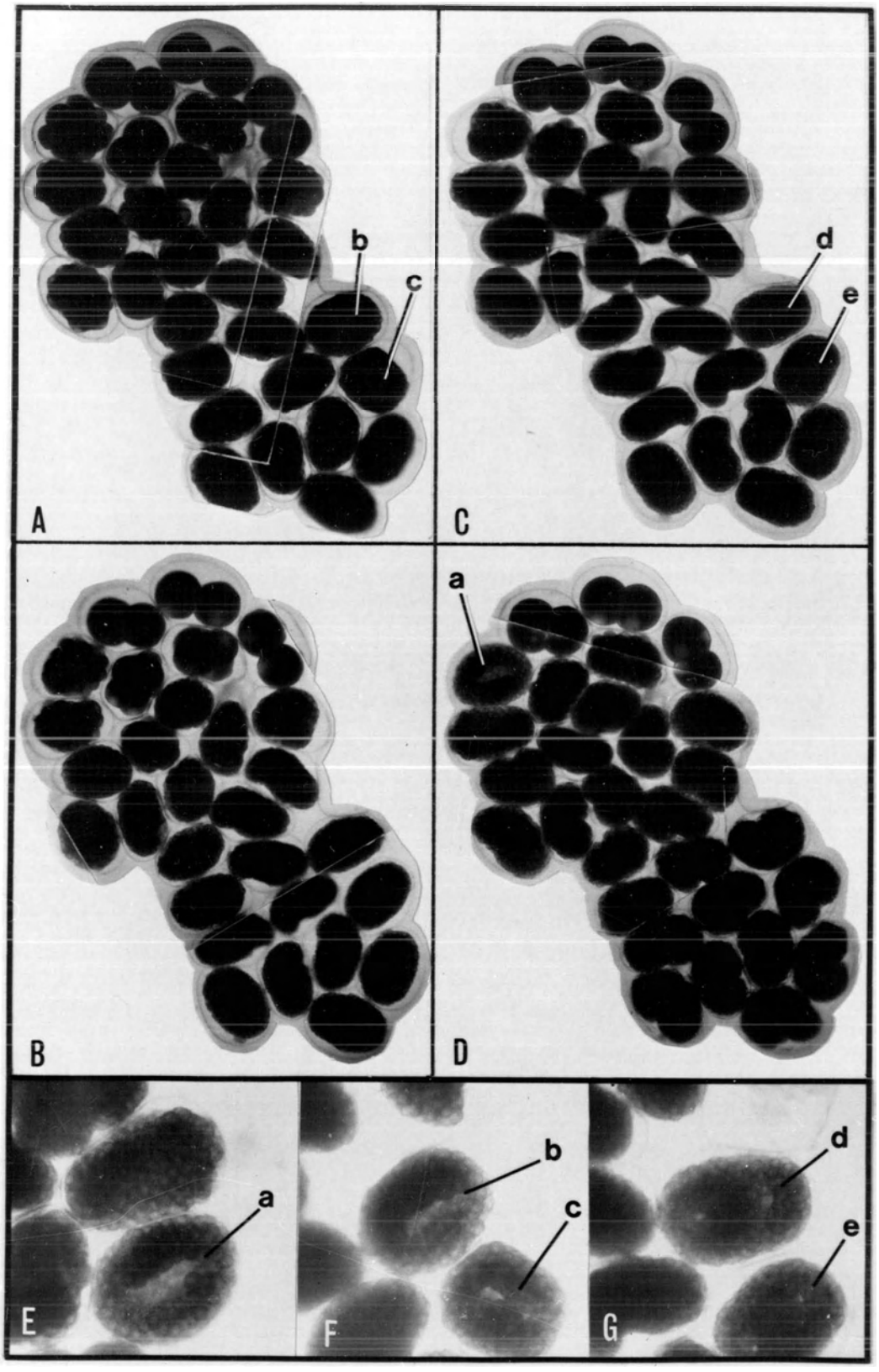

A
b
c
C
d
e
B
D
a
E
a
F
b
c
G
d
e

Figure 11. Embryogenesis of O. vesicarius

n, nucleus

bp, blastopore

A. Embryos showing normal and abnormal development. Normal 2 and 4 celled stages at upper right. Abnormal embryos exhibit unequal blastomeres, incomplete cleavage (3 celled embryo) and abnormal blastomere arrangement. X170

