ATTACHMENT ORGANS OF *COTYLURUS*

THE STRUCTURE AND FUNCTION OF THE ATTACHMENT ORGANS IN *COTYLURUS VARIEGATUS* CREPLIN, 1825 (ODENING, 1969) (TREMATODA: STRIGEIDA)

by

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Previous studies dealing with the structure and function of the attachment organs in the strigeid trematodes have neglected to describe the processes involved in the formation of attachment. A knowledge of these processes is necessary to promote the understanding of the host-parasite relationship.

In the present study, specimens of developing Cotylurus variegatus were examined using light and electron microscopic techniques. It seemed relevant to consider not only the sequence of attachment events, but the growth and structure of the attachment organs in relation to the total parasite body growth and structure. This of course, has led to considerations of the reputative functions of these structures.

PREFACE

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INTRODUCTION

The supersuperfamily of Strigeida* Poche, 1925 (Dubois, 1938) is a group of trematodes sharply separated from other Digenea by the possession of a series of well defined characteristics.

- In most (except superfamily Cyathocotylides Dubois, 1936) a transverse constriction divides the adult bodies into a flattened or cup-shaped forebody, and a cylindrical or conical hindbody.
- Posterior to the ventral sucker, in both the metacercariae and adults, an additional suctorial, glandular structure develops. This is the tribocytic or adhesive organ. It is a variable structure in different genera. For example, in the genera Cyathocotyle and Diplostomum, it takes the form of a comparatively simple, circular chamber in the ventral body wall; while in the genera Strigea, Apatemon and Cotylurus, it is highly modified to form two lobed tongue-like flaps of tissue.
- The genital pore of the adult is posteriorly situated.

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* The classification adopted in the present study is that of Dubois (1953).

- In addition to the protonephridial or flame cell system, a reserve bladder system is present in both the metacercaria and adult.

Cotylurus variegatus Creplin, 1825 (Odening, 1969) is a strigeid trematode. The hosts for the adult stage of this parasite are Larus argentatus and L. delewarensis (Pont.). In previous years, a large mortality of gulls in Southern Ontario has been recorded. The mortality factor has been attributed to infections of C. variegatus (Jensen, personal communication). The tetracotylid stage has been described by Hughes (1928) as Tetracotyle diminuta from Perca flavescens (Mitchill). Experimental studies to determine the complete life-cycle of C. variegatus have been unsuccessful.

As in all other tetracotylids, all the organs of attachment to be found in the Strigeida are present in the forebody of C. variegatus - oral sucker, paired lateral lappets or pseudosuckers, ventral sucker, and the tribocytic or adhesive organ. The adults are attached firmly by the forebody to the villi of the large intestine of the previously mentioned species of Larus. Often, the worms are fixed so firmly that the villi themselves are removed when the parasites are detached. Attachment to the mucosa involves the participation of the complex attachment organs, as well as the fore-body conformation. In addition to providing a permanent adhesion, many of the attachment organs may be of great importance in the acquisition of nutrients.

Cheng (1964) has recently commented upon the relative importance of a firm attachment to the intestinal flatworms and he states that "within their hosts, the parasites must remain permanently and strongly attached so that they can not be sloughed off with the chyme."

In the past, the published works have dealt mainly with descriptions on the size and morphology of the parasites' body, attachment and reproductive organs. These descriptions were included to provide bases for the taxonomic identification of new species. In a few of these papers, some hypotheses were presented concerning the functions of the attachment organs. These early theories have recently been reviewed by several writers, e.g. Erasmus and Ohman (1963) reviewed the early theories on the nature and function of the adhesive organ; Lee (1962) and Ohman (1965), the lappets. In all cases the descriptions and hypotheses were based on the examination of either the metacercariae or the adults fixed in situ.

During the late 1950's and early 1960's, attention was focused on determining the chemical composition of the parasites, as well as the nature of the parasites' secretions. Using histochemical methods, the chemical composition of the trematode tegument was described (see Lee, 1966, for review). Studies on the nature of the secretions revealed that many of the attachment organs secreted various phosphatases and proteolytic enzymes. Based on these results, additional hypotheses concerning the

functions of the attachment organs were presented (see Bogitsh, 1966, and Ohman, 1966, for review).

In the 1960's, the electron microscope was applied to the study of digenetic trematodes. Following the first report of the ultrastructure of the tegument by Senft et al. (1961) came numerous electron microscope studies on the tegument of many species of digenetic trematodes (see Lee, 1966, for review). In addition, a few papers also dealt with the ultrastructure of the attachment organs and related the processes involved in the manufacture and release of the parasite secretions (see Erasmus, 1969a,b, for review).

In all the studies which have dealt with the structure and function of the attachment organs, little or no attention has been paid to the sequence of attachment by a developing metacercaria as it attaches to its host. This aspect of parasite development includes such basic questions as: What are the growth patterns of the attachment organs? Is the growth of an organ related to its function? At what stage, and for what duration of time does an organ function? Answers to these questions are important in providing a full understanding of the nature of completed attachment.

The present work is the first report describing the sequence of events during the attachment process of C. variegatus.

This study, therefore, is two-fold in its purpose, that is, demonstration of the mode of attachment in the developing metacercariae, and a morphological description of completed attachment. In addition, comparisons will be made between the attachment of C. variegatus and those reported previously in other strigeid trematodes.

II. MATERIALS AND METHODS

1. Collection of Materials

(i) Source and Maintenance of Definitive Hosts

The gradual sequence of attachment organ development of Cotylurus variegatus was studied by means of experimental infections. Ring-billed gulls, Larus delewarensis, the natural hosts for this species of strigeid trematode, were utilized as experimental hosts. Eggs were gathered from partially incubated clutches collected from Mohawk Island, near Dunnville, Ontario, and were transferred in large thermal cartons containing warm absorbant material* (Pl. 1, fig. 1). Incubation was completed in a Fisher Isotemp Incubator at 37.5°C.

The young gulls were brooded in cardboard boxes heated with 60 watt light bulbs, and fed canned fish** mixed with water. When the birds were able to feed themselves, they were moved to pens and maintained under continuous daylight conditions. Their diet consisted of only the cat food, and they were given free access to city-supplied water. In this manner, it was assured that the

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* "Eatonia Pet Litter"
** "Puss and Boots Cat Food"

birds were parasite-free before the experimental infections were conducted.

(ii) Source and Collection of Cotylurus variegatus

When the birds were 6 to 8 weeks old, experimental infections commenced. Material for these infections was obtained from frames (filleted carcasses) of yellow perch, Perca flavenscens, Mitchell, supplied by Mr. Steve Powell, a fisherman at Port Maitland, Ontario. The frames were transported in a large metal container to the university. Fresh samples of perch frames were obtained for each series of experiments. The material was always examined within a few hours of delivery, during which time it was stored at 8°C.

The parasites were recovered as encysted metacercariae attached to the endothelial linings surrounding the heart, the closely associated arteries and veins and in the connective tissue surrounding the swim bladder. The majority were collected from the connective tissue of the swim bladder. Of the samples of fish examined 90-100% were infected with this cotylurid parasite in numbers varying from 10 to more than 100.

Using iris forceps, cysts were easily collected by pulling the swim bladder from the remainder of the fish carcass. The bladder and attached cysts were immediately placed in a 0.85% NaCl solution and stored at 8°C.

To check the infectivity of the encysted metacercariae, samples of 10-12 cysts were placed in a dish containing chilled 0.85% NaCl solution. The cysts were then broken open with dissecting needles and the newly released parasites were examined under a dissecting microscope. Of the released metacercariae, 70-80% displayed active body movements (rapid contractions and elongations) and were considered viable.

2. Experimental Infections of Definitive Hosts

Each experimental gull was starved for 24 hours before administration of 200-300 cysts *per os*. The infected birds were closely watched for 10-15 minutes following feeding to ascertain whether regurgitation of the infective food occurred. When, on occasion, this material was regurgitated it was immediately re-fed to the bird. Using the above procedure it was possible to obtain infections in 100% of the experimental birds.

Following infection, the subsequent period of observation and tagging with metal bands for identification purposes, the infected animals were allowed access to their normal diet of cat food.

3. Collection and Preservation of Developing Metacercariae

The infected birds were maintained as described until the infection had attained the desired duration of

development. The gulls were then sacrificed by heart puncture or by fumigation in ether vapours. The birds were immediately necropsied and their intestines removed and placed in chilled 0.85% saline solution. With the early stages of parasite development, sections of the intestine were cut open with the dissecting scissors, and examined under a dissecting microscope. The worms found free within the intestine were removed with a glass pipette and placed (without pressure of a cover glass) in one of the two general fixatives used - either chilled 10% buffered formalin or Zenker's Fluid (Appendix I).

After longer periods of development, the worms were found attached to various portions of the intestine. These were removed by excising sections of the intestine with attached parasites. This material was also fixed in one of the two fixatives used. Autopsy reports were maintained for each animal examined indicating the locations of the parasites within the host's digestive tract.

Specimens of developing metacercariae from the time of introduction to 6 days post infection were obtained and preserved. The greatest number of specimens were taken during the early period of development, i.e. 2 hours-24 hours, as preliminary studies showed a rapid parasite development in relation to attachment at this time. The periods of development studied were as follows: 0 hours (metacercariae removed from cysts), 2 hrs., 4 hrs., 8 hrs.,

18 hrs., 20 hrs., 24 hrs., 2 days, 3 days, 4 days, 5 days, 6 days.

During the preliminary studies an additional fixative, Bouin's (Appendix I) was tried in an attempt to obtain good preservation of body morphology; however, 10% formalin or Zenkers gave the best results. In addition and particularly for the early developmental stages, some recovery samples were too small to segregate into 3 fixatives and still permit a sufficient sample for comparative measurement analysis. For these reasons 10% formalin and Zenkers' fixatives were used exclusively in subsequent experiments.

Following fixation and washing, the tissues were dehydrated in ascending concentrations of ethanol and finally stored in 70% ethanol for further treatment.

4. Measurement Analysis of Preserved Specimens

To investigate the growth of the body regions and attachment organs in the developing metacercariae, the fixed specimens which had been stored in 70% alcohol were used, and whole mounts were prepared as follows:

(1) Over-stained in an aceto-carmine solution (1 part stain:20 parts 70% alcohol). The specimens remained in this mixture overnight while being slowly rotated on a Multi-purpose Rotator (Model 150).

(2) The parasites were washed several times with 70%

alcohol and then,

(3) Differentiated in a solution of 1 part HCl:80 parts 70% alcohol. The time required varied, depending on the size of the worms and the length of time used for staining. A sample was left in this acid solution until the parasites appeared light-pink in colour.

(4) Washed with 70% alcohol.

(5) Dehydrated in an alcohol series (80%, 95% and absolute ethanol).

(6) Cleared in clove oil*. Early studies showed that dehydrated samples placed in pure clove oil underwent extensive shrinkage. To overcome this problem, the tissues were placed in small dishes containing absolute alcohol. To this was added increasing amounts of clove oil over a 48 hour period. Eventually the alcohol was allowed to evaporate, leaving the tissues cleared in pure clove oil. It was found that the best results were obtained from samples which had remained in the clove oil for at least one week. Therefore, all material prepared in this fashion was allowed to clear in the clove oil for at least a week (maximum 2 weeks).

(7) Mounted onto glass slides with Permount** and allowed to air dry for at least two weeks before examining.

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* Fisher Scientific Company
** Harleco Synthetic Resin - Canadian Laboratory Supplies

It is important to point out, at this time, that the above staining procedures were followed for all the specimens examined. Up to the end of 3 days of development, this staining procedure provided specimens suitable for the measurements of attachment organs and body sizes. In the interval 4-6 days, however, the specimens were developed to such an extent that the thick body wall obscured the attachment organs and rendered accurate observations and measurements impossible. In an attempt to overcome this problem, the techniques were modified:

(a) in addition to clearing the specimens in clove oil, terpineol* was tried,

(b) some samples were stained with Mayer's haemalum (Dowry and Wallington, 1967) and cleared in either clove oil or terpineol.

None of the above modifications provided suitable samples for measuring the attachment organs. Consequently measurements of only the body sizes were taken of specimens 4-6 days old. The procedure for these measurements was to place unstained worms in a deep-welled glass slide filled with 70% alcohol.

All measurements were taken under light microscopy using a Leitz Wetzlar Microscope with an occular micrometer. Conversions and calculations were performed on an Olivetti

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* Fisher Scientific Company

Underwood (Model 101) desk computer. Analyses of standard deviation and standard error were applied to the measurements of the body and attachment organs from the different periods of development, using the measurement of length as an indicator. The analyses were performed to determine whether the measurements obtained revealed differential growth or changes in relationships of parts during the period of development.

5. Preparation of Parasites for Light Microscopic Studies

To investigate the morphology of the developing metacercariae whole mounts of specimens 0 to 3 days post infection were examined. These specimens were prepared as outlined in the previous procedure for the measurement analyses. In addition, histological sectioning was performed on preserved metacercariae obtained from 18 hours to 6 days of age.

The material stored in 70% alcohol, was dehydrated, cleared in Terpineol and embedded in Tissuemat (M.P. 52°C). Sections were cut at 5 - 10μ thickness, mounted onto glass slides with egg albumin and routinely stained with Harris's haematoxylin and Eosin technique (Appendix II). Staining with haematoxylin and eosin was found to be adequate to allow examination of the developing metacercariae and was used exclusively for all tissues sectioned. During microscopic examination photomicrographs were taken using a

Leica (Model MDa) 35 mm camera.

6. Preparation of Parasites for Electron Microscopy

Tissues for electron microscopic studies were selected from samples of developing parasites, and included the whole body of the smallest worms and samples of the anterior and posterior bodies of the large worms. Included in the samples taken from the large worms were portions of the anterior and posterior body teguments, the attachment organs and associated adhering host tissues.

Samples of the specimens were cut into small pieces and fixed in freshly prepared 3.5% glutaraldehyde buffered to pH 7.4 with Sorensen's phosphate buffer (Appendix III) for 2-3 hours at 0-4°C in a bath of crushed ice. In this fixative, the tissue was further cut into small blocks 1 mm^3 with a sharp razorblade. Placing the samples in this fixative hardened the tissues sufficiently to allow clean dissection into the small sized blocks.

Following fixation, the tissues were washed several times in the cold Sorensen's phosphate buffer, and then postosmicated at 0-4°C in 2% osmium tetroxide (Palade, 1952) buffered to pH 7.4 with modified veronal-acetate buffer containing 4.50 sucrose/100 ml solution (Appendix IV). The tissues remained in this solution until they turned black (approximately 2 hours).

After fixation,all specimens were washed with cold 50% alcohol,and then quickly dehydrated with 70% and 95% alcohol for 15 minutes each (including 2 changes). Dehydration was completed in absolute alcohol at 0°C for 20 minutes (2 changes). The material was then transferred to 0°C propylene oxide for 20 minutes (2 changes). After the second change the material was allowed to warm up to room temperature.

The tissues were then infiltrated with mixtures of propylene oxide and Epon 812 (Appendix V). Four hours was required to complete the infiltration. During this time the tissues and solutions were slowly rotated on an inclined turntable to aid impregnation.

The tissue was transferred, using small, sharpened wooden sticks, to a capsule containing three large drops of Epon embeddent. The gelatin capsules (#0.00)* had previously been labelled with India Ink. The block of tissue was allowed to settle to the bottom and oriented as necessary with a sharp stick, filled with Epon and heat polymerized. Complete polymerization was obtained at 62°C for 24 hours and curing at room temperature for a minimum of 4-5 days.

Glass strips (400 mm long X 25 mm wide X 6.35 mm thick) were prepared as glass knives using an LKB (Type

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* Parke, Davis and Company

7800B) knifemaker. A small water reservoir was constructed onto the back of the knife. Black electrical tape was originally used in production of reservoirs, but this was later found to give off oil-droplets. To overcome this problem, the electrical tape was replaced by Scotch Brand masking tape. The tape was sealed to the glass with bee's wax.

Sections were cut on a Reichert (Type OMU-2) ultramicrotome and floated onto double distilled water contained in the reservoir.

Silver and grey sections, representing thicknesses of 60-90 and <60 mμ respectively (Kay, 1965), were picked up on uncoated copper grids* (Athena type: 200 mesh). Blue and purple sections, representing relatively thick cuts (190 - 150 mμ loc. cit.) were picked up with a small wire loop. These thick sections were placed on a clean glass slide, (heat) dried, cooled and stained with Toluidine Blue (Appendix VI). Material prepared in this way was examined on a phase contrast microscope and provided information concerning the orientation of thin sections to be examined.

Sections prepared for electron microscopy were stained to increase the contrast using uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

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* Ladd Research Industries, Inc.

This material was examined in a Zeiss EM-9A electron microscope. Electron micrographs were taken at magnifications of 1800X to 18,000X and subsequently enlarged photographically as required.

RESULTS

1. The Destination and Behaviour of the Metacercariae in vivo

A number of experiments were performed to investigate the destination and behaviour of the developing parasites during the first 216 hours after entry into the host. The results of the autopsies carried out after various time intervals are summarized in Table 1.

From these results the following information was obtained:

(a) Not all of the ingested cysts are capable of establishing an infection. Many pass through the gut or die. Some may die in the gizzard.

(b) Two hours following infection, the metacercariae were recovered from the duodenum, e.g. Expt. I, Bird 1. Lysis and escape from the cyst wall had previously been accomplished as the metacercariae were recovered moving actively outside their cysts.

(c) Though excystation is completed at the end of two hours, the first parasites were not recovered from the caecal region of the large intestine until 18 hours after ingestion, e.g. Expt. I, Bird 4.

(d) Between 2 and 24 hours, the metacercariae slowly moved

down the intestine, and as early as 18 hours, juveniles were observed attached to the intestinal mucosa.

(e) From 20 hours to the end of the study period, immature adults and adults were always recovered permanently attached to the large intestine. Only in the cases of severe infections were parasites located in the anterior portions of the large intestine. Otherwise, this region and the remainder of the digestive tract were normally devoid of any permanent infection.

(f) Parasite eggs were recovered from the intestinal lumen 96 hours after infection and are, in this study, considered as an indicator of parasite sexual maturity.