B. Normal 8 and 16 celled embryos X190

C. Eggs during gastrulation (2 days after initiation of cleavage) X125

D. Early larval formation (4 days after initiation of cleavage) X125

E. Later larval formation (7 days after initiation of cleavage) X125

F. Larvae just prior to eyespot development (9 days after initiation of cleavage) X125

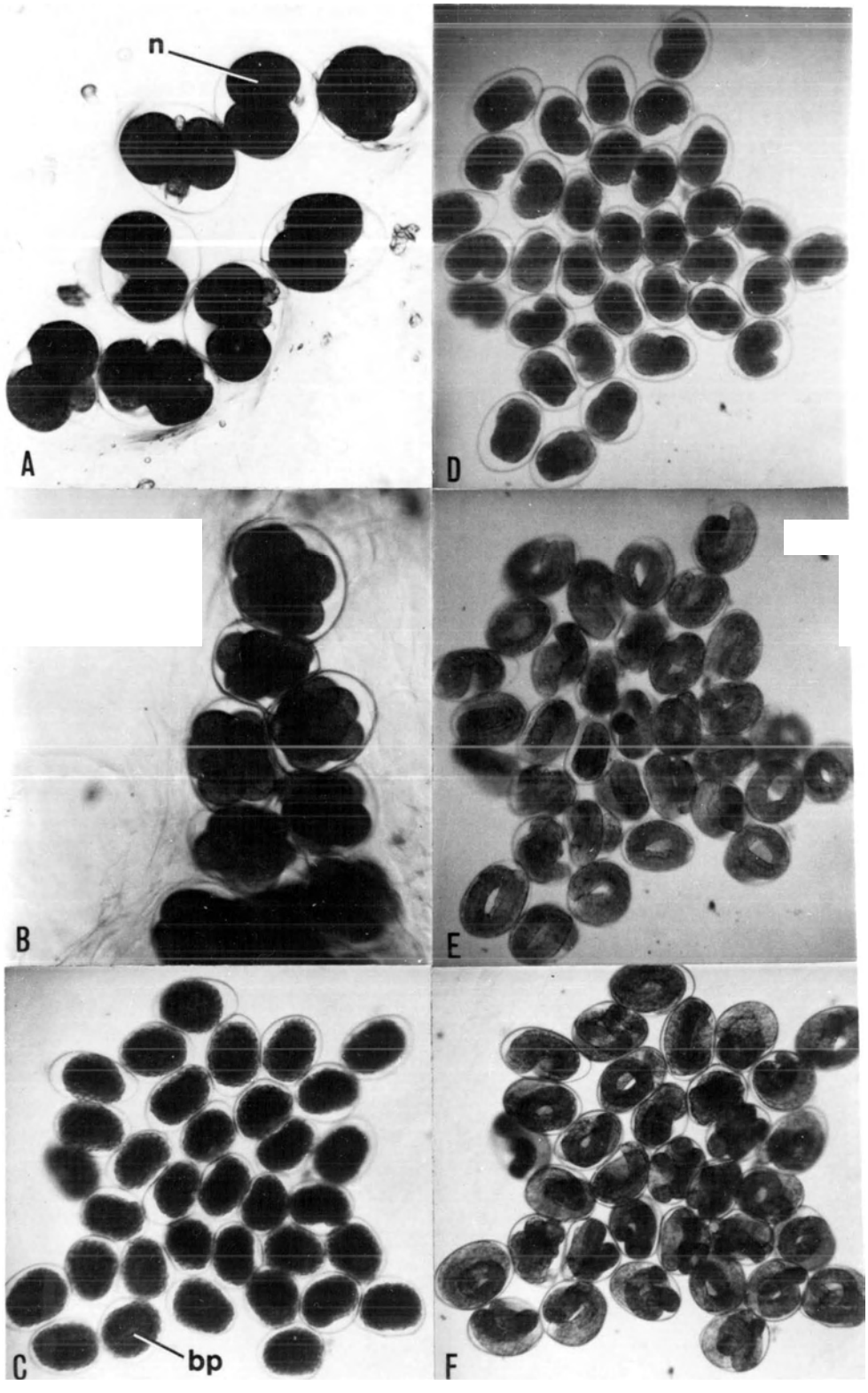

n
A
D
B
E
C
bp
F

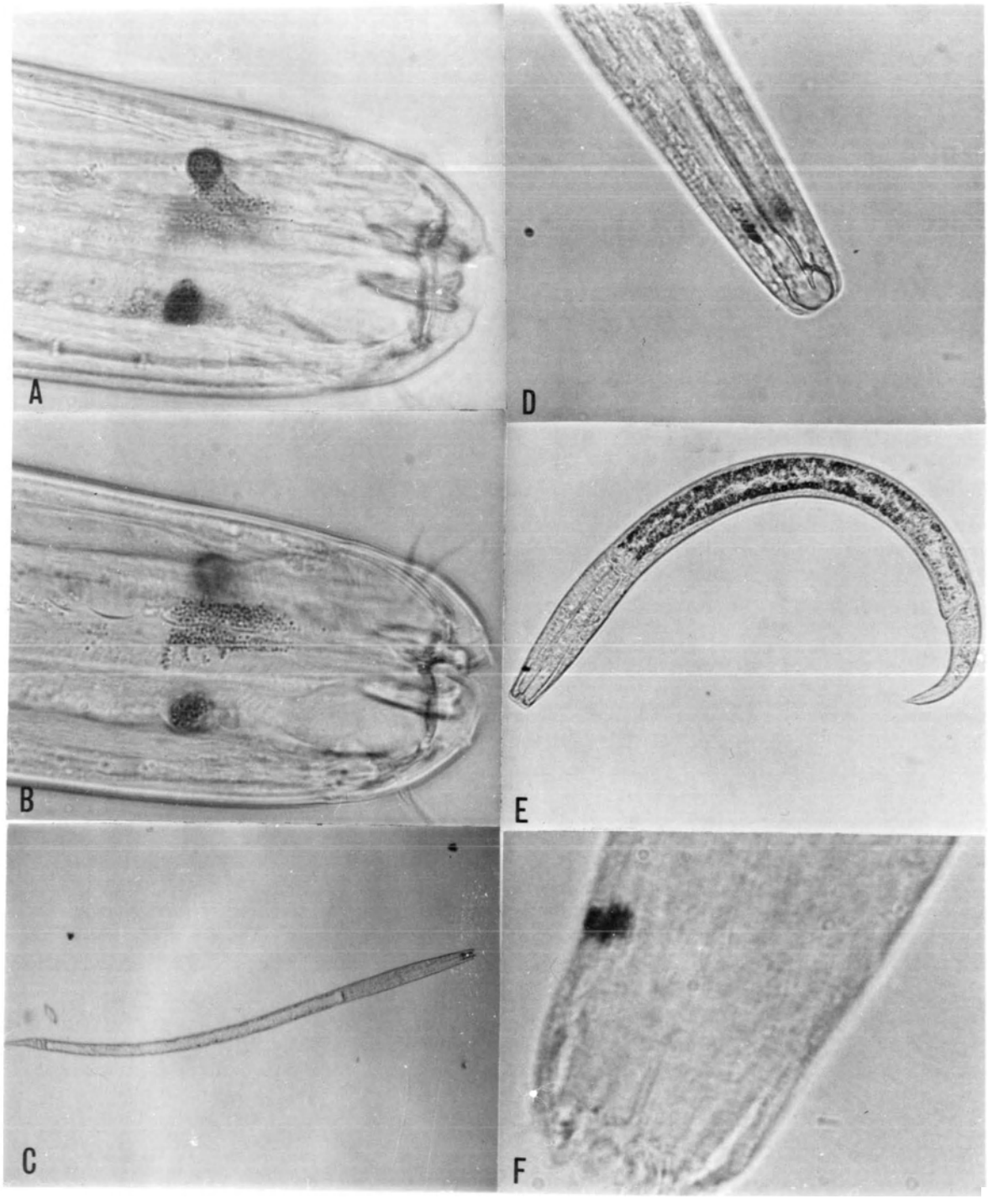
Figure 12. Larval eyespots

A. E. anisospiculus first stage larva, dorsal view X2500

B. E. anisospiculus first stage larva, ventral view X2500

C, D. O. vesicarius first stage larva X100, X 1260

E, F. Seuratiella sp. first stage larva X500, X4000


A
D
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after they were laid. The first two cleavages, as in Enoplus, resulted in four equal blastomeres arranged in a rhomboid. Damage to the eggs during transfer from the agar plates to the microscope slides resulted in abnormal embryos which did not usually develop beyond the second cleavage. The blastomeres in these embryos varied from the normal in size, number and arrangement (Fig. 11A).

In normal eggs the first two cleavages occurred at the same rate as in Enoplus, but subsequent cleavages were more rapid resulting in formation of the coeloblastula within 24 to 48 hours of initiation of cleavage (Fig. 11C). The blastopore had the same shape as in Enoplus and closed in the same manner. Gastrulation was completed within 36 to 60 hours of the initiation of cleavage. Rapid growth and bending over of the embryo in the succeeding 12 hours resulted in a U-shaped embryo (Fig. 11D) followed 24 to 48 hours later by the tadpole stage.

By 7 days after initiation of cleavage, the larvae were active and had developed several flexures (Fig. 11E). Two days later the larvae had increased in length and activity (Fig. 11F) and in the the following two days pigment began to develop in the anterior esophageal muscles. After a further 2 to 3 days the larvae hatched. The time from initiation of cleavage to hatching was 12 to 24 days. No moult was observed prior to hatching.

Newly hatched O. vesicarius larvae had two patches of granular, purple eyespot pigment located laterally in the

anterior esophageal muscle at the base of the buccal cavity (Fig. 12C, D) and sometimes a third smaller patch of identical pigment located dorsally in the esophageal muscle. In newly hatched O. skawensis larvae the distribution of eyespot pigment was the same but the pigment was red. None of the greenish esophageal pigment of the adult was visible at this stage in either species. During subsequent development the eyespot pigment increased in amount and density and esophageal pigment was gradually deposited. In the late larval stages, the eyespot pigment of O. vesicarius sometimes changed in colour from purple to reddish-brown, but the eyespot pigment of O. skawensis remained red (Fig. 6B).

c) Seuratiella sp.

Females of this species usually deposited 5 to 12 round eggs in a cluster. The eggs were 30 to 40 μ in diameter and were generally layed uncleaved. The first two cleavages were the same as in Enoplus and resulted in 4 equal blastomeres arranged in a rhomboid. Cleavage usually began within 24 hours of egg laying and was completed within a further 48 hours. The subsequent developmental stages were the same as in Enoplus. Movement of the embryo was first detected in the tadpole stage which was formed within 4 to 5 days of the initiation of cleavage. The time from initiation of cleavage to hatching was 10 to 12 days. The larvae did not moult prior to hatching.

Development of the eyespot pigment occurred 2 to 3 days

prior to hatching. The newly hatched larvae had two closely spaced pigment spots located dorsolaterally between the cuticle and the esophageal muscle (Figs. 12, E, F). The purple pigment was granular and closely packed and as the larvae matured it increased slightly in density and distribution.

d) Symplocostoma sp.

Eggs of this species were round to oval, 100 to 120 in diameter and deposited in clusters of from 5 to 18 in a gelatinous matrix. The eggs were laid uncleaved or in various stages of cleavage, but like Oncholaimus most eggs in a single cluster were at the same stage of development. The early development was identical to that occurring in Enoplus. Cleavage was usually complete within 48 hours, gastrulation within a further 24 hours and the tadpole stage formed within five days from initiation of cleavage. Subsequent development proceeded more slowly than in the other marine nematodes. The time from initiation of cleavage to hatching was 45 to 50 days. There was no evidence of moulting prior to hatching.

All the newly hatched larvae had the well developed, strongly chitinized type of buccal cavity of the adult female (Figure 4B). None had eyespot pigment, but all had paired refractile bodies laterally located in the esophageal muscle at the base of the buccal cavity. During subsequent development, the female larvae retained the well developed buccal cavity and did not develop eyespot pigment. The male

larvae retained the well developed buccal cavity until the moult to the adult, when they assumed the reduced male-type buccal cavity (Figure 4A). Just prior to this moult, the male larvae developed finely granular reddish-brown pigment spots in the anterior esophageal muscle and a second pair of refractile bodies just anterior to the pigment. During the moult, the well developed buccal cavity and the anterior pair of refractile bodies were shed with the cuticle (Fig. 4C). The pigment spots subsequently increased in size and density. Similar observations have been made on larval S. tenuicolle males (Wieser, 1953).

b) Discussion

All the marine nematodes studied were oviparous and laid their eggs either uncleaved or in early stages of cleavage. In comparison to parasitic species, free-living nematodes produce a relatively small number of large eggs, usually less than 50 per female (Hyman, 1951). The marine nematodes studied here were typical in this respect, the largest number of eggs produced by a single female being 40. Also, the greatest numbers of eggs were produced by the larger species and the fewest numbers by the smaller species.

The development of larvae inside the bodies of dead females observed in Enoplus and Oncholaimus species may be related to the phenomenom described as "Endotokia matricida" observed in some of the Rhabditida (Hirschmann, 1960). In these Rhabditids, old females are unable to discharge

their eggs and the larvae hatch in the uterus. After death of the mother, they break through the uterine wall, feed on the body contents and eventually escape through the cuticle to continue development. In the marine nematodes, the phenomenon differed in that the females died while the eggs were still in an early state of cleavage, larval developemnt did not contribute to the female's death and the larvae did not feed on the body contents after hatching but escaped directly through the disintegrating cuticle.

Although nematode cleavage is not generally considered to follow a typical spiral plan (Hyman, 1951) there is some disaggreement (Crofton, 1966). Among the parasitic nematodes a great deal of variation occurs in the planes of the early cleavages, in the timing and sequence of the blastomere divisions and in the relative sizes of the blastomeres produced. Because of the variation in cleavage planes the configuration of the blastomeres after the second cleavage ranges from a tandem arrangement as is seen in Helicotylenchus vulgaris (Yuen, 1966) and Rotylenchus parvus (Dasgupta and Raski, 1968) to the T-shaped arrangement of Parascaris equorum (Boveri, 1899) and Aphelencoides dactylocercus (Rowse, 1969). Even within a single species, such as Radophilus similis, variations in cleavage planes occur so that both tandem and T-shaped arrangements are found (Van Weerdt, 1960). In contrast, in the few free-living species which have been studied, including Plectus parietinus

(Maggenti, 1961), Diplolaimella schneiderii and Monohystera disjuncta (Chitwood and Murphy, 1964) as well as in the six marine nematodes of the present study, the early cleavages were consistent in plane, timing and sequence of cell division and relative blastomere size. The second cleavage was always oblique to the first and equal and simultaneous in both blastomeres. The rhomboidal arrangement of the blastomeres of the four cell stage indicated that the second cleavage was oblique to the first. In some parasitic nematodes such as P. equorum (Boveri, 1899) and Tylenchorhynchus sp. (Drozdovsky, 1968) a rhomboid configuration occurs at the four cell stage. In contrast to the free-living nematodes, this configuration does not result from oblique cleavage, but rather from migration of the cells after radial cleavage.