2. Anatomy of the Normal Gull Intestine

As the large intestine of the gull is the natural habitat for the developing metacercariae and adult stage of this species of strigeid trematode, it is necessary to consider briefly its structure.

Portions of the uninfected or normal intestinal morphology are shown in Pl. 2, figs. 1 and 2. The mucous membrane lines the intestine and consists of a simple columnar epithelium interspersed with unicellular glands - the goblet cells which secrete mucus. These mucous cells not only border on the lumen of the intestine but extend into the glands of Lieberkuhn. The epithelium is further modified for absorptive functions as evidenced by the so-

called striated border of light microscopy, which in the electron microscope appears as numerous microvilli extending from the distal end of the epithelial cells (Pl. 2, fig. 3).

The epithelium rests on a lamina propria which contains many blood and lymphatic capillaries and gives the appearance of being loosely consolidated. The villi vary in size and shape depending on the portion of the intestine examined. In the large intestine they are broad and somewhat tongue shaped; those of the small intestine are long and finger-like in shape.

The intestinal glands are well developed, unbranched tubules lined with columnar epithelium. The muscularis is very poorly developed and appears to exist of only a few irregularly arranged muscle fibers. Externally the intestinal wall consists of a regularly arranged lamina muscularis - a well developed band of inner circular muscle and a thin, poorly developed longitudinal layer. This description corresponds with Calhoun's (1933) and Farner's (1960) general descriptions of the avian intestine.

3. Pathology and General Observations re Attachment

Examination of the birds' intestines, 2, 4, 6 and 8 hours post infection revealed excysted metacercariae at successively greater distances from the duodenum towards the small intestine. During this period, the parasites were located on the surface of the thick layer of mucus covering

the intestinal mucosa. It was only after 18 hours, that attachment to the intestine occurred. The developing adults adhered, by means of their attachment organs, to the mucosa below the layer of mucus. During the initial phases of attachment, which occurred between 0 - 24 hours, adhesion was not very firm and individuals could be easily loosened from their sites. After 24 hours, adhesion was very firm, and it was impossible to free them without tearing away from the intestine the plug of host tissue buried within the forebody.

During the early phases of establishment, no changes were noted in the external appearance of the host intestine. In severe infections of longer duration, however, a marked change was observed. A heavily infected large intestine became evident as soon as the abdominal cavity was opened and the organs displaced (Pl. 1, fig. 2). In one example (Table 1, Expt. V, Bird 4) the intestine was five times its normal diameter. In addition to the enlarged intestine, the surrounding blood vessels were greatly distended (Pl. 1, fig. 3). Opening the intestine revealed closely massed parasites almost obscuring all of the host tissue (Pl. 1, figs. 4 and 5). When the trematodes were present in large numbers the normally fluid chyme in this region of the intestine was replaced by a large solid core of dehydrated chyme located just anterior to the site of infection (Table 1, Expt. V, Bird 3 and 4).

The parasite infections were always concentrated along 10 - 20 cm of the large intestine, in the area of the openings to the two caeca. Only the hind-bodies of the parasites were visible projecting into the lumen of the gut (Pl. 1, fig. 5). The forebodies, upon closer examination, were seen deeply embedded in the mucosa between the villi. Normally, a pale yellow mucus of host origin partially concealed the fore- and hindbodies of the adult parasites.

The normal columnar epithelium did not undergo any obvious type of alteration beyond the area enclosed within the forebody. Even epithelium in contact with the outer teguments of the fore- and hindbodies remained normal. The capillaries in the villi, to which the parasites were attached, were greatly enlarged. In the early stages of attachment (18-24 hours) a few free host erythrocytes were seen within the cavity of the forebody cup and within the parasite's digestive tract. In the older, firmly attached specimens, host cells were also observed within the parasite. In addition an amorphous eosinophilic material probably of host origin was also found in the parasite digestive tract.

In addition to the enlargement of the capillaries, the normal size and shape of the villus underwent many changes concomitant with the increase in size of the parasite (Pl. 1, fig. 6).

4. General Observations re Body Form and Structure

(i) Body Form

(a) Metacercariae

Metacercariae, removed from their cysts and prepared as whole mounts, were somewhat oval to circular in outline and were discoid in shape (Pl. 2, fig. 4). It was difficult to discern the hindbody primordia in living specimens, however, this area was evident in stained specimens. This primorida was represented by a small concentration of cells, just posterior to the adhesive organ. Also contained within this primordia were the cells which represented the fundaments of the reproductive apparatus. The forebody, in general, resembles that of other strigeid trematodes in possessing the characteristic attachment organs - namely oral and ventral suckers, the lappets and adhesive organ.

Diag. 1 shows the observed stages of developing metacercariae. At the end of the posterior fourth of the body, the ventral body wall is tucked-in forming a long, broad, dorso-ventrally shallow pocket. Faust (1918) referred to this as the "suctorial pocket". Although it was not apparent to any extent in the excysted metacercariae (Pl. 2, fig. 4), it was evident in worms following 2 - 4 hours of development (Diag. 1). The dorsal surface of the body is convex, and the ventral surface decidedly concave. The

ventrally curving anterior rim of this suctorial or forebody cup arises on either side of the forebody adjacent to the lappets and meets in the mid ventral plane posterior to the adhesive organ. In the living specimens it was capable of considerable elongation and contraction. In the younger worms, however, it was usually fixed just anterior to the anterior margin of the ventral sucker (e.g. Pl. 3, fig. 2). In its growth towards the anterior end, it has reached the level of the anterior tip of the forebody in the 24 - 48 hour specimens (Pl. 3, figs. 3 and 4). The ventral-anterior rim is very thin and leaf-like in appearance and the width of its transverse external opening is more than half the width of the parasite. Within this concavity lie the acetabulum and adhesive organs.

(b) Adult

The adult body (Diag. 2) is divided by a transverse constriction into two parts - a cup-shaped forebody containing the attachment organs and a cylindrical hindbody. The body is usually flexed at the junction of the body regions so that it appears roughly L-shaped with the forebody representing the short arm of the L. The forebody seems to be attached to the anterior dorsal side of the hindbody, and when observed <u>in situ</u> the parasite lies with the hindbody projecting into the gut lumen or lying on the surface mucosa.

The deeply cup-shaped forebody lies with the margin of the cup tightly contracted against the projecting lobes of the adhesive organ (Pl. 7, fig. 2; Pl. 16, fig. 1). Between these lobes a mass of host tissue has been drawn. The anterior end of the forebody is sometimes notched where the oral sucker is turned ventral and posterioral. The ventral surface is usually marked by two fairly large inflated areas which are located to the right and left of the median line.

(ii) Body Structure

(a) Light Microscopy

A description of the general body tegument is described in order that a comparison can be made with the specialized morphology of tegument covering the various attachment organs. In addition, the excretory system is briefly outlined in order that a complete understanding of its proposed functions can be discussed.

The forebody of the adults and metacercariae is provided with a relatively thick (2 - 6 μ) tegument. Little differentiation of the tegument can be made under the light microscope (Pl. 9, fig. 2; Pl. 16, fig. 2). The outer surface is thrown into many small folds. Numerous small spines are located on the dorsal body surface. Immediately below the outer surface is a basement membrane (Diag. 3) separating the tegument from the underlying region of muscles.

The muscular system consists of fine outer circular fibers and inner stout longitudinal fibers. Parenchymal tissue lines the inner muscle fibers. In this tissue are numerous large pear-shaped cells which possess large rounded nuclei and contain a refractile granular cytoplasm. Their distal ends project into long thin processes which run towards the tegument.

As in all strigeids, these parasites contain an excretory system which can be divided into two parts:

(1) the primary excretory system consisting of flame cells, their tubules, the larger collecting tubules and finally the excretory bladder,

(2) what has been termed a reserve bladder system, which in the metacercariae consists essentially of a series of large interconnected spaces lying immediately below the whole of the dorsal lateral surfaces of the body.

These excretory lacunae are separated at least partially by numerous traversing delicate strands of tissue which stretch from the subcuticular parenchyma to the main body parenchyma. These strands, themselves appear to be purely parenchymatous, their fibrous matrix containing a few, small, oval, nucleated cells. In living specimens, these excretory lacunae contain a transparent fluid which surges from one part of the body to another in response to the muscular movements.

In the hindbody of adults, a similar set of spaces are present consisting of an intricate network of very large lacunae which surround the organs found in the hindbody. In living specimens, the fluid from the forebody flows from the main trunks of the forebody into the lacunae of the hindbody. Eventually, the fluid empties into the primary excretory system which in turn leads through an excretory pore located in the posterior end of the hindbody.

(b) Electron Microscopy

The general tegument of the forebody shows the basic structure of the cuticle and can be divided into three layers. A thin membrane system on the outer and inner surface and lying between these two membrane systems is a cytoplasmic layer containing many vesicles and few mitochondria (Pl. 4, fig. 1).

The external surface of the tegument is thrown into numerous primary lobes which are in turn divided into 2 - 4 secondary lobes. The external covering is continuous over the surface and exhibits the trilaminate appearance of a plasma membrane. Exterior to the plasma membrane is an extraneous coat consisting of an irregular, medium-dense staining material composed of a parasite secreted mucoid material (Pl. 4, fig. 1) (Lee, 1966).

The main body of the tegument (Pl. 4, fig. 1; Pl. 5, fig. 1) is approximately 2 - 6 μ wide and consists of a

granular cytoplasmic matrix. The matrix is of medium electron density and contains a variety of structures. In the outer two-thirds of the tegument cytoplasm, two types of vesicles are seen:

(1) long and slender rod-shaped vesicles, bounded by a membrane system. They contain material which is more electron dense than that of the tegumentary matrix,

(2) membrane-bound, spherical vesicles. These are fewer in numbers than the rod-shaped type.

In addition, a few mitochondria are present near the base of the matrix, also scattered throughout the cytoplasm are many lipid droplets. No nuclei, lateral membranous partitions, Golgi complexes or endoplasmic reticulum are seen.

The inner surface of the tegument is limited by a plasma membrane. At irregular intervals the membrane is elevated into numerous tubular invaginations which may be long and project as irregular paths into the cytoplasm of the tegument.

A subtegumentary basement layer lies below the basal plasma membrane and rests atop a narrow band of non-striated circular muscle fibers. The basement layer consists of a lamina of loosely arranged fine fibrils and corresponds to the basement membrane of light microscopy. The basement layer is complex in its structure and closely resembles that described for triclad tubellarians (Pederson, 1961). This

fibrous layer determines the limitation of the body shape during movement and provides anchorage for the muscles (Erasmus, 1967b). The layer of circular muscles in turn lie superficial to bundles of smooth muscle fibers oriented parallel to the long axis of the forebody. A third set of muscles radiate from the basal area and extend to the opposite body wall. These muscle systems are membrane bound and contain numerous mitochondria and membranous structures.

Flask-shaped cells lie deep in the body parenchyma. They may be found singularly or in clustered groups. These cells contain a large nucleus, and in the perinuclear cytoplasm are many mitochondria, a Golgi apparatus and numbers of small dense bodies which resemble those found in the matrix of the tegument (Pl. 5, figs. 2 and 3). These cells possess tapering apical extensions of their cell membranes which extend between the muscle layers, through the fibrillar network of the subtegumentary basement layer to the base of the tegument. At these points, the subtegumentary cell membranes and basal (tegumentary) plasma membranes are continuous, and the cytoplasm of the subtegumentary cells merges with the tegumentary matrix. Numerous mitochondria and vesicles are present in the apical processes of the subtegumentary processes. It appears that the vesicles are synthesized within the ribosomes of the endoplasmic reticulum, collected and concentrated in the Golgi vesicles, move through the apical processes and are

eventually deposited in the tegumentary matrix. This interpretation agrees with other investigations (see review by Lee, 1966).

Between the primary lobes, spines occasionally project from the tegument which lines that portion of the forebody within the anterior margin of the suctorial cup (Pl. 6, fig. 1). The spines are deltoid in shape with a thickened basal plate lying close to the basal plasma membrane of the tegument. Their structure appears morphologically similar to that described by Burton (1964). In addition, a number of vesicles differing from those previously described for the general body tegument are found. They are large, double membrane-bound and are either filled with a granular material, or are almost devoid of content. They are believed to be involved in an absorptive process (Bogitsh and Aldridge, 1967) and are considered in greater detail in the DISCUSSION.

5. General Observations on Body Development

(i) Body Development up to 3 Days Post-Infection

The results of the studies of the growth and development of the metacercariae are described in order to ascertain the growth patterns of the parasites. This information will subsequently be correlated with the growth patterns described for the attachment organs.

Table 2 contains the measurements of the general

body dimensions of developing metacercariae from the time of infection to 3 days of age. These results deal with parasites which had been fixed in 10% buffered formalin. The data from Table 2 is presented in Figs. 1 to 4. Included with the measurements plotted on these graphs are bars representing ±2 standard errors.

Figs. 1 to 4 are plots of the developing body size versus the time of development. Time zero represents the measurements of the metacercariae removed from the cysts found in the perch. At this time, the metacercarial total body length (TBL) was 0.288 ± 0.016 mm and its width (TBW) was 0.273 ± 0.014 mm. These measurements also represent the dimensions for the anterior body length (ABL) and anterior body width (ABW). Measurements for the posterior body length (PBL) and width (PBW) are lacking, since this portion of the body does not yet exist as a distinct entity. The small area of cells representing the primordia of the posterior body cannot be measured with any accuracy because of the extreme variation exhibited in its size and shape.

At the end of 24 hours, the total body sizes have increased to 0.411 ± 0.022 mm long and 0.303 ± 0.019 mm wide. During this time interval (0 - 24 hours) growth has not been continuous. Figs. 3 and 4 show these changes in greater detail than Figs. 1 and 2. Up to 24 hours of development the parasites have: (a) decreased in body size, (b) regained this decrease, and (c) continued to enlarge

to a size which is greater than at time 0. The maximum decrease in body size was observed 4 - 8 hours post infection. The overall body length decreased from 0.288 to 0.181 mm or a reduction of approximately 38%; whereas the width dropped from 0.273 to 0.206 mm or approximately 26%.

After 8 hours, the body sizes increased, and between 18 - 20 hours, the TBL and TBW measurements equalled the measurements recorded for the metacercariae at time 0. Between 8 and 24 hours, the TBL increased 101% and TBW 46%. Of this total increase, 80% of the TBL and 75% of TBW occurred between 18 - 24 hours. Not only does the ABL decrease during the first 4-8 hours of development, but a proportionate decrease is observed for the PBL. These two measurements combined give the value for TBL. Associated with the values for PBL is a large standard error. Up to 8 hours post infection, development of this body region is just beginning. The values obtained for 2 and 4 hours represent only those members of the population which showed measurable development of the PBL, and while the averages indicate a decrease for PBL, considering the standard deviation, it is likely that the growth picture is actually one which tends to gradually increase.

A similar phenomenon is observed for the recordings of posterior body width (PBW). However, this value does not influence total body width (TBW), since this parameter is based on the width at the widest point, which at this stage

of development is the anterior body width (ABW).

Figs. 1 and 2 show the body growth from 24 hours to 72 hours, whence the process of attachment is completed. In the interval 1 - 2 days, the TBL has increased to 0.503 ± 0.031 mm. A substantial proportion of this increase is contributed by the PBL. For one unit increase in ABL, there is approximately ten units increase in PBL. The TBW as a result of growth of the ABW,has increased to 0.437 ± 0.039 mm from 0.303 ± 0.019 mm ≈ 30%. During this time, the PBW has increased from 0.125 ± 0.007 mm to 0.230 ± 0.013 mm or 41%.

In the interval 2 - 3 days, there is a rapid increase in TBL (0.503 ± 0.031 mm to 0.733 ± 0.036 mm), which has resulted from the increase of 129% growth of the PBL and only 3% growth of ABL. In fact, the PBL has now exceeded the size of the PBW. The TBW, resulting from the growth of the ABW has increased only slightly (0.437 ± 0.039 mm to 0.470 ± 0.019 mm or 8%). The PBW, in contrast, increased from 0.230 ± 0.13 mm to 0.329 ± 0.020 mm or 43% from the size at 2 days.

(ii) General Relationships of Body Components Up to End of 3 Days Post Infection

Fig. 5 shows the relationships of the two components, ABL and PBL which together determine TBL. The data represents only the development from 0 to 72 hours. Up

until 4 hours of development the ABL constitutes almost all of the TBL. After 4 hours, or when the TBL is greater than 0.40 mm, the contribution of ABL to TBL continues, but is eventually surpassed by the rapidly growing PBL.

As far as TBW is concerned (fig. 6), the ABW is always the measurement of maximum width, but after 4 hours the PBW increases with time and approaches the width of the forebody.

Table 3 shows the calculated values for the comparisons of body components up to 3 days of development. These results are plotted in Fig. 7.

Within the first 4 hours of development, the ratio $\frac{ABL}{PBL}$ dropped from 4.14 to 2.98, while at the end of 8 hours it increased to 3.88. As stated previously, the values for PBL and PBW were difficult to measure. Members of the sample were in varying stages of posterior body development. Considering the large standard error associated with the values, emphasis will be placed only on the ratios following 8 hours of development as these measurements were more homogeneous with a smaller standard error.

From 8 hours to 3 days, the value obtained for $\frac{ABL}{PBL}$ constantly decreased in size, in fact, it dropped from 3.15 to 0.87. This resulted from a rapid increase in the size of the posterior region.

In the interval 8 - 24 hours, the ratio $\frac{ABW}{PBW}$ increased in magnitude, from a low of 1.83 to a high of 2.41. This was

due to the rapid growth observed in the anterior body region during this time. From 24 hours to 3 days, the value decreased to 1.43 as a result of expansion in the posterior body.

The relationship of $\frac{ABL}{ABW}$ remains fairly constant up to the end of 3 days development. There is, however, a slight increase between 24 hours and 2 days (0.92 - 1.24) which may reflect either a change in shape (i.e., a longitudinal lengthening at the expense of the width) or an actual increase in the length of the anterior region. Looking at the data for the growth measurements of ABL and ABW, the increase of their ratio reflects an actual growth increase of the anterior region.

Comparing $\frac{PBL}{PBW}$, we can see that this value increases up to the end of 3 days. This results from a rapid development in the posterior body region.