Subsequent cleavages in the marine nematodes like those in other free-living nematodes and parasitic species occurred rapidly and vaired in planes and sequence of cell division. Some variation occurred in the time from initiation to completion of cleavage in the marine nematodes. Coeloblastula formation occurred in 24 to 48 hours in O. vesicarius and O. skawensis, in 48 hours in Seuratiella and Symplocostoma spp. and in 48 to 72 hours in E. anisospiculus and Enoplus sp. Gastrulation also occurred more rapidly in O. vesicarius and O. skawensis than in the other species. In all the marine nematodes examined, the blastopore appeared as a longitudinal groove on the surface of the embryo which

closed first in the middle and then simultaneously both anteriorly and posteriorly. This is in contrast to some of the parasitic nematodes where the blastopore closes by fusion of the lateral lips, starting first in the posterior region and proceeding anteriorly (Crofton, 1966). The first stage at which bilateral symmetry was determined in the marine nematodes could not be ascertained, but at completion of gastrulation the bilateral symmetry of the embryos was readily apparent (Figs. 8G, 11D). In parasitic nematodes, bilateral symmetry may occur as early as the 4, 5, 6, or 7 cell stage (Wang, 1971 and Clark, 1967).

The later stages of development of the marine nematodes were identical to those of P. parietinus (Maggenti, 1961), D. schneiderii and M. disjuncta (Chitwood and Murphy, 1964) and similar to those of P. equorum and many other parasitic species (Hyman, 1951 and Crofton, 1966). Despite variations in the time required for the very early stages of embryonic development, the tadpole stage was formed in all the marine species within 5 days of the initiation of cleavage. Development from the tadpole stage to hatching however took from 3 to 5 times longer in Symplocostoma sp. than in the other marine nematodes.

In contrast to parasitic species which may undergo one or more moults prior to hatching (Hyman, 1951), all the marine nematodes hatched as first stage larvae. Hatching appeared to occur spontaneously at larval maturity rather than requiring

an environmental stimulus as is the case with many parasitic forms.

The specific time of development of the eyespot pigment and the discrete location in all species examined suggests that the eyespot pigment has a definite function in the nematode. The esophageal pigment of Enoplus and Oncholaimus species in contrast, could well be simply a waste product since it accumulates gradually with age and is diffusely distributed. In all the marine species examined except Symplocostoma sp., the eyespots formed just prior to emergence of the larvae from the eggs. The function of the eyespots therefore probably begins at hatching. At this time, a light sense would be of assistance in finding food and a suitable habitat and later, in finding mates. In Symplocostoma sp., where only the males developed eyespots, formation of the eyespots occurred at the same time as a change in the buccal cavity structure and the maturation of of the reproductive system. Perhaps these changes adapt the adult male for a brief, non-feeding period during which mates are sought. The eyespots, if involved in phototaxis, could assist in this search.

CHAPTER III

PIGMENT IDENTIFICATION

A. Introduction

Relatively little is known of the chemical identity of nematode eyespot pigments although many pigmented species have been reported. In the earliest study, spectroscopic examination of the anterior pigment spot or chromatrope of adult females of the insect parasite Mermis subnigrescens revealed absorption in the red and blue-violet wavelengths, but none in the orange, yellow or green (Cobb 1929). More recently, the alpha and beta absorption bands of oxyhemoglobin were detected in the chromatrope and the presence of hemoglobin confirmed with a positive benzidine test (Ellenby, 1964). Subsequent histochemical examination has shown large amounts of hemoglobin in the hypodermal chord tissue anterior to the trophosome and smaller amounts in the trophosome and hypodermal chords posterior to the chromatrope (Ellenby and Smith, 1966).

Hemoglobin was also suggested as the identity of the eyespot pigment of the fresh water nematode Chromadorina viridis on the basis of solubility tests, benzidine reaction and the demonstration of action peaks of positive phototaxis corresponding to the Soret and alpha absorption bands of hemoglobin (Croll, 1966a). However, no action peak corresponding to the beta absorption band of oxyhemoglobin was found. Recent microspectrophotometric determination of the absorption spectrum of the eyespot pigment of this species has shown that

it is not in fact a hemoglobin (Croll et al., 1972).

The eyespot pigments of a number of marine nematodes, mostly Enoplidae were examined by Ellenby and Smith (1966 a, b), but none were found to contain hemoglobin. A more detailed study, performed on the eyespot pigment of Enoplus communis, showed that the pigment was probably a melanin on the basis of solubility and bleaching tests (Croll, 1966a). The characteristics of the more diffusely distributed esophageal pigment of this species were not reported, but were assumed to be the same as the eyespot pigment (Croll, 1970).

The availability locally of several species of eyespotted, free-living, marine nematodes together with the scanty literature and questions arising out of some of the results reported led to the present study on the chemical identity of the pigment spots. The suggestion that hemoglobin might be involved in a photosensory process was of prime interest.

The eyespot pigments of Chromadorina germanica, Oncholaimus skawensis, Oncholaimus vesicarius, Enoplus anisospiculus, Enoplus sp., Symplocostoma sp. and Seuratiella sp. were characterized through examination of their absorption spectra, fluorescence, solubility and histochemical reactions. The most detailed studies were made on O. vesicarius and the Enoplus spp. since these were the most abundant and easily handled. Observations were also made on the reactions of the esophageal pigment of E. anisospiculus.

During the course of this study, a number of adult Mermis

nigrescens females became available. The absorption spectrum of the anterior pigment of these nematodes was examined micro-spectrophotometrically in order to confirm the presence of oxy-hemoglobin.

B. Materials and Methods

The following procedures were performed on adult nematodes of either sex in all marine species except Symplocostoma sp. where only males were used. Absorption spectra were obtained from newly hatched E. anisospiculus larvae as well as from adults.

1. Solubility Tests

The solubility characteristics of the nematode pigments were determined by treating the in situ pigments with a variety kf organic solvents, acids, alkalies and other reagents as listed in Tables II and III. The heads (anterior ends including pigmented areas) were removed from live nematodes and placed on cavity slives. Several drops of solvent were applied and the pigment response observed microscopically. If there was no immediate colour change or fading of the pigment, the heads were crushed to ensure penetration of the solvent to the pigment site and more solvent was applied. The slides were examined at intervals for up to four hours.

The pigments were considered soluble if they faded within four hours. Though fading in most of the solvents was due to dissolving, in a few solvents it may have been caused by hydrolysis or oxidation. It was impossible to distinguish

between these reactions under the microscope.

Extracts were made of the heads of E. anisospiculus and O. vesicarius with three solvents in which the pigments faded. The extracts were made by crushing 30 to 40 heads in 0.5 ml of 1 N sodium hydroxide and in 0.5 ml of concentrated sulfuric acid and by crushing 100 heads in 0.5 ml of 5% (v/v) concentrated hydrochloric acid in acetone. Visible and UV absorption spectra of the extracts were determined with a Cary 14 spectrophotometer.

An extract was also made of the chromatrope pigment of M. nigrescens by crushing 40 heads in 0.5 ml of distilled water. For comparison, extracts of human oxyhemoglobin and deoxyhemoglobin were prepared in distilled water. The deoxyhemoglobin was prepared by addition of an excess of solid sodium dithionite to the oxyhemoglobin solution (Fernando, 1968). Visible absorption spectra were determined with the Cary 14.

2. Microspectrophotometry:

Absorption spectra of the in situ pigments of the marine nematodes and of M. nigrescens were obtained with a Shimadzu recording microspectrophotometer (Model MPS 50L) at wavelengths from 380 to 700 nm. The heads of live marine nematodes were removed and suspended in filtered sea water on microscope slides. Cover slips were applied with a gentle pressure in order to flatten, but not rupture the heads and were sealed in place. Absorption spectra of the pigment spots of C. germanica adults and E. anisospiculus larvae were recorded with a two micron

sample beam, five micron field diaphragm and photomultiplier dynode voltage of 750 volts. The pigment spots of the other nematodes were all large enough that a five micron sample beam, 20 micron field diaphragm and photomultiplier dynode voltage of 650 volts could be used. The baseline was adjusted with filtered sea water on a microscope slide placed in the sample beam.

Since whole heads were being examined, the sample beam passing through the eyespot recorded not only the absorbance of the pigment, but also the absorption and scattering by tissues above and below the pigment spot. The spectra were corrected for this by determining the absorbance of non-pigmented areas adjacent to the pigments spots and subtracting the values so obtained from the pigment spot absorbance. In this way reproducible results could be obtained from whole head mounts of all species with the exception of E. anisospiculus and O. vesicarius adults. The latter nematodes had much larger head diameters than the other species and large amounts of variably distributed pigments in tissue surrounding the eyespot and esophageal pigments. Even after the heads were compressed in order to minimize the amount of tissue through which the sample beam had to pass, it was difficult to ascertain whether variations in the wavelengths of maximum absorption (λ max) found with different positions of the sample beam within the pigment spots were caused by variations in the composition of the pigment or were actually due to

variations in the distribution of other tissue components. This problem was overcome through use of frozen sections instead of whole head mounts. Fresh frozen sections, eight to ten microns thick, prepared as described below under histochemistry, were mounted in distilled water and the absorption spectra of various sites in the eyespot and esophageal pigment determined.

The absorption spectrum of the chromatrope of M. nigrescens was obtained from whole heads mounted in distilled water with a five micron sample beam, 20 micron field diaphragm and photomultiplier dynode voltage of 650 volts. As a check on functioning of the microspectrophotometer and for comparison of absorption maxima with those of the nematode pigment, the absorption spectrum was also determined of a solution of human oxyhemoglobin in distilled water contained in a glass capillary tube of approximately the same diameter as the nematode head. The concentration of this solution was adjusted so that the maximum absorbance was similar to the maximum absorbance of the nematode pigment. The oxyhemoglobin solution was subsequently deoxygenated by addition of sodium dithionite and the absorption spectrum again determined.