The values obtained for the ratios $\frac{ABL}{PBL}$ (0.867), $\frac{ABW}{PBW}$ (1.431), $\frac{ABL}{ABW}$ (1.284), and $\frac{PBL}{PBW}$ (1.315), are approximately equal at the end of 3 days; although, the ratio $\frac{ABL}{PBL}$ is somewhat lower than the other three. This reflects the observations which can be made from looking at the measurements obtained for the individual components. Comparing ABL and ABW, we see that up to 3 days of development, ABL < ABW, after 3 days ABW < ABL. A similar change takes place when comparing PBL and PBW.

(iii) Body Development Following 3 Days Post Infection

At the end of 3 days post infection, the attachment process is generally completed. The following observations deal with the parasite's development from this stage up to 6 days post infection. Table 4 and Figs 8 and 9 present the measurements of growing parasites from 2 - 6 days. The initial 2 - 3 days has been included to lend continuity to the average measurements of growing parasites following 3 days.

At the end of 6 days growth the TBL is 4.913 ± 0.371 mm; the components, ABL and PBL are each 1.402 ± 0.128 mm and 3.511 ± 0.302 mm respectively. The amount contributed by PBL is 29% and ABL 72% to the TBL. The TBW (1.688 ± 0.109 mm); ABW (1.608 ± 0.076 mm); and PBW (1.549 ± 0.152 mm) are all essentially the same; the PBW equalling the ABW at the end of 3 days development.

In the development period before attachment is completed (i.e., 8 hours to 3 days) the TBL increased by 264%, following the completion of attachment (i.e., 3 to 6 days) the TBL increased by 570%. Similarly the TBW before and after attachment increased by 127% and 259%. Considering the time interval measured before and after attachment to be approximately the same (72 hours before vs 64 hours post attachment) the growth following attachment has been increased by a factor of two times over the observed growth prior to attachment.

Growth is continuous during the time interval measured, and continuous up to 10 days of development. Willey (1941) studying the growth of Zygocotyle lunata and Ulmer (1951a) Postharmostomum helicis have both reported that growth is continuous up to the time of the parasite's death and it is possibly that a similar growth occurs in C. variegatus.

(iv) General Relationships of Body Components Following 3 Days Post Infection

Fig. 10 compares the relationships of ABL and PBL to TBL from 3 to 6 days of development. As growth proceeds the proportion contributed by the rapidly growing PBL to TBL exceeds that contribution of the more slowly growing ABL.

In Fig. 11 the relationship of ABW and PBW to TBW are compared. While the ABW constitutes the maximum width of the developing parasites, growth of the PBW is continuous and ultimately the width dimension of this part approximates that of the anterior body.

The various ratios of body components from 3 to 6 days are presented in Table 5 and Fig. 12. Values obtained prior to attachment (i.e. 0 - 3 days) have been included with the graph to lend continuity to averaged ratios following 3 days of development.

Generally, all the ratios appear to level off at constant values after 4 days development. This indicates that the relationships between any of the two components considered remains constant; however, one cannot conclude from this that body growth has ceased. Rather, the levelling off of the values implies only that the relationships remain constant, even though various body components continue to grow, e.g. consult ratio $\frac{ABL}{ABW}$. While this ratio remains constant ($\sim$0.8), looking at the values for the components, one sees that ABL increased from 0.86 to 1.40 and ABW increased from 1.07 to 1.61; in this case both have continued to grow 0.54 mm.

6. Structures Involved in Attachment

(i) Oral Sucker

(a) Growth

Table 6 and Figs. 15 to 20 contain the measurements obtained from the attachment organs of developing metacercariae.

In the juvenile worms, the oral sucker is somewhat oval in shape; its width being 0.004 mm greater than its length. During the first 4 hours of development following infection, this organ decreases in size in both the length and width dimensions (Table 6, figs. 15 and 16). Originally being 0.051 ± 0.002 mm long and 0.055 ± 0.010 mm wide, at

the end of 4 hours it measures 0.043 ± 0.003 mm and 0.049 ± 0.003 mm respectively. This represents a decrease of 16% in length and 13% in width and corresponds to a similar decrease observed for the general body size.

From 8 hours to 3 days, there is a gradual increase in magnitude, and at the end of the measured interval of development the length has increased to 0.085 ± 0.004 mm and the width to 0.084 ± 0.005 mm. This represents an increase of 67% for length and 53% for width from the original size at time 0.

Figs. 17 and 18 show the relationships of the attachment organs compared with the dimensions of the anterior body. With increasing development of the forebody the relationship of OSL (oral sucker length):ABL increases from 0.18 to 0.23. Similarly, as the metacercariae develop the OSW (oral sucker width):ABW decreases from 0.23 to 0.18.

(b) Orientation

In the fixed metacercariae, the oral sucker is in a ventral sub-terminal position,with the mouth located directly ventral (Pl. 2, fig. 4). The oral sucker is located on the summit of a small prominence which lies on the anterior margin of the forebody cup. In living specimens, under cover glass pressure, the prominence may be thrust forward so that the oral sucker appears terminal in position.

The above orientation is retained up to the time when extensive forebody development (in particular development of the forebody lip) occurs (Diag. 3). Around 18-20 hours post infection, the oral sucker, in a majority of specimens is directed posterioral or even dorsal, in addition to the originally described ventral position (Pl. 3, fig. 2; Pl. 15, fig. 2). Thus, we can see that the oral sucker is capable of extensive movement and capable of orienting in several directions.

Newly emerged metacercariae, when placed in a glass petri dish, attached to the glass by means of the oral and ventral suckers. After attaching with the ventral sucker, the forebody was stretched, and the oral sucker attached. Subsequently, the ventral sucker was released, the forebody contracted, resulting in the ventral sucker coming to rest near the point of attachment of the oral sucker. Repeating this sequence, the worms were able to creep across the surface. This process has been observed in young worms during the early hours following infection as they migrated down the intestine.

At 18 - 24 hours post infection, primary attachment was accomplished by many of the developing parasites. In addition to the oral sucker, the lappets were also involved in the formation of an adherence to the host (Pl. 6, fig. 2; Pl. 7; fig. 1).

The portion of host tissue drawn into the mouth was subjected to the abrasive action of the oral sucker (Pl. 8, figs. 1 and 2). This action stripped off the superficial host intestinal epithelium and ruptured the small underlying blood capillaries. The loosened cellular debris and red blood cells were often seen within the parasite's caeca which arise from a short oesophagus immediately below the pharynx.

This primary feeding was observed from worms recovered from the small intestine and also from the first parasites to be recovered from the large intestine (Pl. 7, fig. 1). It is assumed that the attachment and feeding of juveniles within the small intestine may be only a temporary situation,as the results from infections after 24 hours showed the worms matured only in the lower portion of the large intestine.

From 72 hours after infection to the end of the experimental study period, the aforementioned orientation of the oral sucker adhering to host tissue is lost. Extensive growth of the adhesive organ and in particular the anterior lobe, displaces the oral sucker from the host tissue. The anterior lobe lies between the host tissue and oral sucker (Pl. 9, fig. 1). The oral sucker however, is still functional and in many specimens, it was observed to be clamped onto the adhesive organ (Pl. 7, fig. 2; Pl. 9, fig. 2). This orientation remained for the duration of the parasite's life.

(c) Histology

(1) Light Microscopy

The well developed oral sucker is shown in Pl. 9, figs. 1 and 3. This organ may be described as a highly muscular, protrusible cup, surrounding the mouth cavity. It is surrounded by a ring of specially differentiated muscles which consist of several systems of radial and circular fibers. Scattered ganglion cells, not visible with this staining technique, are present among the fibers (Dogiel, 1966).

This organ is limited by a tegument which is thin in comparison with the general body tegument. The tegument lining the orifice is thrown into large folds containing many smaller secondary folds which probably accommodate the muscular expansion and contraction of this organ. There are no spines or hooks present on the oral sucker of this trematode species.

Peripherally, the oral sucker is limited by a thickened basement membrane which separates this organ from the surrounding body parenchymal tissue. Between the muscle fibers small secretory cells with large, deeply staining nuclei containing one or more nucleoli, duct to the inner tegumentary surface.

(2) Electron Microscopy

Lining the lumen of the oral sucker is a tegument which closely resembles the morphology previously described

for the forebody tegument (Pl. 10, fig. 1; Pl. 11, fig. 1). As described for the forebody tegument an external plasma membrane is continuous over the entire surface; however the folds are not as numerous and regular as seen in forebody tegument. Also present is an extraneous granular coat representative of a mucoid substance (Pl. 11, fig. 1). The matrix of the cytoplasm, presence of membrane bound vesicles, and a basal plasma membrane are similar to those of the forebody tegument. The tubular projections of the basal plasma membrane are fewer in number as compared with those previously described for the general body tegument. A zone consisting of irregularly arranged fibers lies between the basal plasma membrane and the well developed bundles of muscular fibers. The complete system of muscles around the lip of the sucker consists of circular muscles and bundles of longitudinal fibers. A similar series of muscular bundles is located around the limiting "wall" of the sucker (Pl. 12, fig. 1). A third system of muscular bundles radiates from the proximal to the distal ends of the oral sucker and are inserted between the bundles of longitudinal fibers. The limiting "wall" of the sucker resembles previously described zones of irregularly arranged fibers.

The muscle systems are membrane bound and associated with them are numerous mitochondria (Pl. 12, fig. 1).

In addition, numerous tegumentary cells similar to those previously described are found. These cells are

located between the bundles of radiating muscles with their nuclei located in the proximal portion of the sucker. Within their cytoplasm, numerous vesicles similar to those of tegument are found, and small processes (not illustrated) connect the cell cytoplasm with that of the tegument, thus establishing the synctial nature of the tegument.

(ii) Ventral Sucker

(a) Growth

In the metacercariae, the ventral sucker is 0.063 ± 0.002 mm long and 0.067 ± 0.002 mm wide (Table 6, figs. 15 and 16). At the end of 3 days growth, this organ has increased to 0.126 ± 0.013 mm by 0.138 ± 0.007 mm; representing a total increase of 100% in length and 106% in width.

In contrast to the oral sucker, this structure did not appear to decrease in size during the early hours following entry into the host. During the first 8 hours of development the only change noted was a slight (0.004 - 0.008 mm) increase in width. Subsequently, the width is greater than the length (0.012 - 0.033 mm).

In relation to the size of the anterior body (Figs. 17 and 18), the VSL (ventral sucker length):ABL is 0.22 in the small parasites up to 24 hours; while after this time the proportion decreases to 0.134 at the end of 72 hours. Correspondingly, the VSW (ventral sucker width):ABW

increases from 0.21 to 0.29.

(b) Orientation

The acetabolum or ventral sucker of the metacercariae is located on the ventral surface in a small, rounded,median prominence (Pl. 2, fig. 4; Pl. 13, fig. 1). This prominence is found immediately in front of the adhesive organ,in the anterior part of the posterior half of the body. In fixed specimens,its cavity appears quite large and deep, and is usually obscured by the anterior lobe of the adhesive organ.

This organ does not appear to contribute substantially to the development of a permanent attachment. In only one specimen, recovered at 24 hours of development, was tissue found within the sucker's cavity (Pl. 14, fig. 2). In two other cases, host tissue debris was seen within the acetabulum (Pl. 14, fig. 3). In all other specimens examined,the ventral sucker was not attached to host tissue. As the worms increase in size, the ventral sucker tends to be displaced so that it eventually rests at the junction between the base of the anterior lobe of the adhesive organ or in some cases within the cephalic face of the anterior adhesive organ lobe (Pl. 13, fig. 2; Pl. 14, fig. 1). Extensive development of the adhesive organ lobes result in a situation where the ventral sucker is not in the vicinity of host tissue (Pl. 13, fig. 2; Pl. 14, fig. 1).

This orientation remains for the duration of the parasite's existence.

(c) Histology

(1) Light Microscopy

Although much largerin size than the oral sucker, this organ presents a histological picture similar to that previously described for the oral sucker (Pl. 13, fig. 1; Pl. 14, fig. 3).

(2) Electron Microscopy

The ultrastructure of the ventral sucker has not been included at this time. Presumably the morphology of this organ is similar to that previously described for the oral sucker.

(iii) Lappets

(a) Growth

Extreme difficulty was experienced in measuring the dimensions of the lappet organs because the associated glands were extremely diffuse in nature. The lips surrounding the cavity of the sucking cup, on the other hand, were well defined,and the length and width dimensions of this cup were used asan indicator of growth. These dimensions gave accurate and consistent measurements.

As seen in Table 6 and Figs. 19 and 20, the average length of the lappets measured from newly emerged

metacercariae was 0.058 ± 0.002 mm and the width 0.031 ± 0.004 mm. Following 3 days of development within the host, the sizes increased to 0.145 ± 0.010 mm and 0.066 ± 0.009 mm respectively. The width itself has increased 113% and the length 150% during this interval.

The length of the lappets is always greater than the width, and before infection the difference between these two dimensions was 0.027 mm. At the last measured period of development, i.e., 3 days, this difference is 0.079 mm, an increase of 189%. After 18 hours, the development in length is exceedingly rapid with 89% of the total occurring between 18 hours and 3 days, and the remainder between 0 and 18 hours. In the interval 18 hours to 24 hours (total 6 hours) this organ increased by 36%, a rather extensive development within a short period of time.

The proportion of lappet size to forebody sizes (Figs. 17 and 18) is Lap L (lappet length):ABL is 0.20 and Lap W (lappet width):ABW is 0.11 in the larval worms up to 18 hours of development. Following 18 hours to 3 days these proportions alter to new values of 0.40 and 0.14 respectively.

(b) Orientation

Whole mounts of fixed metacercariae show that the lappets are composed of compact masses of deeply staining glandular tissue containing the so-called lateral sucking

cups (Pl. 2, fig. 4; Pl. 15, fig. 1). The zones of tissue are located beneath the ventral forebody surface on either side of the oral sucker. They extend posterioral to the level of the acetabulum.

The lateral sucking cups are a pair of elongated pockets occupying the medial portions of the deeply staining anteriolateral prominences. These sucking cups have their long, narrow, slit-like external openings running along the ventral surface of the anteriolateral prominences. Both the pockets and their external openings are directed obliquely backwards, and extend posteriorad and laterad toward the level of the acetabulum.

In fixed specimens, the lateral sucking cups are generally invaginated (Pl. 15, fig. 1) to form slight depressions. In living specimens, they are extremely labile and exhibit alternating phases of invagination and eversion (Pl. 15, fig. 2).

Information concerning the function of the lappets has been obtained from histological examinations and indicates that by 18 hours the lappets begin to express their attachment function.

As stated previously, the lappets demonstrate alternating phases of invagination and eversion. In the everted stage they appeared as "extended ear-like appendages" (Van Haitsma, 1931b) and were pressed or clasped onto a portion of the gull's intestinal tissue (Pl. 6, fig. 2;

Pl. 9, fig. 3). The earliest period of development when this orientation situation was observed came from specimens recovered from the small intestine after 18 hours of development. This clasping action is believed to have resulted from muscular contractions of the muscles inserted in the lateral walls of the sucking cups. This phenomenon is further supported by the following observations: (a) upon opening the bird's intestine at 18 hours after infection many worms were observed to be already attached while some of those which were previously seen lying free in the lumen proceeded to attach in the above fashion; (b) only a few specimens were found adjacent to the host tissue and many of the worms so attached separated during the histological procedure; (c) many sections revealed the lappet organs fixed in the everted position, an observation previously made by Van Haitsma (1931b). Where host and tissue had separated, the conformation of the adjacent host intestine indicated an earlier attachment (Pl. 6, fig. 2).

The autopsy results (Table 1) indicate that the clasping attachment may be a temporary stage for the metacercariae recovered from the small intestine at 18 hours. Following 18 hours maturing worms were also recovered adhering to the walls of the large intestine. It may be then that the parasites release their hold, travel down the intestine and complete their maturation within the large intestine.

The first specimens recovered from the large intestine were also attached to the intestine by means of the lappets and oral sucker (Pl. 7, fig. 1). This orientation was observed in many specimens up to the end of 48 hours. After this, the lappets invaginated into deep pocket-like cavities drawing in portions of the intestinal tissue within them (Pl. 13, fig. 2; Pl. 16, fig. 1). The host villus was in direct contact with the linings of these pockets. The columnar epithelium of the host mucosa had been completely lost so that the lappet tegument was oppressed to the subepithelial connective tissue. This contact was so intimate that it was often difficult to distinguish the boundary between the parasite and the host by light microscopy.

(c) Histology

(1) Light Microscopy

Parenchymal tissue of the body wall extends into the lappets and is the major component of this tissue (Pl. 15, fig. 1; Pl. 16, fig. 2). Embedded within this tissue are a large number of deeply staining "basophilic" gland cells. These cells contain a granular cytoplasm and fairly large nuclei and can be seen tapering into processes which penetrate the tegumental lining as fine processes.

The tegument lining the walls of the lappet sucking cups is thinner than the rest of the body and is completely devoid of spines.

A complex system of muscles is associated with each lappet. Inserted into the base of each sucking cup is a system of muscle fibers which terminate just below the subtegument of the forebody. In addition, a well developed set of longitudinal fibers, which arise anteriorly in the lappets, run in a posterior direction along the inner walls of the cup to the base of the forebody, and eventually attach to the dorsal and ventral sides of the hind body. These muscle fibers are very strongly developed in the region of the lappets. Their contraction would result in producing the invagination of the lappets, aided of course, by sub-cuticular and dorso-ventral muscles.

(2) Electron Microscopy

An electron microscopic study of the surface of the lappets has revealed a differentiation of the tegument which contrasts markedly with the general tegument (Pl. 17, fig. 1). The outer portions of the tegument possesses a number of irregularly shaped extensions which project into the lumen. These projections have been referred to as "setae" by Erasmus (1969a). The setae are covered with an external plasma membrane continuous with the tegument, and contain an extension of the tegumentary cytoplasm. In addition, supporting fibrils similar to those described by Erasmus (1969a) for Apatemon gracilis minor have been observed.

The matrix of the cytoplasm, the membrane bound vesicles, basement layer, bundles of muscular fibers, and subtegumentary cells, etc. are morphologically similar to those characteristic features previously described for general tegument. The concentration of membrane-bound, spherical vesicles is greater in the lappet tegument than in the general body tegument.

In addition to the projections of the subtegumentary cells, the apical portions of the pear-shaped gland cells are also interspersed between the muscles. The apical portions penetrate the basement membrane, basal plasma membrane and the matrix of the tegumentary cytoplasm. In the sections examined, the apical portions of the gland cells have not been observed to pass through the external plasma membrane. In contrast, Erasmus (1969a) found that the gland cells of A. gracilis minor open directly to the exterior.

Within the cytoplasm of the processes, large densely staining masses of secretion bodies replace most of the cell cytoplasm. In the distal portions of the processes near the outer plasma membrane, these bodies appear to break down and may be secreted as fine granular material (Pl. 17, fig. 1). Also present in the peripheral cytoplasm of the gland cell processes are a number of circularly outlined microtubules. Erasmus has also described similar tubules found in the gland

cell cytoplasm of A. gracilis minor, and he believes that these tubules may "help to stabilize and maintain the rigidity of the apical portion of the cell in a region which exhibits considerable muscular activity." The microtubules are not present in the proximal portions of the cell (Pl. 18, fig. 2).

Although not investigated in the present study, it appears that the nuclear portions of these gland cells may be the sites of synthesis of the secretory particles. Erasmus has found that the cytoplasm of the lappet gland cells contains mitochondria, Golgi complexes and numerous endoplasmic reticulum, suggestive of active synthesis.

The tegument lining the lip of the lappets contrasts markedly with that previously described for the tegument lining the sucking cups (Pl. 18, fig. 1). In contrast, the morphology of the lip tegument closely resembles that previously described for the general body tegument. Gland cells and setae are absent. The outer plasma membrane is thrown in numerous, large, irregular folds. The cytoplasm of the tegument contains numerous membrane bound vesicles, and is in communication with the subtegumentary cells by means of apical processes. These features and the morphology of the basement layer and muscular bundles are similar to those previously described for the general body tegument.

The surface of the lappet sucking cups undergoes many

changes after attachment to the host is completed. In an example taken from a 6 day old specimen, the lappet glands had disappeared. Numerous, long, irregular projections of the tegument replaced the setae (Pl. 19, fig. 1 and 2). The extensions of the tegument extended deep within the host tissue. The host tissue also underwent many morphological changes. The layer of epithelial and mucous cells was replaced by a zone of medium-electron dense granular material. This band of altered tissue appears under the light microscope as a "thickened basement membrane". Within the lamina propria of the host tissue, threads of the aforementioned material were also observed.

In the present study, no ultrastructural studies have been performed on specimens between 0 and 6 days post infection. Future investigation during these times, would probably reveal the formation of this altered tissue, which presumably comes about as a result of the lappet gland secretions. Further interpretations of the present results are considered in the DISCUSSION.

(iv) Adhesive Organ

(a) Growth

The largest attachment organ measured is the adhesive organ (Table 6, figs. 19 and 20). Between 0 and 3 days this structure grows from 0.085 ± 0.003 mm long and 0.092 ± 0.002 mm

wide to a length of 0.270 ± 0.029 mm and a width of 0.292 ± 0.038 mm. This represents an increase of 218% and 217% respectively. These increases are the result of the rapid growth observed after 18 hours, and represents the greatest increase observed of all the attachment organs (Table 7). The large standard errors associated with each of the values as seen in Figs. 19 and 20 indicate the extreme plasticity of this organ; an important observation which will be further elaborated upon in another section.

The proportion that this organ occupies in comparison with the anterior body has been determined to be: AOL (adhesive organ length):ABL 0.30 and AOW (adhesive organ width):ABW 0.35 in the young metacercariae (Figs. 17 and 18). During 3 days of development the respective values increase to 0.74 and 0.62.

(b) Orientation

The adhesive organ is seen in mounted specimens of excysted metacercariae as a large, irregular prominence, somewhat circular in outline (Pl. 2, fig. 4; Pl. 13, fig. 1). This heavily staining structure is attached to the ventral surface near the posterior end of the forebody.

In living specimens, the adhesive organ consists of two broad lobes which appear to arise from the base of the forebody. In specimens 18 hours after development these

lobes are situated on the dorsal and posterior walls of the forebody pocket (Diag. 1). There are two main lobes - one dorsal (also referred to as the anterior lobe) and one ventral (posterior). They are extremely flexible showing alternating phases of extension and contraction. In the extended position, in worms as early as 18 hours after development, either or both may project above the rim of the forebody or they may be fully contracted into it (Pl. 20, figs. 1 and 2).

The anterior lobe is broad and thick at its base, being somewhat thinner and broader distally. On its anterior surface, near its base, the acetabulum is located (Pl. 13, fig. 2; Pl. 14, fig. 1). The lateral margins of this lobe are normally turned posteriorly, thus forming a deep groove (Pl. 13, fig. 2). In addition, the distal ends of the lateral margins are curled when the lobe is in the contracted phase (Pl. 8, fig.1).

The posterior lobe appears as a thick muscular cup capable of considerable flexibility and is attached to the forebody near the base of the ventral forebody cup (Diag. 2). The base of the posterior lobe is approximately as broad as the base of the anterior lobe, but is somewhat thinner. Distally, the posterior lobe expands forming a thin fan-shaped structure which normally fits the curve of the forebody cup (Pl. 7, fig. 2; Pl. 16, fig. 1). The margins of this, as well as the anterior lobe, may be variously

folded or contracted so that at times either or both lobes may appear to be notched (Pl. 13, fig. 2).

Approximately 48 hours post infection, the lobes of the adhesive organ uncurl in such a way that the inner surfaces (bearing the gland cells) are in contact with the host tissue. Prior to this time, the adhesive organ, in all specimens examined, remained contracted. Even though the lappets were firmly attached to the host tissue, the distal areas of the adhesive organ lobes were always observed only touching the intestinal tissue without embracing it (Pl. 8, fig. 1 and 2).

During the course of growth and eversion, the anterior lobe extends up to, and finally beyond, the level of the oral sucker. In many specimens, a portion of this lobe had been drawn within the cavity of the oral sucker, as previously described (Pl. 9, figs. 1 and 2).

In examining specimens 72 hours post infection, the aforementioned process leading to the apposition of the adhesive organ lobes to the host villus is completed (Pl. 7, fig. 2; Pl. 16, fig. 1). In whole mounts, one or both lobes may be seen to extend beyond the level of the forebody cup (Diag. 2). An intimate host-parasite interface is established along the inner walls of the lobes, and in these areas of contact, the columnar epithelial and mucous layer of the host villus is completely destroyed. The adhesive organ lobes are in direct contact with the

underlying villar lamina propria (Pl. 7, fig. 2; Pl. 16, fig. 1). Within this lamina propria the blood vessels enlarge and many are immediately adjacent to the surface of the adhesive organ lobes (not illus). This intimate association of the adhesive organ and host tissue has been observed up to the end of the parasites life.

(c) Histology

(1) Light Microscopy

Sections of the adhesive organ show considerable glandular tissue present in the basal portions of both lobes. This tissue also extends to the tips of the lobes (Pl. 7, fig. 2; Pl. 16, fig. 1, etc). Contained within the parenchymal tissue, cells are concentrated to form a compact mass, generally arranged in clusters ressembling segments of an orange (Szidat, 1929). These gland cells are small, pear-shaped, and contain large nuclei and granular, basophilic cytoplasm.

Krause (1941) concluded that the adhesive organ lacked a tegumental covering; however, light microscopic studies show that the two lobes are covered by a tegument which is continuous with that lining the forebody cup. The inner opposing surfaces of the lobes appear to be covered by a thin tegument devoid of spines. The outer portions of both lobes are covered with many minute spines.

The lobes are not solid, rather each contains a large excretory space. These spaces are continuous with the excretory lacunae of the anterio-dorsal parts of the hindbody (Pl. 7, fig. 2). Within each lobe the central space is surrounded by, and communicates with, numerous smaller spaces occupying the more peripheral regions of the lobes. Strong muscle fibers, whose arrangement is difficult to trace, pass between the excretory lacunae. In addition, the lobes contain subtegumentary circular and longitudinal fibers.

(2) Electron Microscopy

The inner surfaces of the two adhesive organ lobes consists of an outer membrane which is elevated into microvilli (Pl. 21, figs. 1 and 2). The adhesive organ surface has a plicate appearance and in the folded areas the microvilli are densely packed, while on the outer regions they are separated. The morphology of the adhesive organ surface is similar to that described for Apatemon gracilis minor (Erasmus, 1969b).

The microvilli are covered by a plasma membrane which is continuous with that the general tegument. The microvilli are varying in length and may branch of fuse with neighbouring villi. The apex may taper or be swollen to form a bulbous tip.

Beneath the layer of microvilli is a fibrous basement membrane and between the two is a space. In this space,

secretion granules from the gland cells accumulate (Pl. 21, figs. 1 and 2). A similar observation was reported by Erasmus and Ohman (1965).

A cytoplasmic layer is also present in portions of the sectioned material. It contains numerous mitochondria and scattered ribosomes, and is bounded by a plasma membrane which in many places penetrates the cytoplasm (see diagram, Erasmus, 1969b, p. 250). The underlying morphology of the gland cells etc. has not been described in the present study. Presumably they are similar to those described by Erasmus (1969b) in A. gracilis minor.

The outer surface of each lobe is covered with a tegument resembling that described for the general body tegument. On the inner surface, near the edges of the lobes a transition occurs where the microvillar surface is replaced with general tegument (Pl. 22, fig. 2). This region and the outer surface of the lobes contains numerous spines, resembling those previously described.

The epithelium lining the reserve bladder lacunae in the lobes of the adhesive organ is shown in Pl. 21, fig. 1 and Pl. 22, fig. 1. The surface of the cytoplasm adjacent to the lumen of the lacuna is elevated to form stacks of lamellae which are bounded by a plasma membrane. In many areas, these lamellae appear to fuse with one another, but, in other places, they are clearly separated (Pl. 22, fig. 1). The cytoplasm of the epithelium is rich in

mitochondria and is involved in the release of fat droplets into the lumen of the lacuna (Erasmus, 1967a, 1969b).

In the sections removed from a 6 day old parasite, the host tissue is in direct contact with the microvillar surface of the adhesive organ (Pl. 21, fig. 1). The proximity of the host tissue and the excretory lacunae of the reserve bladder system, as well as the surface specializations of the adhesive organ surface and lacuna epithelium, may be important for the absorption of host nutrients. Further consideration of this is described in a subsequent section.

Table 1

The Results of a Series of Experiments Carried out to Determine the Destination and Behaviour of the Metacercariae of Cotylurus variegatus in vivo

Expt No	Bird No	Time after ingestion	Gizzard	Results			
				Duodenum	Small Intestine	Large Intestine	Cloaca and Rectum
I*	1	2 hrs	dead specimens present	- 5 cm below gizzard extending for a distance of 26 cm posteriorad one encysted, and remainder excysted moving actively - dead specimens present			
	2	4 hrs		- 5 cm below gizzard extending 26 cm posteriorad 70 unattached worms			
	3	8 hrs		- below gizzard extending 25 cm posteriorad 100 unattached worms	present in anterior portion		

Table 1 (continued)

Expt No	Bird No	Time after ingestion	Gizzard	Results			
				Duodenum	Small Intestine	Large Intestine	Cloaca and Rectum
I	4	18 hrs		- 10 cm back of gizzard to end	- throughout	- present in anterior portion	
II**	1	20 hrs		- 10 cm back of gizzard to end	- throughout - posterior region worms attached	- 17 attached 8 cm anterior to caeca	
	2	24 hrs			- posterior region - unattached and attached	- anterior to caeca - unattached and attached	
	3	48 hrs		- posterior region - attached and unattached	- throughout - unattached and attached	- anterior to caeca - attached	
	4	72 hrs			- posterior region - attached	- anterior to caeca - attached	

Table 1 (continued)

Expt No	Bird No	Time after ingestion	Gizzard	Results			
				Duodenum	Small Intestine	Large Intestine	Cloaca and Rectum
III***	1	120 hrs				- 12 cm anterior and 10 cm posterior to caeca - attached - parasite eggs	
	2	144 hrs			- last 8 cm	- extensive infection around caecal region	
	3	168 hrs				- caecal region - parasite eggs	- Cloaca harbored 6
	4	192 hrs				- caecal region - parasite eggs	- cloaca
	5	216 hrs				- 12 cm of caecal region greatly distended - 530 worms - anterior to infection hard core of dehydrated digestive material	- through-out cloaca

Table 1 (continued)

Expt No	Bird No	Time after ingestion	Gizzard	Results			
				Duodenum	Small Intestine	Large Intestine	Cloaca and Rectum
IV****	1	24 hrs		- throughout - attached and unattached	- throughout attached and unattached	- 10 cm anterior to caeca - attached	
	2	48 hrs			- lower portion attached	- 15 cm anterior to caeca - attached	
	3	72 hrs				- 10 cm anterior and 2 cm posterior to caeca - attached (extensive infection)	
	4	96 hrs				- 20 cm anterior to caeca - parasite eggs	
V*****	1	120 hrs				- caecal region - parasite eggs	
	2	144 hrs				- 35 worms - clustered 2 cm posterior and 10 cm anterior to caeca - parasite eggs	

Table 1 (continued)

Expt No	Bird No	Time after ingestion	Gizzard	Results			
				Duodenum	Small Intestine	Large Intestine	Cloaca and Rectum
V	3	168 hrs				- intestine greatly distended, heavily infected - anterior to infection semi-solid faecal material - parasite eggs	- cloaca harbored 6
	4	192 hrs				- intestine enlarged 5X - packed with parasites - hard dry core anterior to infection - parasite eggs	

* Bird host - 6 weeks old
** Unfed except for water
*** Fed food and water
**** Bird host - 1 year old
***** Bird died
Oesophagus, proventriculus and caecae always free of parasite infection.

TABLE 2

Measurements of the Body Components of Developing Metacercariae (Cotylurus variegatus) from 0 - 72 hours

		Period of Development (Hours)								
		0	2	4	8	18	20	24	48	72
ABL	Average	0.2879	0.2598	0.1805	0.1807	0.2108	0.3139	0.3412	0.3544	0.3662
	Sample Size	34	16	10	25	14	20	8	16	17
	Standard Deviation	0.0458	0.0348	0.0370	0.0253	0.0331	0.0375	0.0695	0.0420	0.0281
	Standard Error	0.0079	0.0087	0.0117	0.0051	0.0089	0.0084	0.0246	0.0105	0.0068
ABW	Average	0.2729	0.2622	0.2160	0.2069	0.2309	0.3054	0.3031	0.4374	0.4697
	Sample Size	34	17	10	29	14	20	8	16	17
	Standard Deviation	0.0402	0.0503	0.0239	0.0210	0.0226	0.0315	0.0268	0.0778	0.0399
	Standard Error	0.0069	0.0122	0.0075	0.0039	0.0060	0.0070	0.0095	0.0195	0.0097
PBL	Average		0.0894	0.0622	0.0507	0.0669	0.1000	0.1232	0.1866	0.4283
	Sample Size		8	5	21	14	20	8	16	17
	Standard Deviation		0.0504	0.0286	0.0184	0.0114	0.0272	0.0161	0.0293	0.0603
	Standard Error		0.0178	0.0128	0.0040	0.0030	0.0061	0.0057	0.0073	0.0146
PBW	Average		0.1190	0.0878	0.1023	0.1273	0.1508	0.1250	0.2295	0.3292
	Sample Size		8	5	21	14	20	8	15	17
	Standard Deviation		0.0241	0.0190	0.0173	0.0145	0.0179	0.0110	0.0259	0.0414
	Standard Error		0.0085	0.0085	0.0038	0.0039	0.0040	0.0039	0.0067	0.0100

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TABLE 2 (continued)

		Period of Development (Hours)								
		0	2	4	8	18	20	24	48	72
TBL	Average	0.2879	0.2608	0.2041	0.2213	0.2450	0.3736	0.4111	0.5030	0.7330
	Sample Size	34	17	10	26	13	20	8	16	17
	Standard Deviation	0.0458	0.0342	0.0261	0.0195	0.0270	0.0456	0.0313	0.0612	0.0741
	Standard Error	0.0079	0.0083	0.0082	0.0038	0.0075	0.0102	0.0111	0.0153	0.0180
TBW	Average	0.2729	0.2622	0.2160	0.2069	0.2309	0.3054	0.3031	0.4374	0.4697
	Sample Size	34	17	10	29	14	20	8	16	17
	Standard Deviation	0.0402	0.0503	0.0239	0.0210	0.0226	0.0315	0.0268	0.0778	0.0399
	Standard Error	0.0069	0.0122	0.0075	0.0039	0.0060	0.0070	0.0095	0.0195	0.0097

TABLE 3

Relationships between the Dimensions of the Body Components of Developing Metacercariae (Cotylurus variegatus) from 0 - 72 hours

		Period of Development (Hours)								
		0	2	4	8	18	20	24	48	72
ABL/PBL	Average		4.1418	2.9395	3.8812	3.1449	3.3809	2.7628	1.9331	0.8666
	Sample Size		8	5	21	14	20	8	16	17
	Standard Error		0.8466	0.5077	0.3295	0.1401	0.2468	0.1193	0.0770	0.0250
ABW/PBW	Average		2.5788	2.5504	2.1478	1.8271	2.0391	2.4144	1.9425	1.4305
	Sample Size		8	5	21	14	20	8	15	17
	Standard Error		0.3606	0.2675	0.0957	0.0539	0.0496	0.0760	0.1093	0.0326
ABL/ABW	Average	0.9605	1.0232	1.2396	1.1879	1.1113	0.9776	0.9205	1.2395	1.2838
	Sample Size	34	16	10	25	14	20	8	16	17
	Standard Error	0.0263	0.0339	0.0877	0.0431	0.0385	0.0180	0.0707	0.0502	0.0198
PBL/PBW	Average		0.7201	0.7425	0.5067	0.5303	0.6614	0.9971	0.8193	1.3151
	Sample Size		8	5	21	14	20	8	15	17
	Standard Error		0.1057	0.1696	0.0462	0.0280	0.0351	0.0687	0.0256	0.0521

TABLE 4

Measurements of the Body Components of Developing Metacercariae (Cotylurus variegatus) from 2 - 6 days