All absorption spectra after correction for variations in the baseline and for absorbance by substances other than the eyespot or esophageal pigment were normalized to the absorbance maximum in order to facilitate comparison of the positions of absorption peaks.

3. Fluorescence

The pigments of all seven species of marine nematodes were examined with a Zeiss fluorescence microscope for fluorescence excited by UV light. Observations were made on whole heads suspended in sea water, 0.01 N sodium hydroxide and 0.01 N hydrochloric acid. Observations were also made on unfixed frozen sections of E. anisospiculus and O. vesicarius adults prepared as described under histochemistry and suspended in the above solutions.

4. Histochemistry

Histochemical tests to detect the presence of hemoglobin, carotenoids, hematoidin and bile pigments, melanins, lipofucsins and hemosiderin were performed on fresh frozen sections of the heads of E. anisospiculus and O. vesicarius adults. The heads were removed from live nematodes, rinsed in distilled water to remove adhering debris and salt crystals and mounted in groups of ten to 20 in Tissue-Tek OCT embedding medium (Ames Co., Elkhart, Indiana). The heads were then frozen in a cryostat at -20° C and serial sections eight to ten microns thick were cut and mounted on albuminized slides. Sections of E. anisospiculus adhered well to albuminized slides, but those of O. vesicarius did not. Sections of the latter, after mounting on slides and drying were coated with a thin layer of 0.25% formvar in ethylene dichloride and carried through the staining procedures with uncoated and similarly coated sections of E. anisospiculus.

The Lepehne-Pickworth benzidine test (Lynch et al., 1969) performed on sections post-fixed in 10% neutral buffered formalin was used to detect hemoglobin. This test is based on the ability of hemoglobin, acting as a peroxidase, to catalyze the oxidation of benzidine by peroxide to yield a blue quinhydrone. Thermolabile peroxidases and catalases were inactivated prior to testing by heating the sections to 100° C (Lee and Smith, 1965). The presence of carotenoids was tested by the murexide test (Lillie, 1965) in which carotenoids will sometimes yield a transient blue colour when treated with concentrated sulfuric acid. Hematoidin and bile pigments were tested for by the Gmelin reaction (Pearse, 1961) in which progressive oxidation or dehydrogenation by concentrated nitric acid yields various pigments ranging in colour from red to purple, green and blue.

The following tests were performed to detect and distinguish between melanins, lipofucsins and hemosiderin. Reductive capacities of the pigments were determined by the Masson-Fontana silver nitrate reduction method and Schmorl's ferricyanide reduction test (Pearse, 1961). The ability to take up fat stains was tested by Lillie and Ashburn's Oil Red O method (Culling, 1963) and by the Sudan Black B method (Pearse, 1961) performed on sections post-fixed in 10% formol-calcium. Susceptibility to bleaching was tested by exposure to 10% hydrogen peroxide for 48 hours (Pearse, 1961). Ferrous iron was determined by the Turnbull blue method (Lynch et al., 1969 and free ferric iron by Perl's Prussian blue method (Gomori,

1964). Protein bound ferric iron was determined by Perl's test performed on sections previously treated for 30 minutes with alkaline 30% hydrogen peroxide to unmask the iron by denaturing the protein (Pearse, 1961). The ability to chelate ferrous ions was tested by the ferrous iron uptake method (Lillie, 1965).

In order to ascertain whether or not the pigment granules were lysosomes the presence of acid phosphatase was determined by the standard coupling azo dye technique (Pearse, 1961) and by Gomori's lead nitrate method (Gomori, 1964) performed on sections post-fixed in 10% neutral buffered formalin. Fresh frozen sections of frog liver known to contain acid phosphatase were used as a control.

C. Results

1. Solubility tests

The results of the solubility tests on the in situ nematode pigments are summarized in Tables II and III. The eyespot pigments of all marine species were insoluble in water and rapidly soluble in 1 N sodium hydroxide, but showed varying degrees of solubility in the hydrochloric acid solutions. The chromatrope pigment of M. nigrescens, in contrast to the marine nematode pigments was rapidly soluble in distilled water. All pigments tested were rapidly faded by concentrated HCl, HNO_3 and H_2SO_4 with the exception of the eyespot pigment of E. anisospiculus and Enoplus sp. which was not affected by concentrated HCl. Each of the four pigments tested with the

Table II

Solubility of *in situ* Nematode Pigments in Water, Acid and Alkali. + = soluble, - = insoluble, NT = not tested

	water	1 NaOH	1 N HCl	conc. HCl.	conc. HNO_3	conc. H_2SO_4
C. germanica	-	+	+ fast	+	NT	NT
Enoplus spp.						
eyespot pigment	-	+	-	-	+	+
esophageal pigment	-	+	+ slow	+	+	+
O. skawensis	-	+	+ fast	+	NT	NT
O. vesicarius						
major component	-	+	+ slow	+	+	+
minor component	-	+	+ fast	+	+	+
Seuratiella sp.	-	+	+ slow	+	NT	NT
Symplocostoma sp.	-	+	+ fast	+	NT	NT
M. nigrescens	+	NT	NT	NT	NT	NT

Table III

Solubility of *in situ* Marine Nematode Pigments in Organic Solvents and Other Reagents
+ = soluble, ± = partly soluble, - = insoluble,
NT = not tested

	O. vesicarius		*Enoplus* spp.	
	major component	minor component	eyespot pigment	esophageal pigment
acetone	-	+	-	±
acetone - 5% HCl	+	+	-	±
butanone	NT	NT	-	-
ethanol	NT	NT	-	-
methanol	-	+	-	-
butanol	NT	NT	-	-
benzene	-	+	-	-
chloroform	-	+	-	±
cyclohexane	-	+	-	±
dimethyl sulfoxide	NT	NT	-	-
diethyl ether	NT	NT	-	-
dioxane	-	-	-	-
hexane	NT	NT	-	-
petroleum ether	NT	NT	-	-
pyridine	NT	NT	-	-
xylene	NT	NT	-	-
1% aqueous digitonin	-	-	-	-
1% methanolic digitonin	-	-	-	-
Triton-X-100	-	-	-	-
4 M urea	-	-	-	-
4 M urea and Triton-X-100 (1:1)	-	-	-	-

organic reagents (Table III) showed different solubility characteristics.

Although the pigments of O. vesicarius and E. anisospiculus were completely faded by treatment with either 1 N sodium hydroxide or concentrated sulfuric acid, the extracts in these solvents when read in the Cary 14 had no significant absorption except for a small peak at 280 nm probably caused by aromatic amino acids. The lack of pigment absorption may have been due to protein hydrolysis and pigment breakdown caused by the solvents or the pigment may not have been sufficiently concentrated in the extracts. The eyespot pigment of O. vesicarius was completely faded by acetone-HCl, but upon extraction in this solvent, again only a small peak at 280 nm was obtained. The extract of E. anisospiculus in acetone-HCl, however, had a small absorption peak at 360 nm in addition to the peak at 280 nm. The former peak was probably due to some component of the esophageal pigment since this pigment was partially removed by acetone-HCl while the eyespot pigment was completely unaffected.

The chromatrope pigment of M. nigrescens was extracted into distilled water. The positions of the absorption peaks and the corresponding absorbances obtained with the Cary 14 of this pigment and of human oxy and deoxyhemoglobin are shown in Table IV. The absorption spectrum of the pigment of M. nigrescens was similar to that of human oxyhemoglobin except that the absorbance of the beta peak was so much higher than that of the

Table IV

The wavelengths of maximum absorption and corresponding absorbances of aqueous extracts of human oxy- and deoxyhemoglobin and of the chromatrope pigment of M. nigrescens as determined with the Cary 14.

Absorption peaks	Oxyhemoglobin nm	absorbance	Deoxyhemoglobin nm	absorbance	M. nigrescens nm	absorbance
Soret	415	1.81	429	1.80	410	0.120
beta	540	0.64			543	0.054
visible			555	0.645		
alpha	575	0.635			shoulder	

alpha peak that the position of the alpha peak appeared as only a shoulder.

2. Microspectrophotometry

The corrected and normalized absorption spectra obtained with the microspectrophotometer from the in situ eyespot pigments of whole head mounts of C. germanica, O. skawensis, Seuratiella sp. and Symplocostoma sp. and from frozen sections of O. vesicarius are shown in Figure 13. The positions of the absorption peaks and the corresponding corrected absorbances for these spectra and the ones described below are summarized in Table V. The absorbances given are from single nematodes, but are typical of each species. Variation was found in the absorbances of individual eyespots, but within each species the spectra were qualitatively the same.

The absorption spectra of the eyespot pigment of whole head mounts of E. anisospiculus larvae and of the eyespot and esophageal pigments of fresh frozen sections of adults are shown in Figure 14. Regardless of the position of the sample beam within the pigment spot, the larval eyespot pigment gave a consistent absorption spectrum with a single broad peak at 500 - 515 nm. The adult eyespot however, showed some variation depending on whether anterior or posterior portions of the spot were being examined. The absorption spectrum shown here with peaks at 450 - 460 and 500 - 515 nm was obtained from the anterior of the adult eyespot. The adult esophageal pigment also showed variations in absorption depending on the location

Figure 13. Corrected and normalized absorption spectra of *in situ* eyespot pigments of *C. germanica*, *O. skawensis*, *O. vesicarius*, *Symplocostoma* sp. and *Seuratiella* sp. Obtained with microspectrophotometer.