		Period of Development (days)				
		2	3	4	5	6
ABL	Average	0.3544	0.3662	0.8588	1.0651	1.4019
	Sample Size	16	17	20	20	10
	Standard Deviation	0.0420	0.0281	0.1088	0.2008	0.2024
	Standard Error	0.0105	0.0068	0.0243	0.0449	0.0640
ABW	Average	0.4374	0.4697	1.0713	1.3493	1.6082
	Sample Size	16	17	20	20	10
	Standard Deviation	0.0778	0.0399	0.1564	0.2074	0.1204
	Standard Error	0.0195	0.0097	0.0350	0.0464	0.0381
PBL	Average	0.1866	0.4283	2.0313	3.3933	3.5111
	Sample Size	16	17	20	20	10
	Standard Deviation	0.0293	0.0603	0.2533	0.3610	0.4780
	Standard Error	0.0073	0.0146	0.0566	0.0807	0.1512
PBW	Average	0.2295	0.3292	0.9073	1.3887	1.5493
	Sample Size	15	17	20	20	10
	Standard Deviation	0.0259	0.0414	0.0675	0.1295	0.2396
	Standard Error	0.0067	0.0100	0.0151	0.0290	0.0758

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TABLE 4 (continued)

		Period of Development (days)				
		2	3	4	5	6
TBL	Average	0.5030	0.7330	2.8775	4.4584	4.9131
	Sample Size	16	17	20	20	10
	Standard Deviation	0.0612	0.0741	0.2895	0.0508	0.5861
	Standard Error	0.0153	0.0180	0.0647	0.1135	0.1853
TBW	Average	0.4374	0.4697	1.0713	1.4061	1.6882
	Sample Size	16	17	20	20	10
	Standard Deviation	0.0778	0.0399	0.1564	0.1578	0.1724
	Standard Error	0.0195	0.0097	0.0350	0.0353	0.0545

TABLE 5

Relationships between the Dimensions of the Body Components of Developing Metacercariae (Cotylurus variegatus) from 2 - 6 days

		Period of Development (Days)				
		2	3	4	5	6
$\frac{ABL}{PBL}$	Average	1.9331	0.8666	0.4281	0.3137	0.4021
	Sample Size	16	17	20	20	10
	Standard Error	0.0770	0.0250	0.0157	0.0109	0.0196
$\frac{ABW}{PBW}$	Average	1.9425	1.4305	1.1853	0.9756	1.0563
	Sample Size	15	17	20	20	10
	Standard Error	0.1093	0.0326	0.0444	0.0200	0.0475
$\frac{ABL}{ABW}$	Average	1.2395	1.2838	0.8171	0.8013	0.8741
	Sample Size	16	17	20	20	10
	Standard Error	0.0502	0.0198	0.0343	0.0395	0.0395
$\frac{PBL}{PBW}$	Average	0.8193	1.3151	2.2419	2.4685	2.3234
	Sample Size	15	17	20	20	10
	Standard Error	0.0256	0.0521	0.0572	0.0508	0.1681

TABLE 6

Measurements of the Attachment Organs of Developing Metacercariae (Cotylurus variegatus) from 0-72 hours

		Period of Development (Hours)								
		0	2	4	8	18	20	24	48	72
OSL	Average	0.0509	0.0494	0.0432	0.0512	0.0533	0.0567	0.0603	0.0751	0.0851
	Sample Size	34	16	9	28	13	18	8	14	16
	Standard Deviation	0.0045	0.0045	0.0045	0.0032	0.0055	0.0071	0.0062	0.0105	0.0077
	Standard Error	0.0008	0.0011	0.0015	0.0006	0.0015	0.0017	0.0022	0.0028	0.0019
OSW	Average	0.0552	0.0514	0.0485	0.0542	0.0544	0.0690	0.0635	0.0819	0.0841
	Sample Size	34	16	9	28	13	18	8	14	16
	Standard Deviation	0.0285	0.0045	0.0045	0.0055	0.0032	0.0089	0.0055	0.0105	0.0095
	Standard Error	0.0049	0.0011	0.0015	0.0010	0.0009	0.0021	0.0019	0.0028	0.0024
VSL	Average	0.0626	0.0613	0.0672	0.0650	0.0662	0.0658	0.0673	0.0918	0.1255
	Sample Size	31	17	8	28	14	16	7	12	17
	Standard Deviation	0.0063	0.0071	0.0089	0.0071	0.0110	0.0134	0.0100	0.0237	0.0266
	Standard Error	0.0011	0.0017	0.0032	0.0013	0.0029	0.0034	0.0038	0.0068	0.0065
VSW	Average	0.0673	0.0701	0.0753	0.0734	0.0829	0.0936	0.0996	0.1197	0.1377
	Sample Size	32	17	8	29	14	16	8	12	17
	Standard Deviation	0.0071	0.0077	0.0045	0.0084	0.0084	0.0089	0.0063	0.0281	0.0145
	Standard Error	0.0012	0.0019	0.0016	0.0016	0.0022	0.0022	0.0022	0.0081	0.0035

Continued on Page 74

TABLE 6 (continued)

		Period of Development (Hours)								
		0	2	4	8	18	20	24	48	72
Lap L	Average	0.0575	0.0537	0.0467	0.0502	0.0580	0.0918	0.0891	0.1328	0.1453
	Sample Size	33	17	10	25	8	20	8	16	11
	Standard Deviation	0.0071	0.0100	0.0032	0.0100	0.0228	0.0126	0.0084	0.0245	0.0167
	Standard Error	0.0012	0.0024	0.0010	0.0020	0.0081	0.0028	0.0030	0.0061	0.0050
Lap W	Average	0.0305	0.0298	0.0262	0.0294	0.0301	0.0403	0.0382	0.0610	0.0663
	Sample Size	33	17	10	26	9	20	8	15	12
	Standard Deviation	0.0045	0.0032	0.0032	0.0089	0.0045	0.0089	0.0063	0.0219	0.0152
	Standard Error	0.0008	0.0008	0.0010	0.0018	0.0015	0.0020	0.0022	0.0057	0.0044
AOL	Average	0.0847	0.0962	0.0969	0.0798	0.0821	0.1400	0.1303	0.2038	0.2695
	Sample Size	34	17	7	24	11	17	8	16	17
	Standard Deviation	0.0095	0.0134	0.0141	0.0170	0.0077	0.0305	0.0170	0.0402	0.0607
	Standard Error	0.0016	0.0033	0.0053	0.0035	0.0023	0.0074	0.0060	0.0101	0.0147
AOW	Average	0.0920	0.1083	0.0921	0.0945	0.1102	0.1533	0.1443	0.2210	0.2919
	Sample Size	34	17	8	28	12	19	8	16	17
	Standard Deviation	0.0063	0.0095	0.0077	0.0100	0.0105	0.0214	0.0114	0.0322	0.0379
	Standard Error	0.0011	0.0023	0.0027	0.0019	0.0030	0.0049	0.0040	0.0081	0.0092

TABLE 7

Percentage Increase of the Attachment Organs of the Developing Metacercariae (*Cotylurus variegatus*)

Organ	Dimension	0 - 72 hrs. % increased	8 - 72 hrs. % increased
Oral sucker	L	67	67
	W	53	56
Ventral sucker	L	100	94
	W	106	89
Lappets	L	150	190
	W	113	128
Adhesive organ	L	218	238
	W	217	207
Anterior body	L	27	102
	W	72	127

Fig. 1

Relationship between the anterior body length and width, posterior body length and width, and the age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.

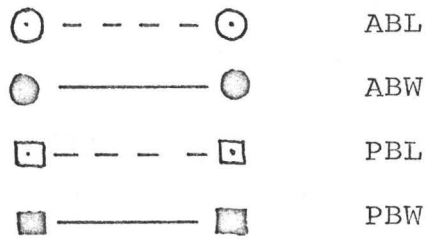


Fig. 2

Relationship between the total body length, total body width and the age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.

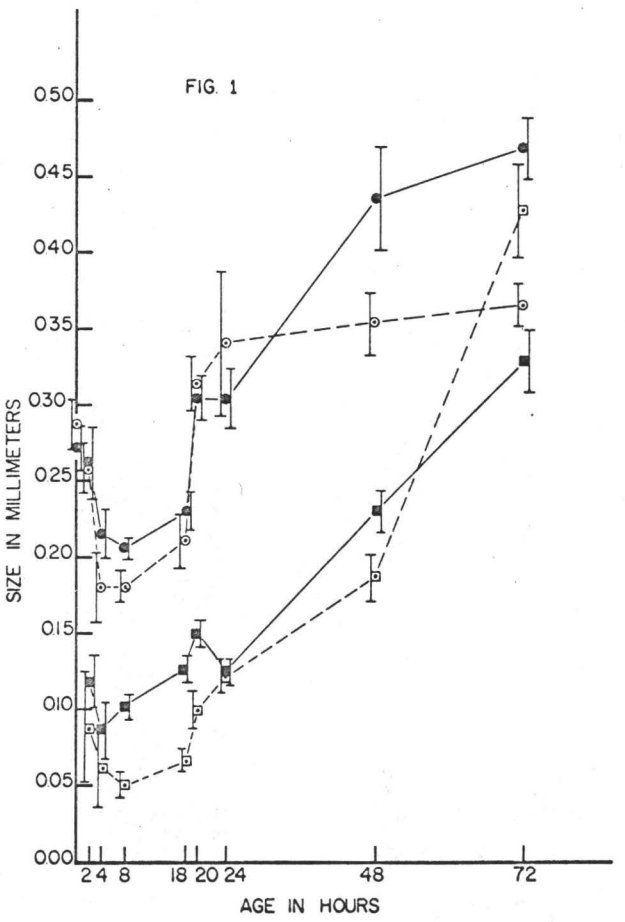


FIG. 1
SIZE IN MILLIMETERS
000
005
010
015
020
025
030
035
040
045
050
2 4 8 18 20 24 48 72
AGE IN HOURS

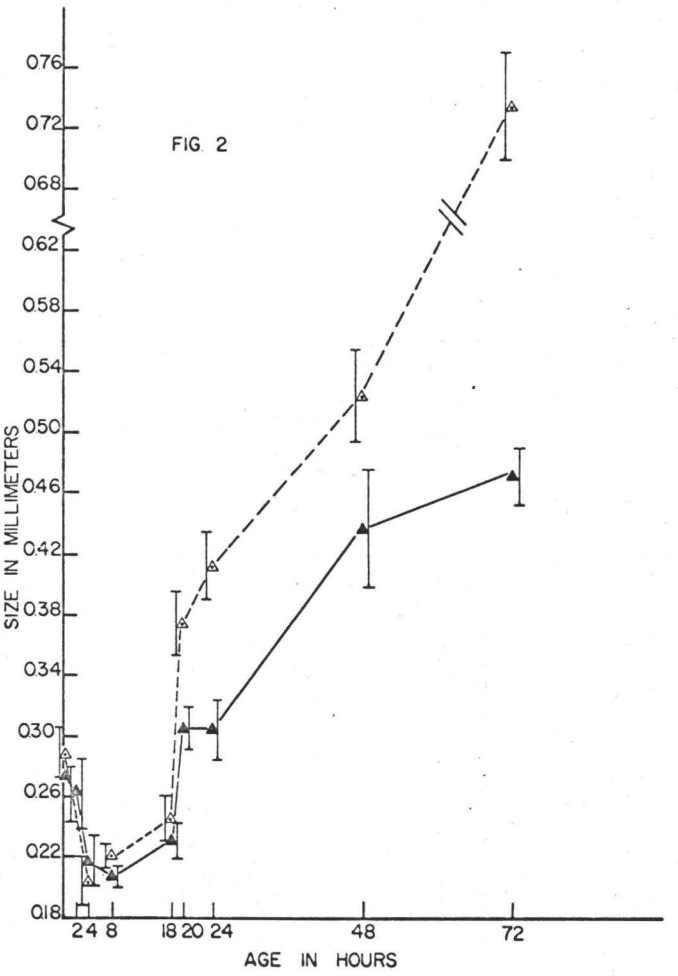


FIG. 2
SIZE IN MILLIMETERS
018
022
026
030
034
038
042
046
050
054
058
062
068
072
076
2 4 8 18 20 24 48 72
AGE IN HOURS

Fig. 3

Relationship between the anterior body length and width, posterior body length and width and the age of the developing metacercariae (0 - 24 hours) of Cotylurus variegatus.

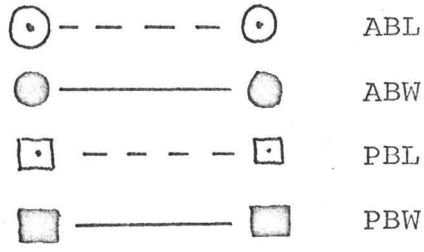


Fig. 4

Relationship between the total body length, total body width and age of the developing metacercariae (0 - 24 hours) of Cotylurus variegatus.

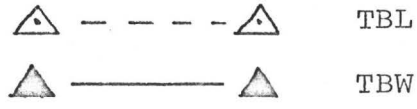


FIG. 3

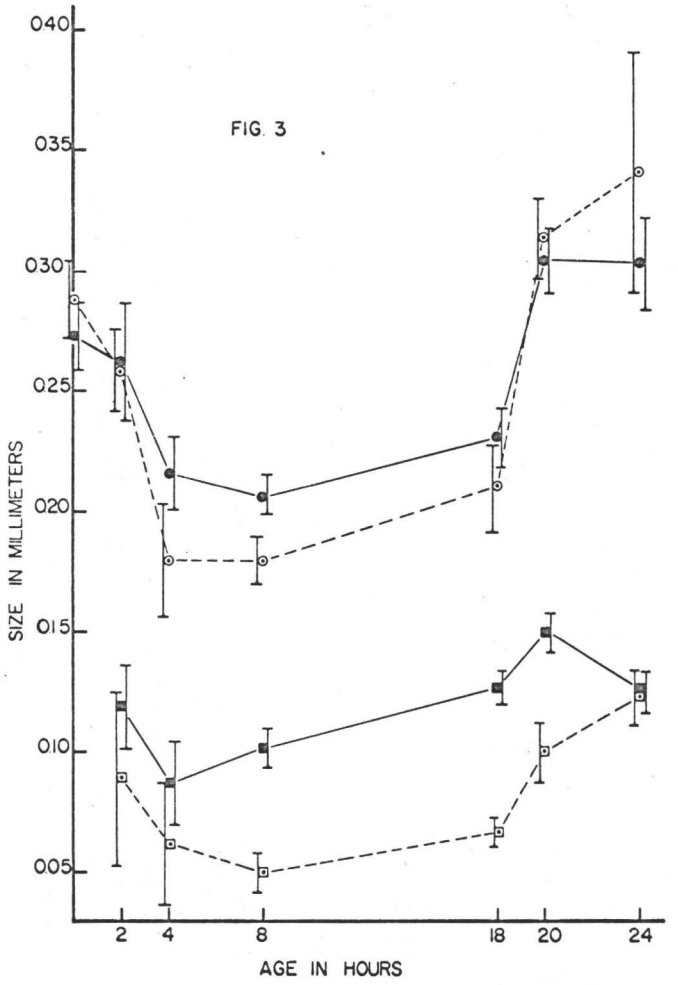

040
035
030
025
020
015
010
005
SIZE IN MILLIMETERS
2
4
8
18
20
24
AGE IN HOURS

FIG. 4

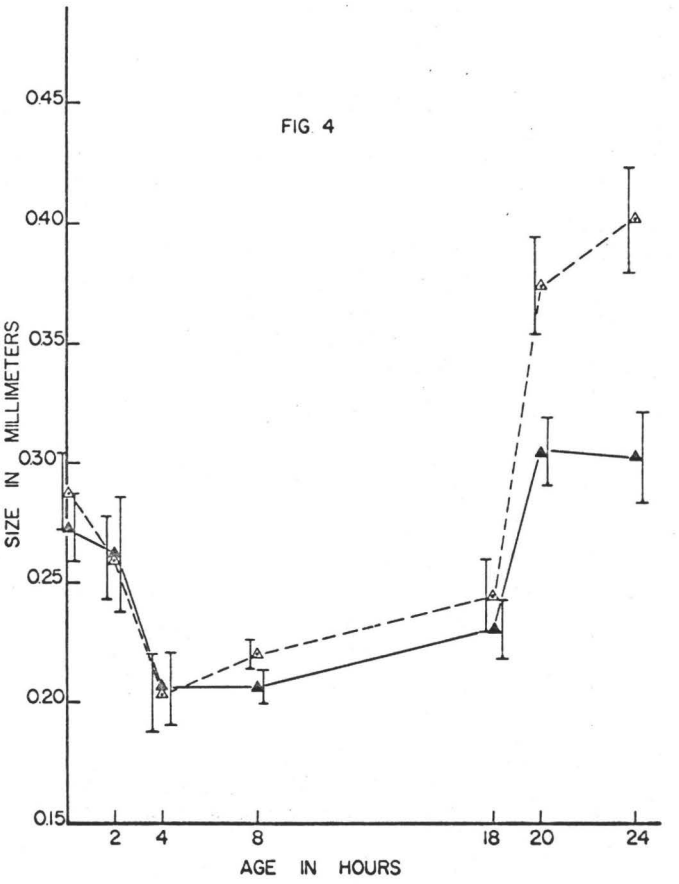

045
040
035
030
025
020
015
SIZE IN MILLIMETERS
2
4
8
18
20
24
AGE IN HOURS

Fig. 5

Relationship between the total body length and anterior body length, posterior body length of developing Cotylurus variegatus metacercariae recovered 0 - 72 hours post infection.

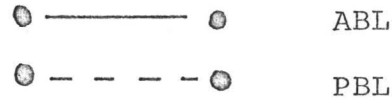


Fig. 6

Relationship between the total body width and anterior body width, posterior body width of developing Cotylurus variegatus metacercariae recovered 0 - 72 hours post infection.