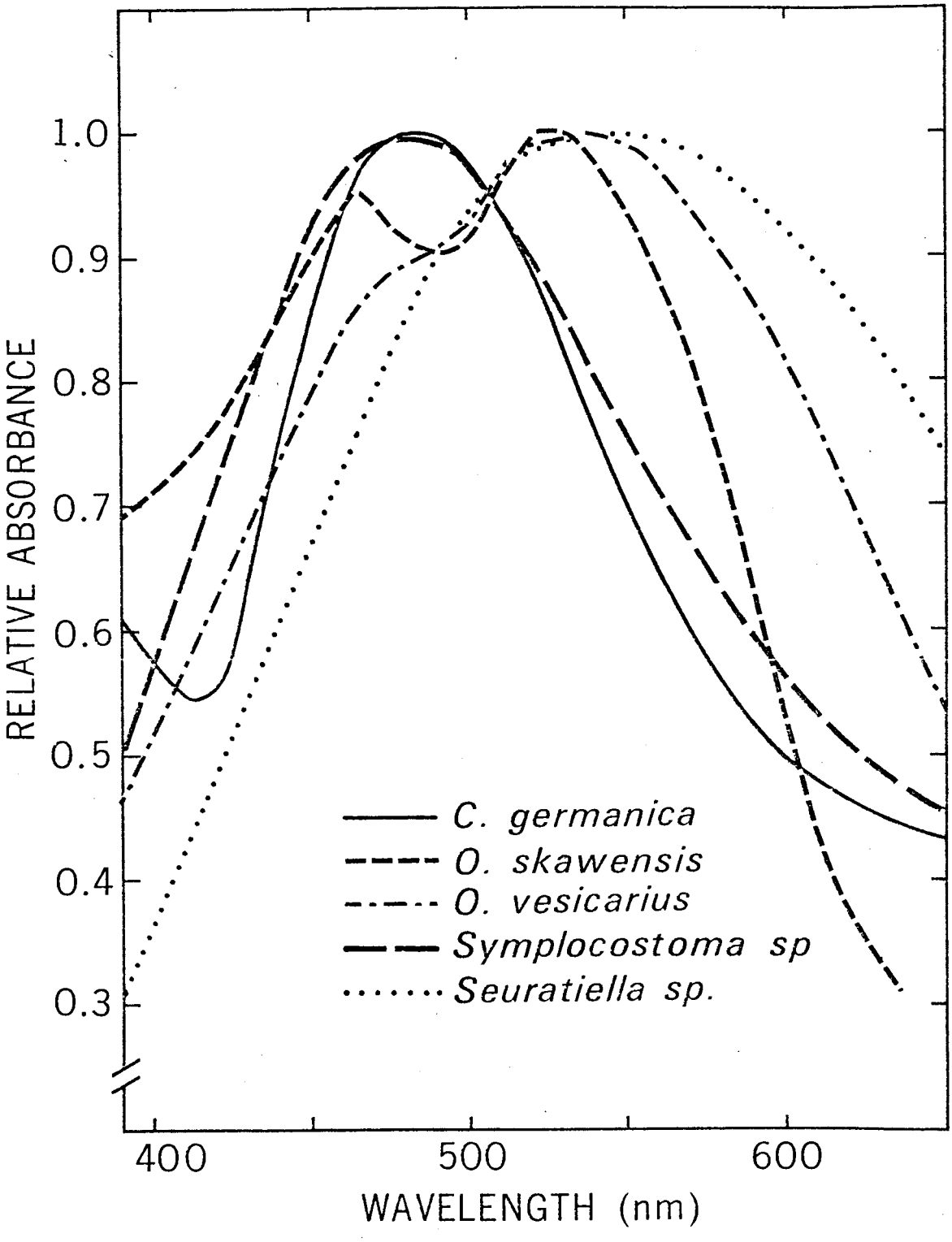
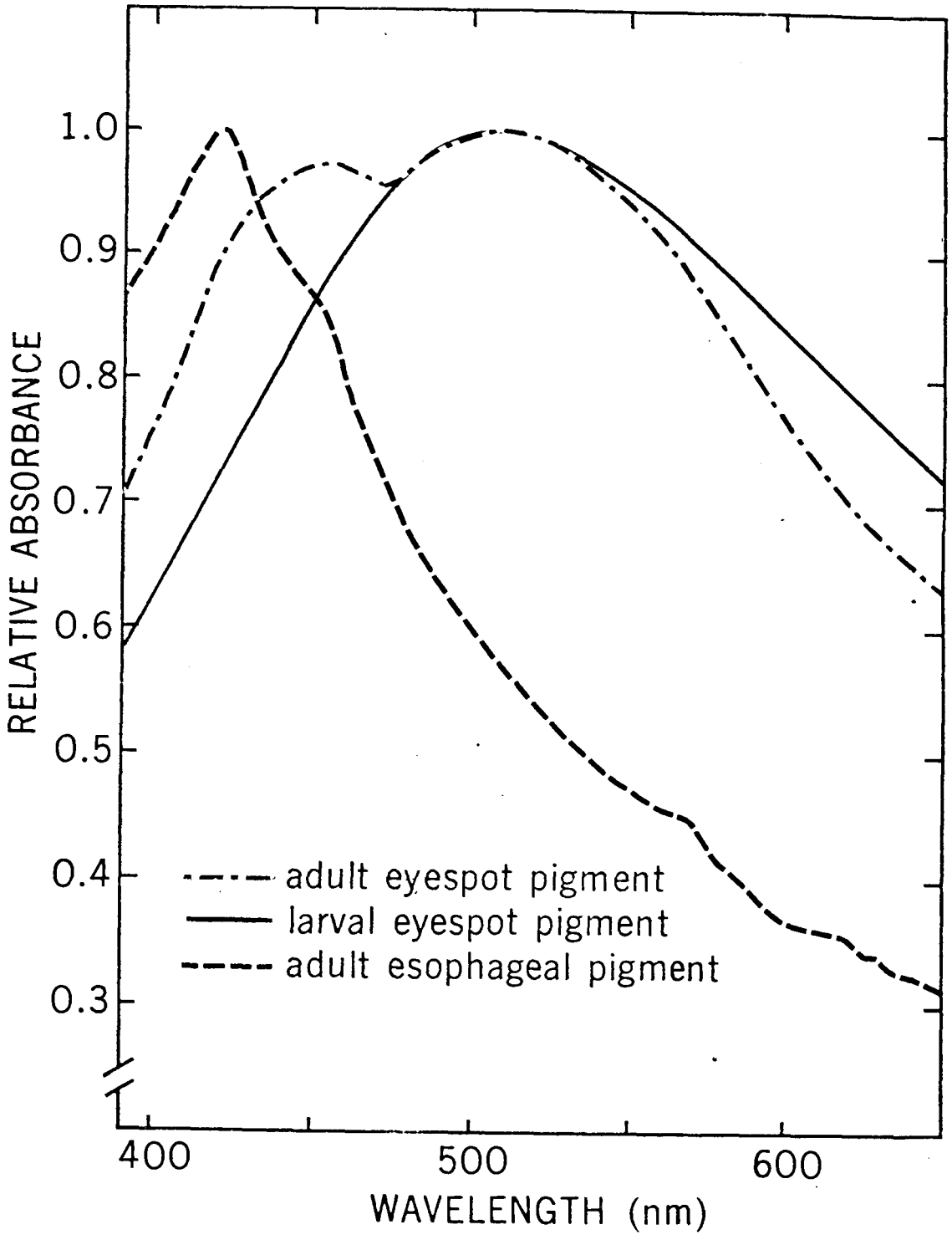

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
RELATIVE ABSORBANCE
400
500
600
WAVELENGTH (nm)
C. germanica
O. skawensis
O. vesicarius
Symplocostoma sp
Seuratiella sp.

Table V

Wavelengths of Maximum Absorption and Typical Corrected Absorbances for Pigment Absorption Spectra Obtained with the Microspectrophotometer

	nm	absorbance
C. germanica eyespot	485	0.78
O. vesicarius eyespot	535	1.41
	480 (shoulder)	0.88
O. skawensis eyespot	465	0.39
	525-530	0.41
Seuratiella sp. eyespot	540-550	1.54
Symplocostoma sp. eyespot	485	1.66
E. anisospiculus		
larval eyespot	500-515	1.36
adult eyespot	450-460	1.45
	500-515	1.50
adult esophageal pigment	420	1.21
	450 (shoulder)	1.05
	550 (shoulder)	0.58
	570 (shoulder)	0.55
	610 (shoulder)	0.44
	630 (shoulder)	0.41
adult hypodermal chord	415	1.51
	535	0.45
	570	0.44
M. nigrescens chromatrope	413	2.74
	543	0.63
	583 (shoulder)	0.48
Human oxyhemoglobin	415	1.98
	540	0.44
	573	0.43
Human deoxyhemoglobin	429	1.90
	555	0.44

Figure 14. Corrected and normalized *in situ* absorption spectra of pigments in *E. anisospiculus*. Microspectrophotometry on whole larvae and frozen sections of adults.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
RELATIVE ABSORBANCE
adult eyespot pigment
larval eyespot pigment
adult esophageal pigment
400
500
600
WAVELENGTH (nm)

of the pigment in the nematode. The spectrum shown for this pigment with a peak at 420 nm and five shoulders was obtained from esophageal pigment located at some distance posterior to the eyespot. Absorption spectra obtained from areas in the posterior of the eyespots and in the anterior portions of the esophageal pigment gave results intermediate to the two types shown. The shoulders suggested at 450, 550, 570, 610 and 630 nm in the posterior esophageal pigment were reproducible.

Microspectrophotometric examination of hypodermal chord material adjacent to the eyespot pigment in whole head mounts of E. anisospiculus adults yielded the absorption spectrum shown in Figure 15 which is typical of an oxyhemoglobin.

The absorption spectrum obtained with the microspectrophotometer from the in situ pigment of M. nigrescens (Fig. 16) compared favourably with that obtained with the Cary 14 from extracted pigment. A good agreement was also found between the absorption spectra of capillary tubes of human oxy- and deoxyhemoglobin solutions obtained with the microspectrophotometer (Fig. 16) and of the same solutions with the Cary 14.

3. Fluorescence

No fluorescence under UV light was visible in any of the pigmented areas of the marine nematodes, although other areas of the body such as the gut contents, ova and teeth did fluoresce. In O. vesicarius and E. anisospiculus adults, there was a large amount of general body fluorescence which in whole mounts could have been sufficient to obscure pigment fluorescence.

Figure 15. Corrected and normalized *in situ* absorption spectrum of hypodermal chord hemoglobin in *E. anisospiculus* adults. Obtained with the microspectrophotometer.

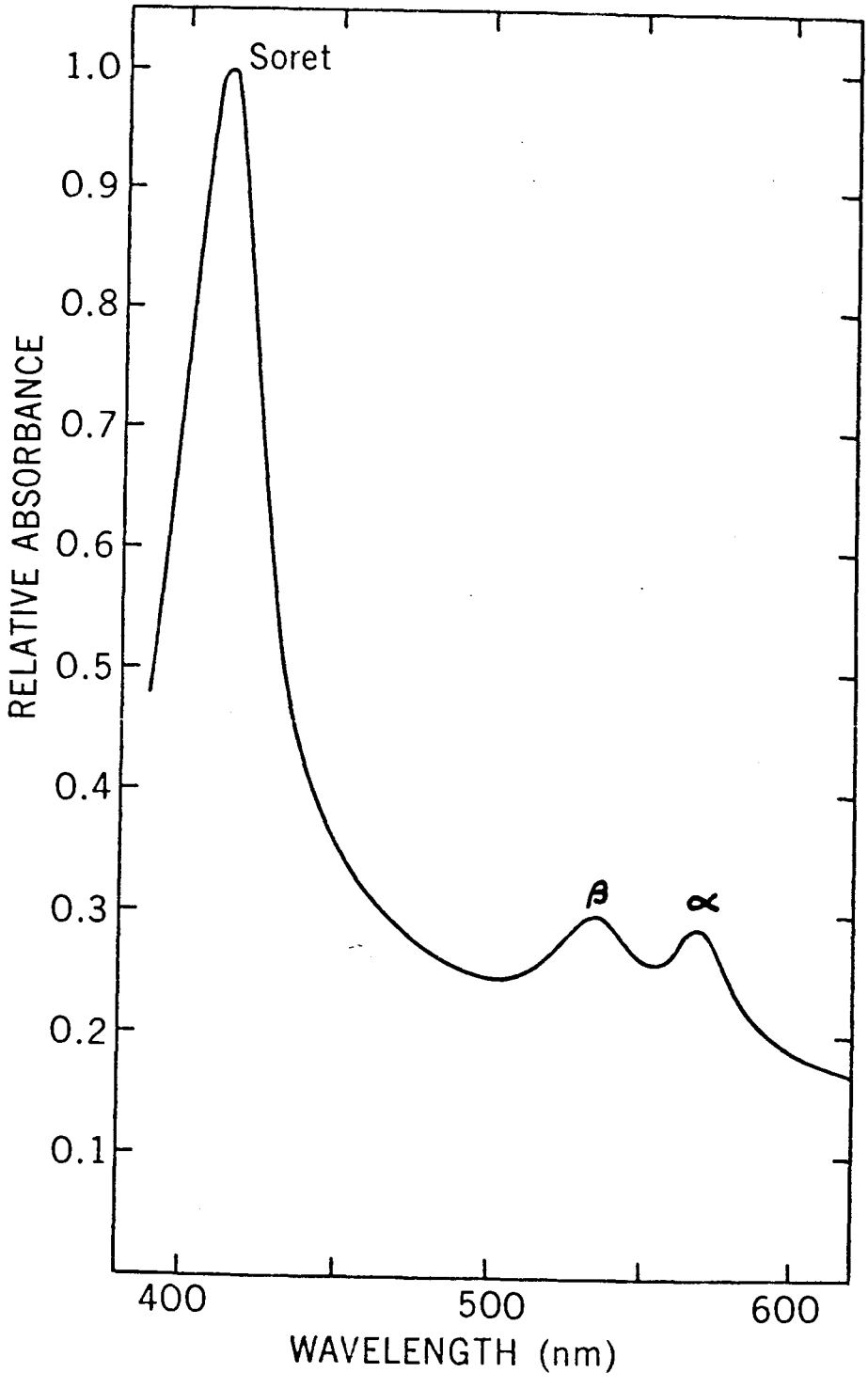
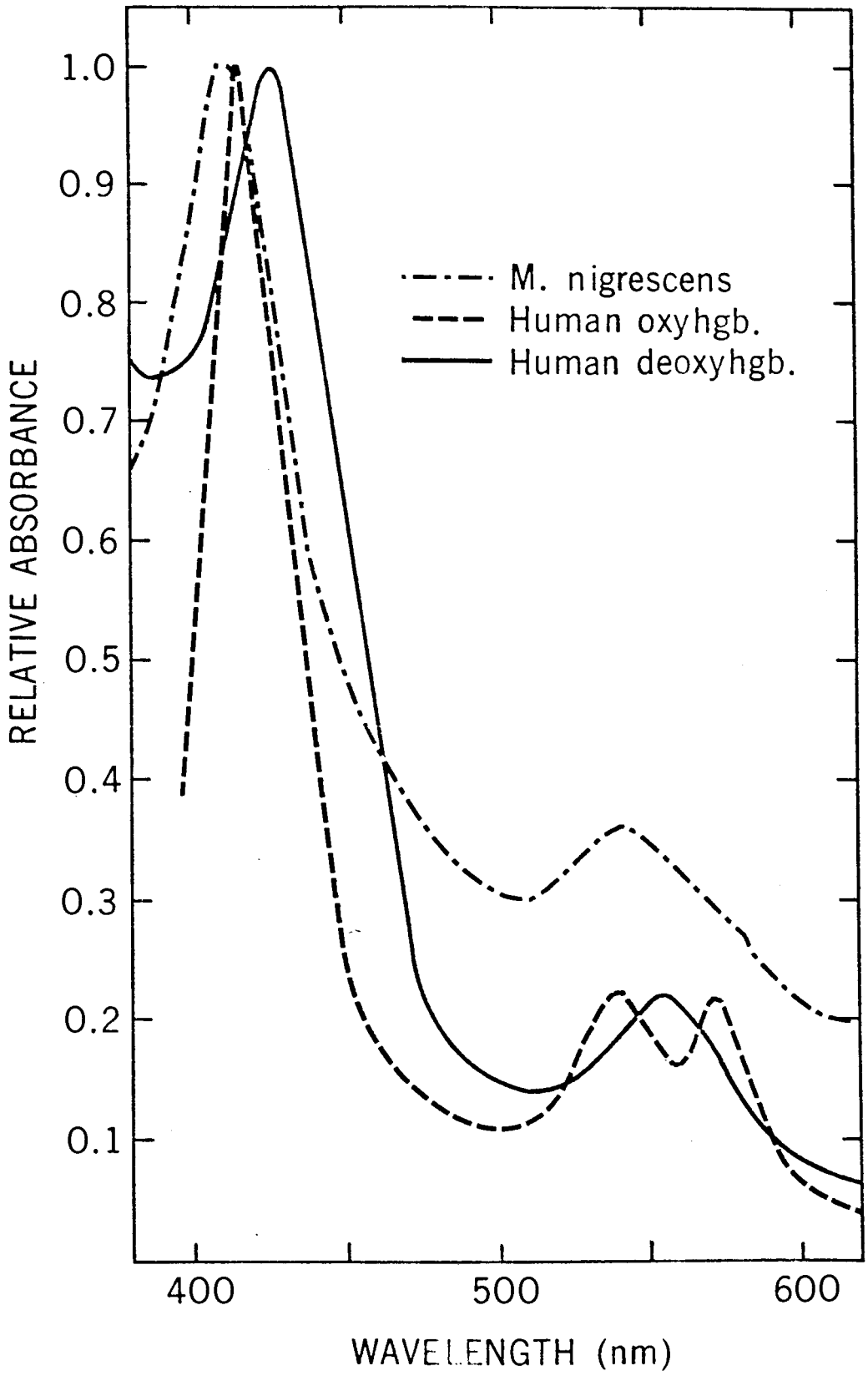

Soret
β
α
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
RELATIVE ABSORBANCE
400
500
600
WAVELENGTH (nm)

Figure 16. Corrected and normalized absorption spectra of the *in situ* chromatrope pigment of *M. nigrescens* and of human oxy- and deoxyhemoglobin solutions in capillary tubes. Obtained with the microspectrophotometer.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
RELATIVE ABSORBANCE
M. nigrescens
Human oxyhgb.
Human deoxyhgb.
400
500
600
WAVELENGTH (nm)

Frozen sections of these species were examined, but no pigment fluorescence was observed.

4. Histochemistry

The eyespot pigment of O. vesicarius adults and the eyespot and esophageal pigments of E. anisospiculus adults all gave negative reactions to the tests for hemoglobin, carotenoids, bile pigments and hematoidin. The hypodermal chords and non-pigmented esophageal muscle of both species gave positive reactions for hemoglobin. The results of the tests for melanins, lipofucsins and hemosiderin are summarized in Table VI. Typical reactions of the latter pigments are shown in Table VII of the discussion section.

None of the histochemical tests except those for acid phosphatase were adversely affected by formvar coating of the sections. In sections of frog liver the acid phosphatase reaction was inhibited by the formvar coating possibly because of enzyme inactivation by the ethylene dichloride in which the formvar was dissolved or because the reagents could not penetrate the formvar layer. The negative reaction given by formvar coated sections of O. vesicarius did not therefore indicate the absence of acid phosphatase.