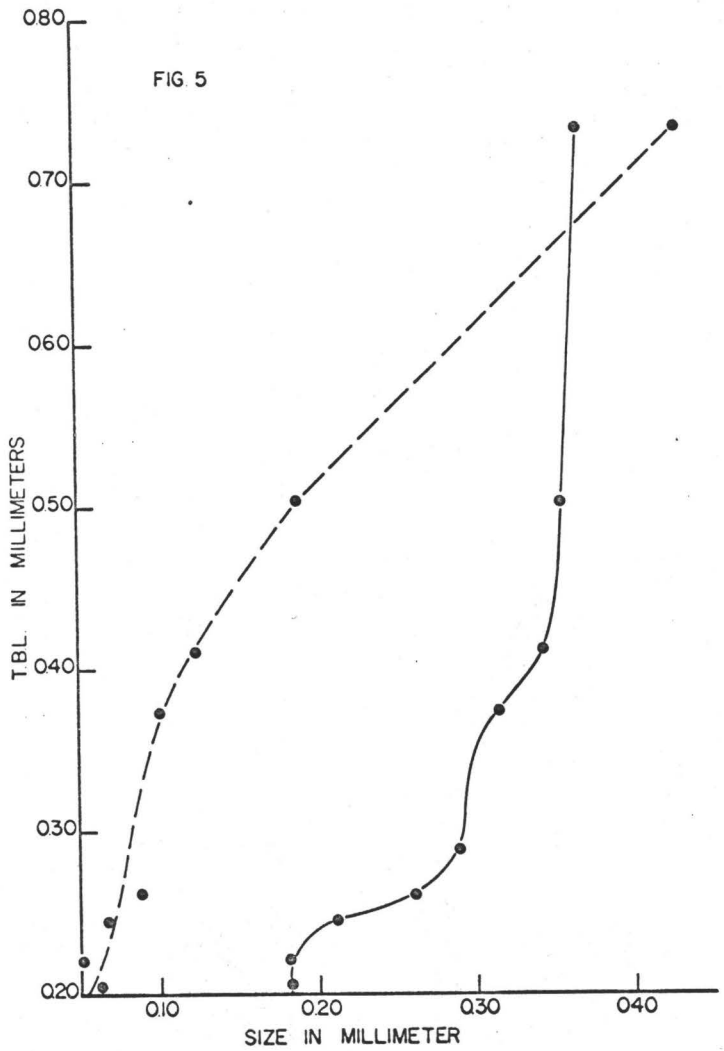


FIG. 5
0.80
0.70
0.60
0.50
0.40
0.30
0.20
T.BL. IN MILLIMETERS
0.10
0.20
0.30
0.40
SIZE IN MILLIMETER

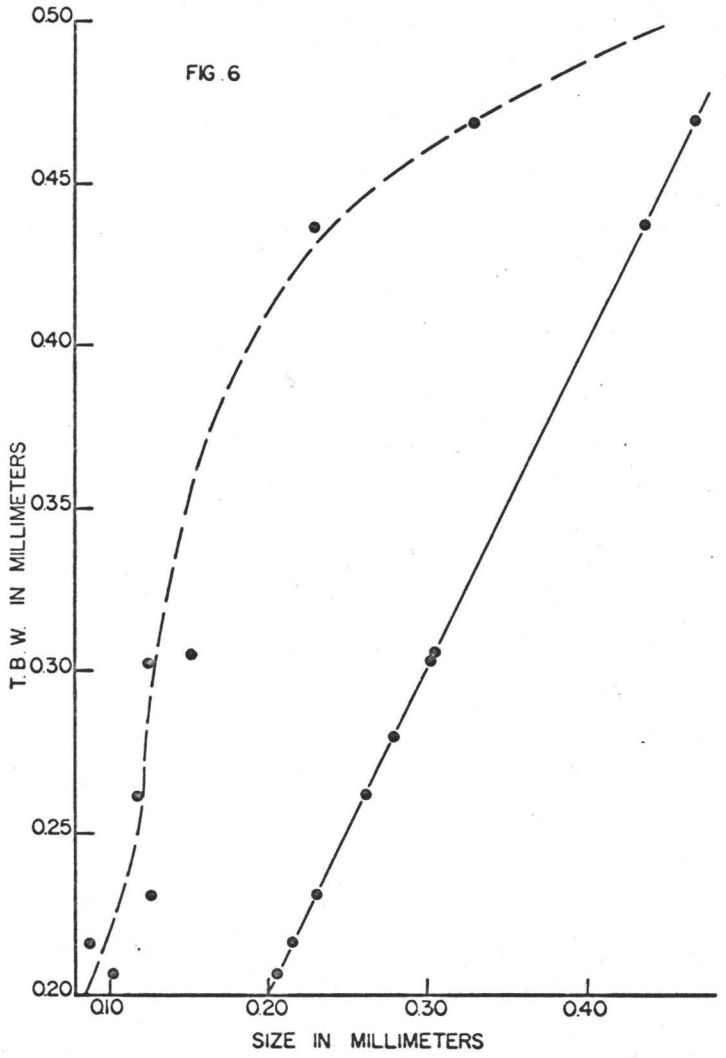
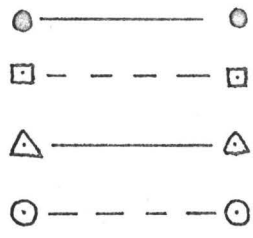


FIG. 6
0.50
0.45
0.40
0.35
0.30
0.25
0.20
T.B.W. IN MILLIMETERS
0.10
0.20
0.30
0.40
SIZE IN MILLIMETERS

Fig. 7

Relationship between the dimensions of the body components and age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.



ABL/PBL

ABW/PBW

ABL/ABW

PBL/PBW

FIG. 7

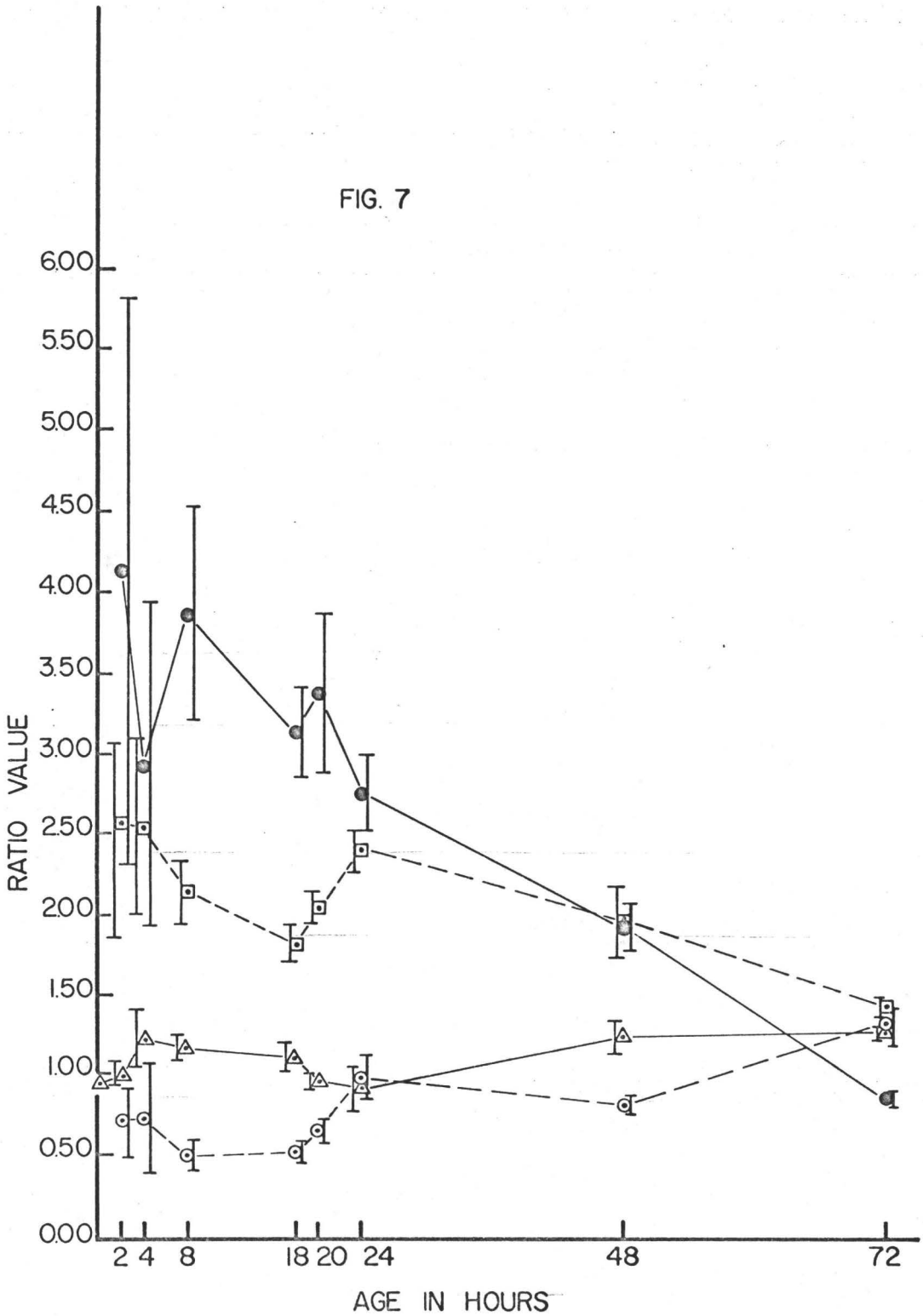

6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
RATIO VALUE
2 4 8 18 20 24 48 72
AGE IN HOURS

Fig. 8

Relationship between the anterior body length and width, posterior body length and width, and the age of the developing metacercariae (2 - 6 days) of Cotylurus variegatus.

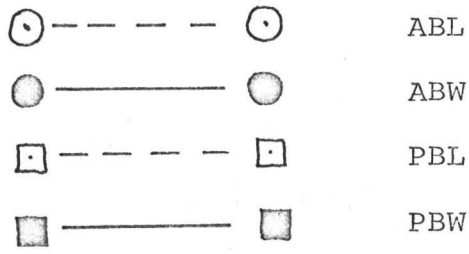


Fig. 9

Relationship between the total body length, total body width and age of the developing metacercariae (2 - 6 days) of Cotylurus variegatus.

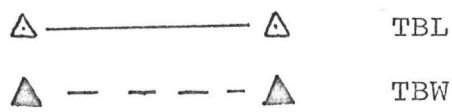


FIG. 8

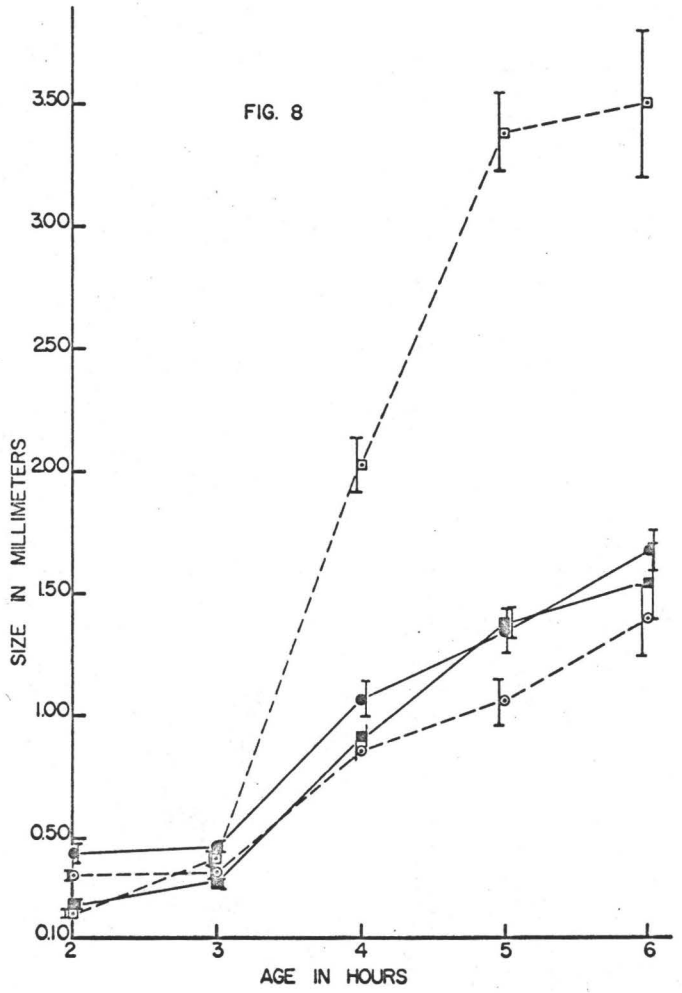

3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.10
SIZE IN MILLIMETERS
2
3
4
5
6
AGE IN HOURS

FIG. 9

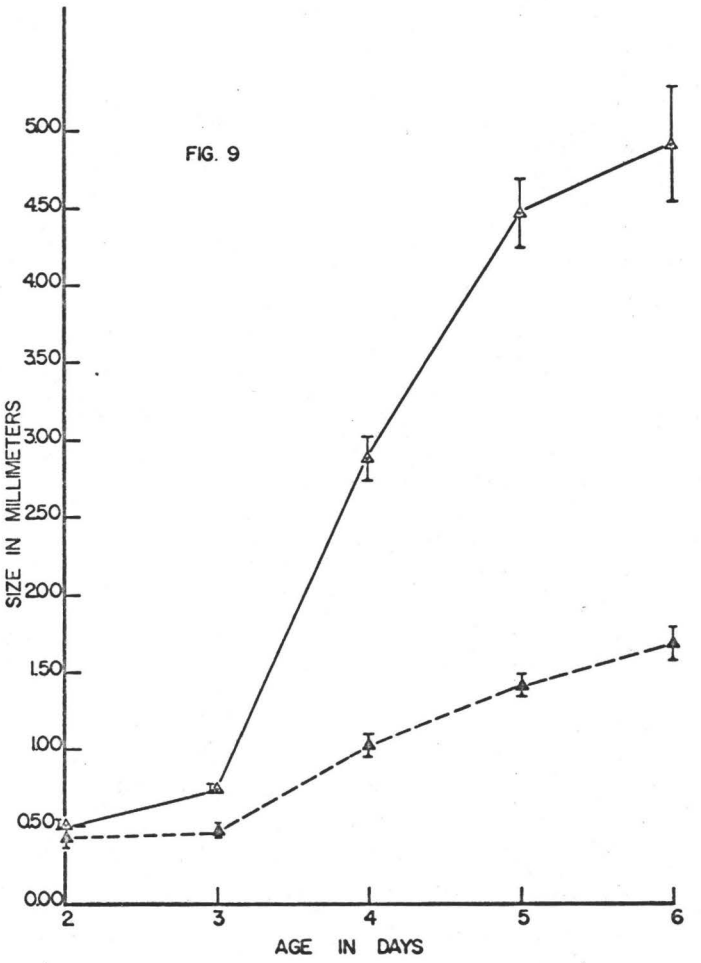

5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
SIZE IN MILLIMETERS
2
3
4
5
6
AGE IN DAYS

Fig. 10

Relationship between the total body length and anterior body length, posterior body length of developing Cotylurus variegatus metacercariae from 3 - 6 days post infection.

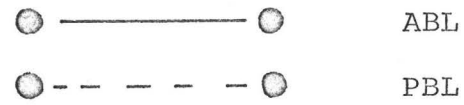
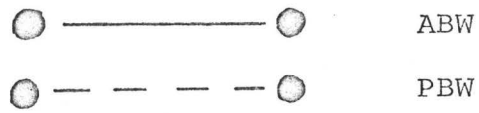


Fig. 11

Relationship between the total body width and anterior body width, posterior body width of developing Cotylurus variegatus metacercariae recovered from 3 - 6 days post infection.



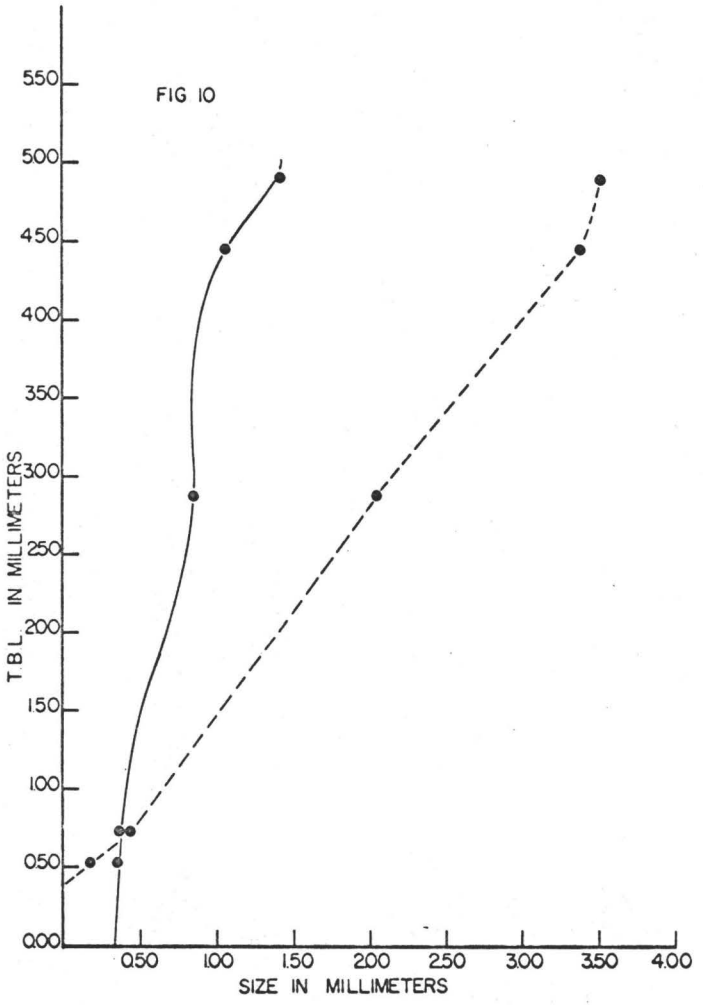


FIG 10
T.B.L. IN MILLIMETERS
SIZE IN MILLIMETERS
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50

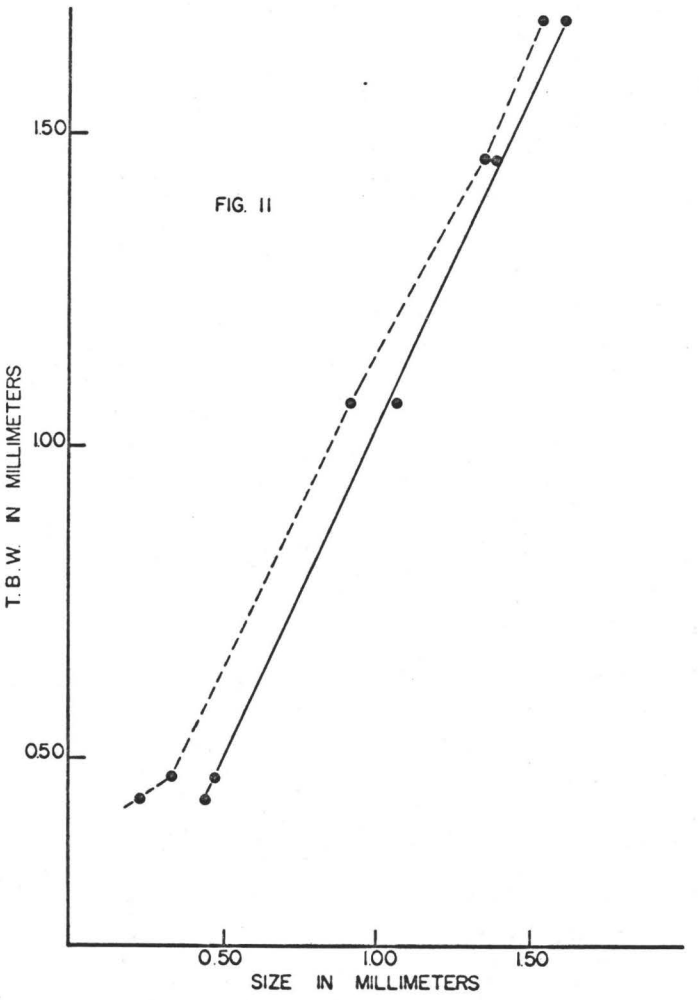


FIG. 11
T.B.W. IN MILLIMETERS
SIZE IN MILLIMETERS
0.50
1.00
1.50
0.50
1.00
1.50

Fig. 12

Relationship between the dimensions of the body components and age of the developing metacercariae (0 - 6 days) of Cotylurus variegatus.

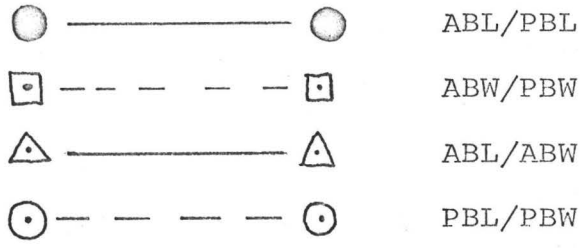


FIG. 12

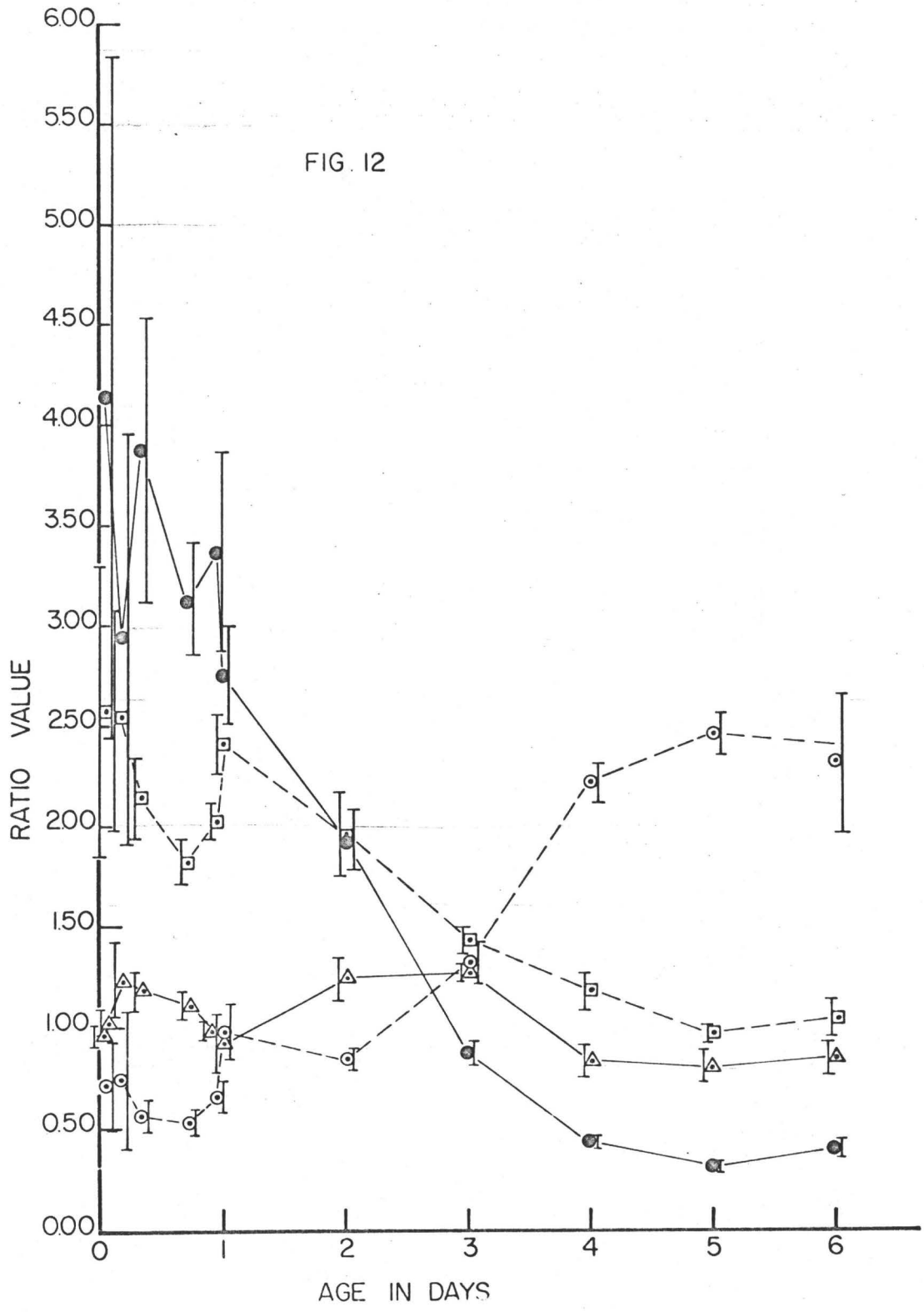

6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
RATIO VALUE
0
1
2
3
4
5
6
AGE IN DAYS

Fig. 15

Relationship of oral sucker length, ventral sucker length and age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.

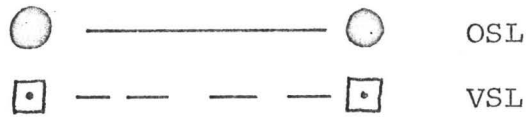
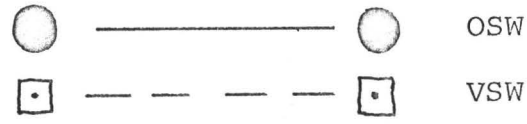


Fig. 16

Relationship of the oral sucker width, ventral sucker width and age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.



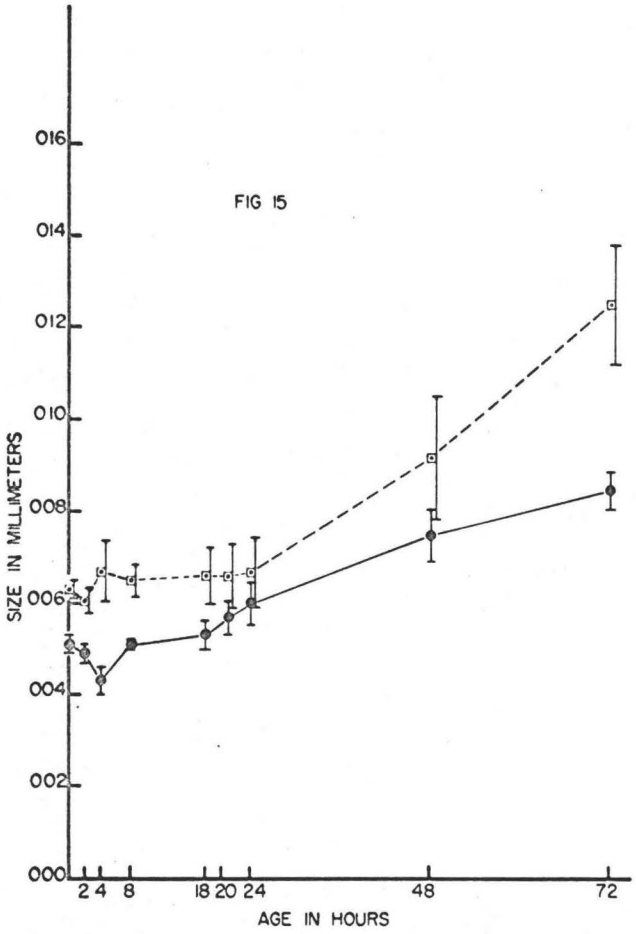


FIG 15
SIZE IN MILLIMETERS
016
014
012
010
008
006
004
002
000
2 4 8 18 20 24 48 72
AGE IN HOURS

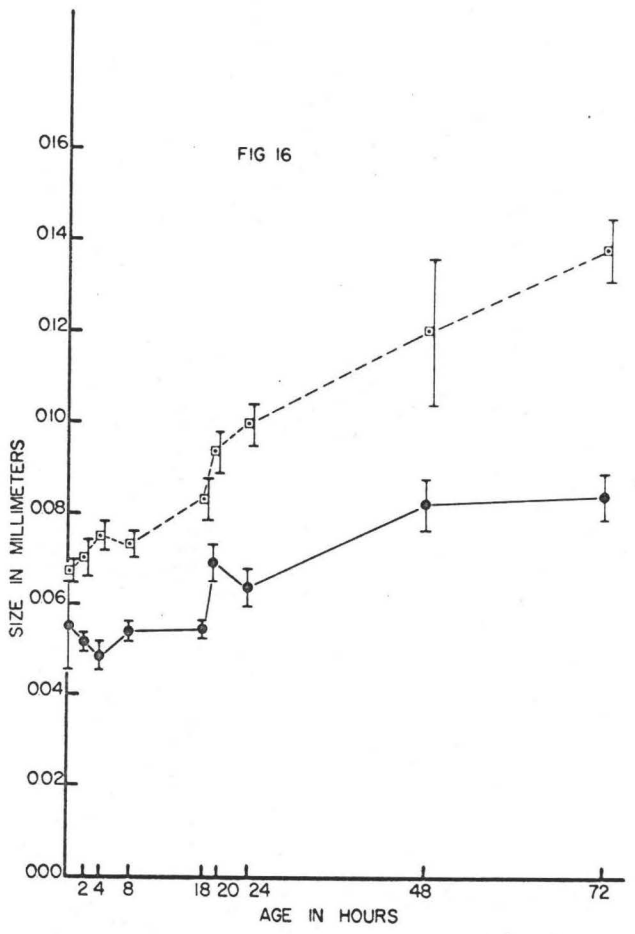


FIG 16
SIZE IN MILLIMETERS
016
014
012
010
008
006
004
002
000
2 4 8 18 20 24 48 72
AGE IN HOURS

Fig. 17

Relationship between the total body length and the length of the attachment organs of Cotylurus variegatus.

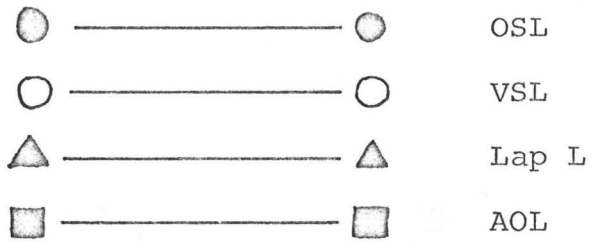
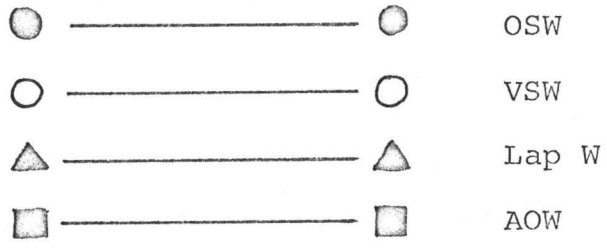


Fig. 18

Relationship between the total body width and the width of the attachment organs of Cotylurus variegatus.



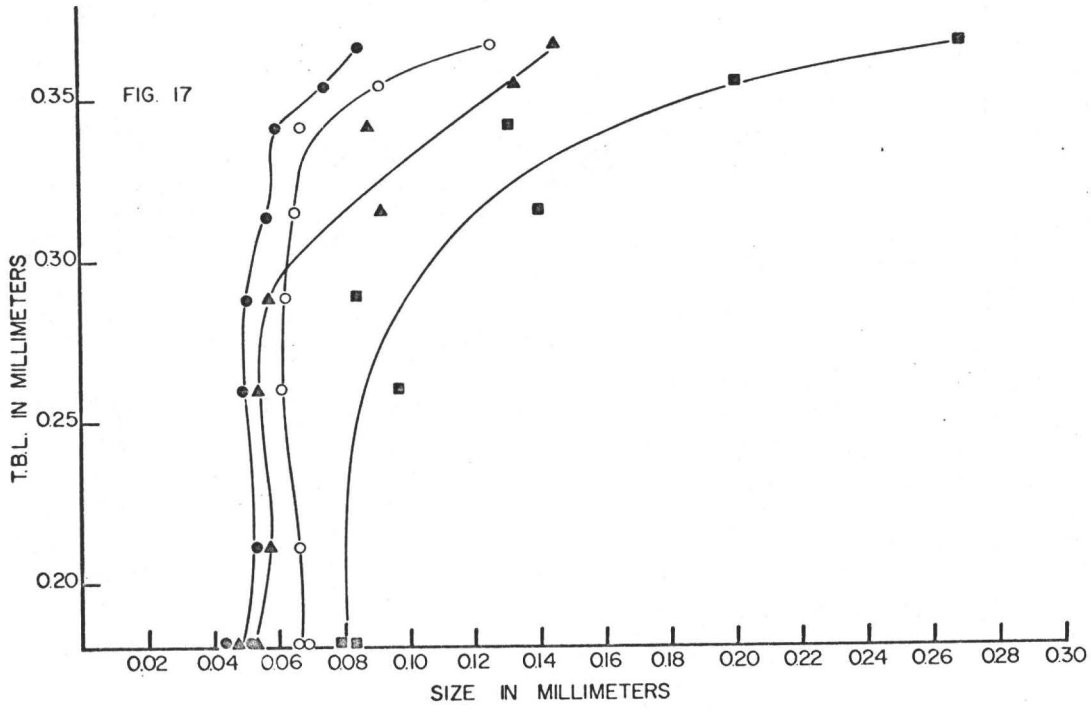


FIG. 17
T.B.L. IN MILLIMETERS
0.35
0.30
0.25
0.20
0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20 0.22 0.24 0.26 0.28 0.30
SIZE IN MILLIMETERS

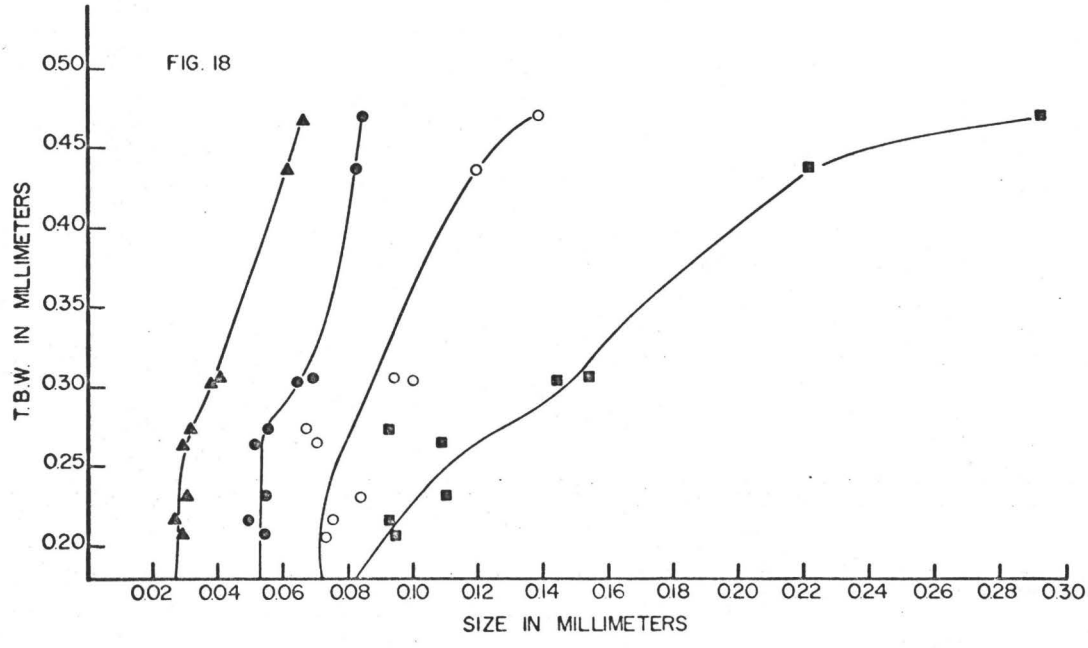


FIG. 18
T.B.W. IN MILLIMETERS
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20 0.22 0.24 0.26 0.28 0.30
SIZE IN MILLIMETERS

Fig. 19

Relationship between the lappet length, adhesive organ length and age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.

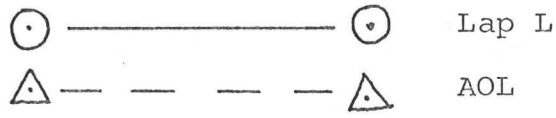


Fig. 20

Relationship between the lappet width, adhesive organ width and age of the developing metacercariae (0 - 72 hours) of Cotylurus variegatus.