D. Discussion:

Differences shown in the chemical characteristics of the nematode pigments indicated that there must be several types of pigment involved. The pigments of some species did however exhibit similar characteristics. In the following section the

Table VI

Histochemical Reactions of Pigments in *O. vesicarius* and *E. anisospiculus*
+ = positive reaction - = negative reaction NT = not tested

	O. vesicarius eyespot pigment	*E. anisospiculus* eyespot pigment	*E. anisospiculus* esophageal pigment
Silver nitrate reduction	-	+ in 24 hrs	-
Ferricyanide reduction	-	-	-
Lipid staining			
Oil Red O	NT	-	+
Sudan Black B	-	slight +	+
Ferrous iron	-	-	slight +
Ferric iron - free	-	-	-
bound	-	slight +	+
Ferrous ion uptake	-	-	-
Bleaching by 10% H_2O_2	+ in 4 hrs	+ in 24 to 48 hrs	+ in 24 hrs
Acid phosphatase		-	-

results are discussed species by species with those having similar pigment characteristics grouped together.

1. E. anisospiculus and Enoplus sp.

The eyespot and esophageal pigments of E. anisospiculus adults were initially thought to be the same because of the apparent similarity in colour, size and shape of the granules. However, the solubility and histochemical tests and the absorption spectra revealed that they had different chemical characteristics. Most of the pigment in the eyespots was much more insoluble than the esophageal pigment, but in the periphery of the eyespots and in esophageal pigment just posterior to them, both soluble and insoluble pigments were found. The eyespot and esophageal pigments apparently merge in these areas without discrete boundaries.

Further evidence of the presence of two pigments was obtained from the absorption spectra (Fig. 14). In the eyespots, the pigment consisted of two components, one absorbing maximally at 500 to 510 nm and the other at 450 to 460 nm. The peak at 450 nm was relatively larger in the periphery of the eyespots than in the central portion of the spots indicating a higher concentration of this component at the periphery. The posterior esophageal pigment absorbed maximally at 420 nm and had shoulders at 450, 570, 610 and 630 nm. At locations between these two, the pigment gave intermediate types of absorption spectra. Larval eyespot pigment had only one type of absorption spectrum with a peak consistently at 500 to 510

nm the same as the major component of the adult eyespot pigment.

The extreme stability and difficulty of extraction of the eyespot pigment together with its absorption spectrum suggested that it might be either a melanin or a lipofucsin. Both pigments are complex highly oxidized polymers of varying composition whose stability is due at least in part to a tightly bound protein component and which exhibit a broad absorption in the visible range (Pearse, 1961 and Nicolaus, 1968).

Animal melanins consist of two types, the brown to black eumelanins derived from tyrosine and the yellow, red or brown phaeomelanins derived from both tyrosine and cysteine. Both are formed by similar processes involving quinone formation, and subsequent oxidation, polymerization, and incorporation of free radicles, but in phaeomelanins there are thought to be fewer active centers for polymerization, resulting in the lighter colour and slightly higher solubility of the latter pigments (Nicolaus, 1968). The protein component of melanin often becomes visible as colourless granules when the pigment is decolourized through oxidation (Pearse, 1961).

Lipofucsins, also known as "wear and tear" or "aging" pigments, chromolipid, haemofucsin, lipochrome and ceroid, are a large class of compounds derived mainly or partly from lipid precursors through oxidation and polymerization. Their characteristics change as oxidation and polymerization proceeds from those of the lipid precursors to those of partially oxidized lipids and finally to those of fully oxidized lipids

and are therefore extremely variable. Lipofucsins are thought to be formed in or from lysosomes and are often found associated with lysosomal enzymes such as acid phosphatases or esterases (Pearse, 1961 and Barka and Anderson, 1965).

The typical reactions of melanins and of lipofucsins (Table VII) are obtained mainly from studies of mammalian pigments (Pearse, 1961 ; Lillie, 1965; Barka and Anderson, 1965). Relatively little work has been done on pigment from other sources (Pearse 1961; Nicolaus, 1968).

A comparison of the results obtained for the eyespot pigment of E. anisospiculus (Table VI) with the typical reactions of melanins and lipofucsins (Table VII) shows that the eyespot pigment did not exhibit all the typical reactions of either of the latter pigments. It did however, exhibit more of the reactions of melanins that of lipofucsins. The solubility, reduction of alkaline silver nitrate, peroxide bleaching, and acid phosphatase results were all indicative of melanins. The ferrous ion uptake test, when positive, is specific for melanins but melanins do not always give a positive reaction (Nicolaus, 1968). The negative reaction of the eyespot pigment does not therefore rule out melanins. The only reactions of the eyespot pigment which suggested the presence of lipofucsin were the slight uptake of Sudan Black B and the slight positive reaction to the bound ferric iron test. Both of these reactions, however, were also given by the esophageal pigment (Table VI) though more strongly. As previously discussed, there is evidence

Table VII

Typical Histochemical Reactions of Melanins, Lipofucsins and Hemosiderins
(based on Pearse, 1961; Lillie, 1965; Burka and Anderson, 1965)
+ = positive - = negative v. = variable

	melanins	lipofucsins			hemosiderins
		unoxidized lipids	partially oxidized lipids	fully oxidized lipids	
solubility in:					
organic solvents	-	+	v	-	v
strong acids	+ except HCl	?	?	-	v
1 N NaOH	+	?	?	-	+
$AgNO_3$ reduction	+ in 24 hrs	-	+	+ after 48 hrs	-
ferricyanide reduction	+	-	+	v	-
lipid staining	-	+	v	-	+
presence of ferric iron	-	-	v	v	+
ferrous iron uptake	+	-	-	-	-
bleaching by 10% H_2O_2	+ in 24 to 48 hrs	+	+	+ after 48 hrs	+
acid phosphatase	-	+	v	v	v
fluorescence	-	v	v	+	-

that a small amount of esophageal pigment is present in the adult eyespots. The lipid and iron reactions of the eyespot pigment can therefore be attributed to this esophageal pigment component. The lack of UV fluorescence and the presence of colourless granules after peroxide bleaching provided further evidence that most of the eyespot pigment is melanin and not lipofucsin.

The eyespot pigment of Enoplus sp. is probably also a melanin since as far as it was tested it gave the same reactions as the eyespot pigment of E. anisospiculus. Melanin has previously been detected in the eyespot pigment of the closely related species, E. communis (Croll, 1966c).

The esophageal pigment of E. anisospiculus did not exhibit the solubility, absorption spectrum or histochemical reactions of either a melanin or a lipofucsin, but instead had the characteristics of the class of iron storage pigments known as hemosiderins. This heterogeneous group of brown granular pigments contains ferric hydroxides in varying concentrations and physical forms, protein carrier substances, lipids, mucopolysaccharides , guanine and 2-deoxy-D-ribose (Wells and Wolken, 1962; Ludewig, 1957). Their histochemical reactions, summarized in Table VII include solubility in strong acids and alkalies, varying solubilities in organic solvents, positive reactions for lipid and ferric iron, bleaching by peroxide and lack of fluorescence under UV light (Pearse, 1961; Lillie, 1965; Barka and Anderson, 1965). The esophageal pigment of E. anisospiculus

exhibited all these characteristics. In addition, the ferric component of hemosiderin can be removed by acid extraction (Lillie, 1965) and has an absorption peak between 330 and 360 nm (Sandritter et al., 1966). The absorption peak at 360 nm in the acetone-HCl extract of E. anisospiculus heads can therefore be attributed to a ferric component removed from the esophageal pigment.

In microspectrophotometric studies of natural and induced mammalian hemosiderin granules, absorption spectra similar to that of the esophageal pigment of E. anisospiculus were obtained (Wells and Wolken, 1962; Sandritter et al., 1966). A Soret peak between 405 and 415 nm and several small peaks and shoulders between 450 and 637 nm were attributed to absorption by porphyrins derived from hemoglobin degradation. The progressive deposition with age of the esophageal pigment of E. anisospiculus suggests that it is a storage product of some metabolic process, possibly the degradation hemoglobins detected in the esophageal muscle and nearby tissues. Similar iron containing granules have been observed in the esophageal muscle and intestinal cells of the free living marine nematode, Pontonema vulgaris (Jennings and Colam, 1970) and in the intestinal cells of many parasitic nematodes (Fernando, 1968). In the animal parasitic nematodes, it has been suggested that the granules result from hemoglobin degradation (Fernando, 1968).

2. Seuratiella sp.

The broad absorption spectrum of the eyespot pigment of

this species (Fig. 13) and the few solubility tests done (Table II) showed that the pigment was neither a hemoglobin nor a carotenoid. Although fully oxidized lipofucsins exhibit a similar broad absorption spectrum, unlike the eyespot pigment, they are insoluble in 1 N NaOH and are usually fluorescence (Pearse, 1961). Fully developed eumelanins also exhibit a broad absorption spectrum, but in contrast to lipofucsins, are soluble in 1 N NaOH and non-fluorescent (Nicolaus, 1968; Pearse, 1961). The characteristics of the eyespot pigment was therefore more like those of a eumelanin than a lipofucsin. Fully developed eumelanins absorb maximally at 530 to 540 nm (Nicolaus, 1968) but are not usually soluble in concentrated HCl (Pearse, 1961). Incompletely polymerized eumelanins also absorb maximally at 530 to 540 nm but in contrast are more soluble (Nicolaus, 1968). The eyespot pigment is therefore most likely an incompletely polymerized eumelanin or a phaeomelanin.

3. C. germanica and Symplocostoma sp.

The eyespot pigments of these species were similar in both solubility (Table II) and absorption characteristics (Fig. 13) and do not appear to contain hemoglobin, melanin or lipofucsin. It is possible that they contain carotenoids since carotenoids absorb maximally between 400 and 500 nm. Also suggestive of a carotenoid, the pigments of both species faded after nematode death (Ch. II). Since the fading occurred during exposure to either light or darkness, it was not caused by photolysis, but probably by an oxidative process. Many pigments, including

carotenoids, lose their colour upon oxidation (Fox and Vevers, 1960). Differences observed in the rates of fading of the two species can be attributed to differences in the rates of inward diffusion of oxidizing substances from the environment after nematode death. Since C. germanica is a smaller nematode and has a thinner cuticle that Symplocostoma sp., such inward diffusion could occur more rapidly in the former species resulting in faster fading of the pigment.

In a recent microspectrophotometric study of the eyespot pigment of C. viridis, a species closely related to C. germanica, an absorption spectrum very similar to that of C. germanica and Symplocostoma sp. eyespots was obtained (Croll et al., 1972). This result invalidated a previous report (Croll, 1966a) that the pigment of C. viridis was a hemoglobin. In the more recent study no suggestion was made as to the actual identity of the pigment.

4. O. skawensis and O. vesicarius

The eyespot pigment of O. skawensis had the same solubility as the C. germanica and Symplocostoma sp. pigments (Table II) and like these pigments did not appear to be a hemoglobin, melanin or lipofucsin. The absorption spectrum, however, differed from those of C. germanica and Symplocostoma sp. (Fig. 13) and was more typical of a carotenoid spectrum than were the latter. The pigment of O. skawensis also faded in light or darkness after nematode death, providing further evidence that it may be a carotenoid.

The solubility characteristics of the eyespots of O. vesicarius unlike those of the closely related O. skawensis indicated the presence of two different types of pigment (Tables II, III). Most of the pigment was slowly soluble in 1 N HCl and insoluble in organic solvents, while a minor component in contrast was rapidly soluble in 1 N HCl and soluble in some organic solvents. Neither of the components had the typical histochemical reactions of a hemoglobin, bile pigment, hematoidin or lipofucsin. The solubility characteristics of the major component of the pigment, like those of Seuratiella sp. pigment indicated that it also is probably a phaeomelanin or incompletely polymerized eumelanin. The rapid bleaching by peroxide and inability to reduce alkaline silver nitrate confirmed that it was not a fully developed eumelanin. The minor component, like all the pigment of O. skawensis, C. germanica and Symplocostoma sp., had solubility and fading characteristics indicating that it may be a carotenoid. The fact that the O. vesicarius eyespots did not give a positive reaction to the histochemical test for carotenoids does not rule out the presence of carotenoids since these pigments do not always react with the test (Lillie, 1965). The absorption spectrum shown for O. vesicarius eyespots (Fig. 13) resembles that of both Seuratiella sp. and O. skawensis and can be explained by the presence in the eyespots of both a melanin and a carotenoid.

Recent electron microscopic investigation of the eyespot pigment of O. vesicarius has confirmed the presence of granules

that resemble developing melanin (Burr and Webster, 1971). Little morphological evidence was found to support the hypothesis that there are two types of pigment present in the eyespots, however this can not necessarily be expected to be evident in the fine structure.

5. M. nigrescens

Unlike the marine nematode pigments, the chromatrope pigment of M. nigrescens was water soluble and had an absorption spectrum typical of a hemoglobin (Fig. 16). Superficially the spectrum resembled that of a deoxygenated hemoglobin in that it only had one clearly defined peak in the visible region. The Soret peak, however, occurred at too short a wavelength for a deoxyhemoglobin and there was a shoulder on the visible peak which suggested the presence of a small alpha absorption peak. More recently, it has been shown that deoxygenation of Mermis hemoglobin produces a typical deoxyhemoglobin spectrum and subsequent reoxygenation produces the original spectrum (Burr, unpublished). The pigment is therefore undoubtedly an oxyhemoglobin. This result substantiates the conclusion of Ellenby and Smith (1966b) who identified oxyhemoglobin in the chromatrope pigment using a spectroscope and the benzidine test.

The chromatrope oxyhemoglobin spectrum is unlike that of vertebrate oxyhemoglobin in that the absorbance of the beta peak is much higher than that of the alpha peak. This characteristic is found in some animal parasitic nematode oxyhemo-

globins and is associated with a very high oxygen affinity (Lee and Smith, 1965). It is interesting that the absorption spectrum of the hypodermal chord hemoglobin of the free-living marine nematode E. anisospiculus more closely resembled the vertebrate oxyhemoglobins.

E. Summary

A summary of the pigments identified in the various species of nematodes is given in Table VIII.

Table VIII

Summary of Findings on Pigment Spot Identity
+ = present - = not present

	melanins	carotenoid-like pigments	oxyhemoglobin	hemosiderin
E. anisospiculus				
eyespot pigment	+	-	-	-
esophageal pigment	-	-	-	+
hypodermal chord	-	-	+	-
Seuratiella sp.				
eyespot pigment	+	-	-	-
C. germanica				
eyespot pigment	-	+	-	-
Symplocostoma sp.				
eyespot pigment	-	+	-	-
O. skawensis				
eyespot pigment	-	+	-	-
O. vesicarius				
eyespot pigment	+	+	-	-
M. nigrescens				
chromatrope	-	-	+	-

CHAPTER IV

GENERAL DISCUSSION

In the introduction, the following three questions were suggested: (1) Are the anterior pigment spots of nematodes involved in any way in phototaxis? (2) If so, are they directly involved in the photoreceptive process? and (3) What is their chemical composition? The third of these questions has now been at least partially answered with the findings of this thesis that the eyespots of several marine nematode species contain either or both melanins and carotenoid-like pigments and that the chromatrope of M. nigrescens does indeed contain oxyhemoglobin. The answers to the remaining two questions are still open to speculation.

In phototactic responses, pigments can function in two different ways, either directly, as primary photopigments like the rhodopsins found in many photoreceptors or indirectly, as shading pigments. Shading pigments are thought to mediate a directional response in semi-transparent organisms such as Euglena by shading an adjacent photoreceptor when the organism has a certain orientation with respect to the light source. The organism reorients itself according to whether or not the photoreceptor is illuminated.

If a behavioural response to light is mediated by only a primary photopigment, the action spectrum of the response will depend on and therefore be superimposable on the absorption spectrum of the pigment. If, on the other hand,

a shading pigment is operative in addition to the primary photopigment, the action spectrum of the response may be a composite of the absorption spectra of both pigments. However, both absorption spectra must overlap.

The chemical compostition of eyespot pigments of the marine nematodes indicates that they are not likely to be involved in photoreception. All photoreceptor pigments so far identified have been of the retinal-protein type. Although melanins undergo photopolymerization reactions and free carotenoids undergo photo-oxidation reactions, these pigments are not known to function as photoreceptors. It would be surprising if the nematodes were unique in utilizing these pigments for photoreception. It is unlikely that they are simply waste products in view of the specific time of development of the eyespots and their discrete location. The broad absorption band of these pigments, particularly the melanins, would make them especially suitable as shading pigments and, indeed, would appear to be the only requirement for this function. The variations in composition of the marine nematode pigments could possibly represent differences in the photic environment, diet or metabolic capabilities of the different species which have resulted in evolution of different pigments to perform the same function. The difference of one pigment between the closely related species, O. vesicarius and O. skawensis, is interesting.

Behavioural evidence of light sensitivity in eyespotted free-living nematodes is scanty. The single study in which both the action spectrum of a behavioural response to light and the absorption spectrum of the eyespot pigment were determined was on C. viridis. The action spectrum of positive phototaxis (Croll, 1966a) did not coincide with the absorption spectrum of the eyespot pigment (Croll et al., 1972) indicating that this pigment could not be the primary photoreceptor. The character of the response in this species and in D. schneideri, where a negative phototaxis was observed (Chitwood and Murphy, 1964) would be difficult to explain without invoking a shading mechanism.

Ultrastructural studies of C. viridis (Croll et al., 1972), D. californicum (Siddiqui and Viglerchio, 1970a,b) and O. vesicarius (Burr and Webster, 1971) provide further evidence that the eyespot pigment is not the photoreceptor, but instead acts as shading pigment for an adjacent photoreceptor. In the latter species, there were no structures in the pigmented areas resembling the characteristic light sensitive organelles of known photoreceptors, but in an enlargement of the amphidial organ just anterior to the pigment, a presumptive photoreceptor organelle was found which would be shaded by light from the posterior and posterolateral directions.

In M. nigrescens, the role of the chromatrope pigment is not so apparent. The oxyhemoglobin of the chromatrope

could theoretically function directly in photoreception since it has been shown to be photosensitive (Gibson and Ainsworth, 1957). The mechanism involved might be a conformational change in the protein moiety induced by photodissociation of oxygen leading to initiation of a nervous impulse in much the same way as a conformational change in retinal-protein pigments, induced by photoisomerization initiates the visual impulse in known photoreceptors. However, the retinal-protein pigments have been highly evolved as photopigments, while hemoglobin is a highly evolved oxygen carrier. It therefore seems unlikely that in M. nigrescens, hemoglobin would function in the dual role of photopigment and oxygen carrier or that it should have evolved instead of a retinal-protein pigment as a photoreceptor pigment in this species.

Although light sensitivity has been shown in this nematode, there is no conclusive evidence that the chromatrope is involved. An approximate correlation has been claimed between the absorption spectrum of hemoglobin and the action spectra of both oviposition (Croll, 1966b) and phototaxis (Cobb, 1926; Croll, 1966b), but only with more accurately determined action spectra can this correlation be convincing. The mechanism actually involved in these light responses might be the same as that involved in the light sensitivity of other parasitic nematodes without chromatropes (Croll, 1970) which has not yet been elucidated. The narrow absorption

bands of hemoglobin would make it unsuitable as a polychromatic shading pigment, however it could, by shading, indicate the direction of a short wavelength light source such as the sky.

If the chromatrope is at all involved in mediating behavioural responses, it is probably in a role more in keeping with the oxygen binding capacities of hemoglobin. Perhaps the chromatrope functions as an oxygen sensor, which in combination with temperature or other sensors, ensures that the adult nematode emerges from the soil under suitable environmental conditions for oviposition.

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