FIG. 19

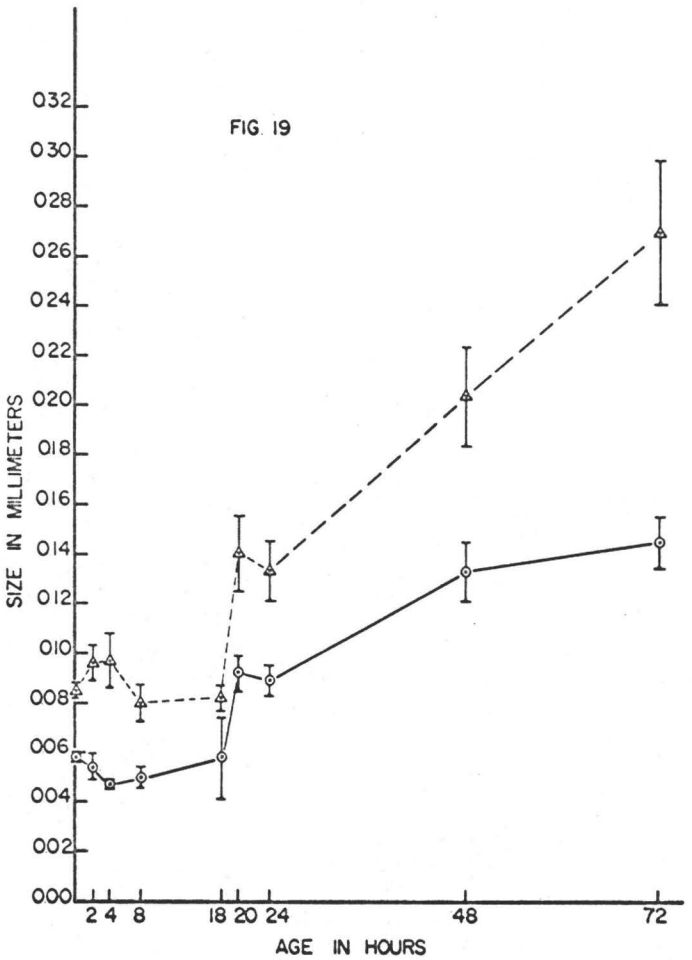
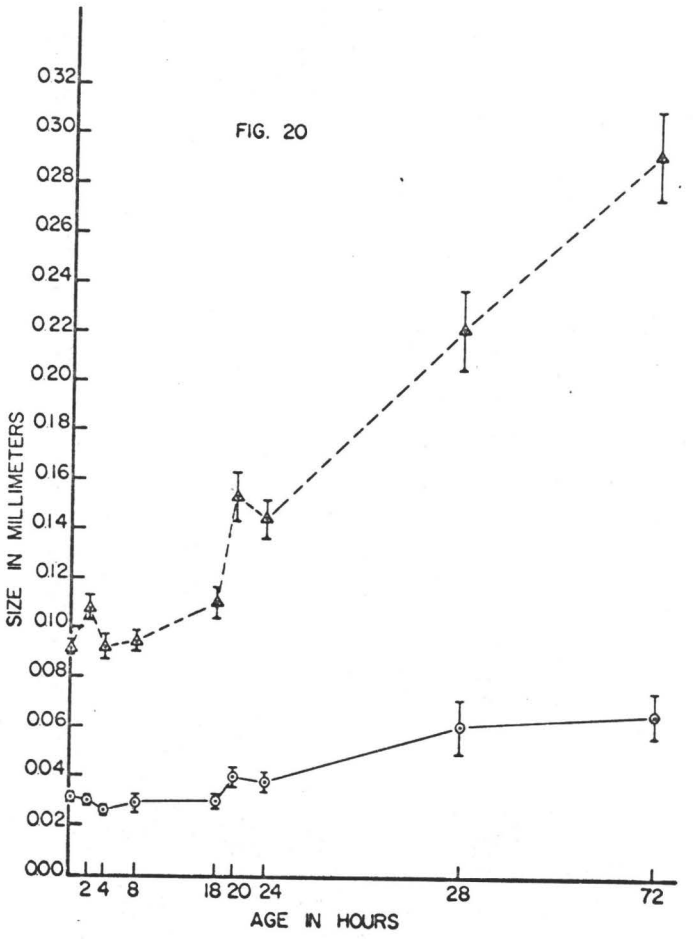

032
030
028
026
024
022
020
018
016
014
012
010
008
006
004
002
000
SIZE IN MILLIMETERS
2 4 8 18 20 24 48 72
AGE IN HOURS

FIG. 20


032
030
028
026
024
022
020
018
016
014
012
010
008
006
004
002
000
SIZE IN MILLIMETERS
2 4 8 18 20 24 28 72
AGE IN HOURS

DISCUSSION

1. Growth of the Attachment Organs

A detailed study on the growth and related development of the parasite's body components has been described (subsequent section), and serves as a standard to which the growth and related development of the attachment organs can be compared. Measurements for the attachment organs have been included until 3 days of age. Further measurements are lacking because (a) after this stage of development, the body sizes are so large, as to obscure the contained organs and do not permit accurate measurements, and (b) the mechanical process of attachment is completed by the end of 3 days. At this time, the organs appear to be in the positions maintained for the duration of the parasite's life. Sectioned material shows that growth continues, but the present study deals with the role of the organs involved in attachment and we need not explore growth in great detail beyond this stage.

During the first 24 hours of development, the observed changes in the sizes of the attachment organs compare with the changes described in other trematodes. Although not commented upon, the results which Ulmer (1951a)

presented for Postharmostomum helicis, show a similar decrease for the attachment organs measured, namely oral sucker width, acetabulum length and width; which occurs as the metacercariae develop until the end of 24 hours post infection. Similarly, a decrease in the width of the acetabulum can be seen in Table VII of Willey's (1941) report. Unfortunately Miller (1939) did not include any measurements of the organs, enabling one to determine if any of the body components of Postharmostomum laruei showed a similar decrease as reported for the general body sizes.

If one considers the range of values for the attachment organs of C. variegatus (best shown in Figs. 15, 16, 19, and 20), one sees that with the 95% confidence intervals, the above mentioned decreases and/or increases of the dimensions may in actuality not have resulted from growth changes during the first few hours. A good example can be seen from considering the values obtained for the adhesive organ lengths and widths. The adhesive organ is extremely flexible. In the living, unattached individuals, this organ was observed to change its shape, constantly stretching and retracting. Fixation preserves the structure in one of the many possible configurations, and thereby results in large differences in the measurements of individuals from the same sample. In addition, one was unable to take into account the total

changes since measurements dealt only with two of the three dimensions. A similar occurrence probably accounts for the fluctuations observed from the results of other investigators. Had they included some indication of statistical variability, a clearer comparison could be made.

An attempt was made to determine the relative proportional relationship of an attachment organ to body size in the developing worms. However, no consistent proportional relationships were maintained with time. The results obtained from the present study and the data of Table VII from Willey (1941) show only that most of the organs increase and/or decrease in size in a pattern similar to that of the body sizes. The growth of the attachment organs during the first 72 hours following infection does not proceed with a proportional relationship when comparing one to another, or to the overall body sizes. In contrast Willey (1941) concluded that "it is apparent that the size of the body and the size of the contained organs increases proportionately up to sexual maturity." Dawes (1956), p. 534, points out, that "Prior to the attainment of maturity the growth of the body and its parts are claimed as being proportionate by Willey, but his figures show distinctly that the gonads increase in relative size up to the time of maturity, and that this probably holds good for organs like the ventral sucker." Beaver (1937) also found that for

Escinostoma revolutum before maturity "none of the structures or regions measured and plotted can be described as having a constant proportion to any other part of the body in all sizes of worms ...." It appears then, especially in the light of this study, that the development of the attachment organs does not proceed with a constant proportional relationship to the increase of body size and/or in general comparison to the increase of one organ to another, up to the time of sexual maturity.

Development after maturity is not included in this study, however, it probably follows a pattern similar to that outlined by Dawes (1956) p. 534, namely "in sexually mature specimens, the relative sizes of organs like the ventral sucker and gonads diminishes, so that the proportions of the mature body are inconsistent."

It is obvious that the attachment organs do not maintain with time a consistent proportional relationship, with respect to body sizes or with other organs. One suggestion may be that the rate of growth of an organ is correlated with its function in the attachment process. The references, which have been considered in this DISCUSSION, have attempted only to establish growth in proportion to the growth of another structure; none has considered the fact that rapid growth may indicate a correlation to that organ's specific or temporal function. This holds true not only for the reproductive organs, but may be especially true

for the attachment organs. If we consider the juvenile metacercariae, the attachment organs appear to be extensively developed before infection takes place, and soon after infection these organs display further rapid development. At these early stages, the posterior body is extremely small and contains only the fundaments of the reproductive apparatus. Only after attachment has been completed, do the cells proliferate and rapidly develop the reproductive apparatus. In general, it appears that the primary objective of an infecting metacercariae is to attach itself, feed extensively, grow and then reproduce. It is "armed" at the juvenile stage to perform its first function and later on as a result of this attachment develops further so that is may accomplish its second role, namely to reproduce.

In the metacercariae the relative sizes of the oral and ventral suckers are essentially the same. In the development interval measured from 8 hours on, both organs increase in size, the oral sucker 67% in length, 56% in width, while the ventral sucker increased 94% and 89%. Thus at the end of 72 hours, the ventral sucker is 1 1/3 times as large as the oral. In relation to the increase of the anterior body, the amount of growth displayed by each of these organs is substantially less. Taking into account the functions of the suckers, an explanation of the decreased proportions which result from slow growth of suckers is revealed.

The suckers are fully developed in newly emerged metacercariae, and are involved in initial travelling movements, and in the establishment of a primary attachment. Soon after this, their attachment functions are lost and they apparently are not used in maintaining a permanent adherence to the host. It seems therefore, that their growth patterns reflect their biological roles as indicated by the small percentage increase of the dimensions.

In the metacercariae, one of the smallest attachment organs measured was the lappets; yet with 72 hours, they increased in size more than the oral or ventral suckers and almost as much as these two organs combined (Table 6). The rapidity of the lappets' growth might indicate their importance in functioning as attachment organs. In the early stages of attachment, the lappets function after primary attachment has been accomplished by the oral sucker. In contrast to the oral or ventral suckers, the lappets appear to function for the duration of the parasite's life. It appears that the importance of the lappets is reflected therefore, by the rapid and extensive development.

The adhesive organ is the largest structure measured in the metacercariae. If we consider growth from 8 hours on, the total increase in growth is 238% in length and 207% in width (Table 6). The actual amount of growth, may, in fact, be somewhat greater than that stated here. The fixation technique, as discussed previously, may influence the size.

In any case, this organ shows rapid development.

In comparison with the other attachment organs during 72 hours of development, this organ develops approximately 3.5 times more than the oral sucker, 2.5 times that of the ventral sucker and 1.3 times greater than the lappets. The period of rapid growth of the adhesive organ overlaps the period of primary attachment. This rapid growth reflects the importance of adhesive organ to the development of the strigeid parasite.

Further consideration will be left until the histological analysis has been presented.

2. Growth of the Body Dimensions

The growth patterns previously described, for the developing metacercariae up to the end of 24 hours, represent a phenomenon which has been under much discussion. Miller (1939) studied the growth of Postharmostomum laruei in mice, and observed a decrease for body length and width, within the first 30 hours of development. After this decrease, there was a considerable recovery, especially for body length. Miller concluded that the decrease in body size for this species and probably for other digenetic trematodes would be "accounted for on the basis that the parasite does not feed during its passage down the alimentary canal to its final site of infection, the cecum. A loss

in weight, and therefore size due to loss of water content and a failure to feed during this passage is not only possible, but highly probable."

Willey (1941) observed the opposite trend while studying Zygocotyle lunata in ducks and rats. He found that the size of the body and contained organs increased proportionately up to sexual maturity. Ulmer (1951a) studied the growth rate of Postharmostomum helicis in mice. He found no increase in size one hour post infection and certainly no decrease; and a gradual increase in total body length and width occurring from 6 hours on.

The results presented in this study show a similar pattern to those reported by Miller, and it may well be that upon entry of these parasites into the final host, with the associated temperature, chemical, pH changes etc., that the worms utilize stored material until they are in the proper environment for feeding to take place. The following points are considered in conjunction with this problem.

Miller's observations were based on measurements of 27 specimens at 0 hours, 15 at 30 and 17 at 96 hours, whereas Willey studied only 10 at 0 hours, 10 at 15 hours and 10 at 2 days. Ulmer considered 50 at 0 hours, 40 at 1 hour, 14 at 6, 10 at 12, 25 at 18 hours and 17 at 24 hours. The samples used in this study have been 0 hours - 34, 2 - 16, 4 - 10, 8 - 25, 18 - 14, 20 - 20, 24 - 8. It therefore

seems, that only the work of Ulmer and the present study can be considered to have investigated in depth the critical early hours of infection. Unfortunately all the reports, except for this study,have omitted any reference indicating the range of values obtained for the samples considered. In his book, "The Trematoda", Dawes (1956) draws attention to the extreme flexibility which flukes demonstrate, and that extreme caution must be emphasized in attaching much importance to the average sizes obtained.

One approach to standardize the results in this study has been to include the standard deviation and more important the standard error with each sample, thereby describing among other things the flexibility of the worms. Taking these values into consideration, it still appears that a decrease in total body size (length and width) occurs during the early hours of infection.

Each worker has used a different technique and has used his technique throughout his individual study. My procedure has been previously outlined, and it too, like the others, was consistently used. Thus, variation within the samples, due to the technique followed is highly unlikely. Ulmer (1951a) is quoted as saying "Miller's techniques used for measurements are unfortunately not listed by him, so that one is in doubt as to whether he dealt with flattened worms." The reason for this statement is uncertain, for Miller in his paper states: "All flukes

were killed .... under pressure of a cover glass."

One important consideration which has been previously overlooked is the time required for the parasites to reach the site of preference and the location of the site of preference. Willey (1941) found that Z. lunata excysted only in the cecum of rats. He observed that 15 hours after feeding no encysted or young worms were found in the intestine of the rats; while many free and some encysted were contained in the caeca. No evidence on this point was available for ducks. From feeding experiments, Ulmer (1951b) found that P. helicis reached the cecum of mice in a very short time. "One hour after 125 metacercariae had been fed only 5 were recovered from the stomach, 75 at various points along the length of the small intestine, and none were found in the cecum. Six hours post-feeding, however, the parasites are found within the cæcum, already actively feeding on blood." Results for the development of P. larvei in mice are lacking from Miller's (1939) paper. From these observations it appears that in the first two cases, the worms are recovered after a short period of time in the final location of development, and that for Z. lunata the metacercariae remain encysted, i.e. protected until the final site of attachment is reached. The result being that there is no decrease in size.

In contrast to this, the autopsy observations previously described reveal that the juvenile cotylurid is

unprotected and subjected to the "new environment" soon after infection. Two hours post infection, the worms had excysted and were located in the duodenum posterior to the gizzard. Feeding apparently does not begin until after 18 hours (subsequent discussion) and the site is not the definitive location of final attachment. At 18 hours, an increase in body size has been recorded, and this is probably correlated with the primary feeding. In general then, the worms, up to 18 hours, can be found slowly moving down the digestive tract. Up to this time when primary feeding begins, the juveniles may be subjected to the greatest "stress", which may lead to the observed decreases in body sizes.

Extensive body development occurs from 24 hours to 6 days. This correlates with the observations that during these hours, the worms reach the cæcal region of the intestine, the final site of attachment, and commence to feed. After this, a rapid increase in the posterior body region was observed. The posterior body length increased by 2754% and the width by 1139%. The anterior body increased only 311% and 431%, suggesting that subsequent to the completion of attachment and the juxtaposition of attachment organs and host tissue, there is an extensive development of the posterior body and the reproductive organs.

As outlined in the observations, body growth is continuous for at least 10 days of analysis. Other workers have found that growth of the digenetic trematodes investigated

is continuous after sexual maturity and up to the death of the parasite. Presumably the growth pattern of Cotylurus variegatus is similar up to the end of 20 - 25 days when the parasite infection ends, and follows a kinetic expulsion (Jarret et al., 1968), probably as a result of a host "self-cure".

3. Mode of Attachment

The suckers (oral and ventral) have been described as the first organs associated with the process of attachment. Creeping movements, using these suckers, doubtlessly aids the developing parasites (following the release from their cysts) to travel down the host's intestine, enabling them to reach their final sites of attachment within the large intestine.

Adhesion, to the substrate by the suckers results from the negative pressure produced by the contractions of the well developed systems of muscles.

The suckers may also play an important role in establishing a primary attachment. Eighteen hours after entry into the host, metacercariae were recovered attached to the mucosa of the small intestine. Sections of parasites fixed in situ revealed that the oral sucker firmly adhered to and contained a portion of an intestinal villus. The results indicate that the ventral sucker is seldom or never attached, which may suggest that this structure is not used

consistently when the parasites are at rest following primary attachment.

The attachment of a strigeid trematode to its host by means of the suckers, in particular the oral suckers, is only a temporary phase of complete attachment. Growth and development of the anterior lobe of the adhesive organ prevents any possible contact between the oral and ventral sucker and the host mucosa. In mature, firmly attached specimens the oral sucker was never applied to the host tissues, although, in some cases, it was attached to a portion of the adhesive organ lobe. The release of the oral sucker from the attachment process may be important from a parasite's nutritional point of view, since this organ has been observed to ingest host tissue debris. This will be dealt with in greater detail in another section.

The views here expressed relating to the method of attachment involving the suckers is probably similar to other species of Strigeida. La Rue (1932) investigating the morphology of Cotylurus communis, and Van Haitsma (1931a) Cotylurus flabelliformis, described a similar arrangement of the suckers during early phases of infection, and believed that the oral sucker as well as the acetabulum were involved in establishing primary adhesion. In firmly attached specimens, both authors have reported that neither organs were apparently functioning as organs of attachment. On the other hand, Van Haitsma (1931b) fround that the suckers

of Diplostomum flexicaudum apparently did not function and that only the lappets served to attach young worms to the host. However, he did state that probably the suckers cooperate with the lappets of the parasite during the process of attachment, and that the lappets and acetabulum are contracted so as to hold the ventral surface of the parasite closely against the intestinal wall of the host. A similar conclusion was noted by Ohman (1965) in her description of Diplostomum spathacium attached to small intestine of Larus ridibundus.

The positions and sizes of the suckers in C. variegatus would not appear to be effective as a means of providing a firm attachment to keep the parasite in situ. These results further support the interpretation that the suckers function only during the early phases of infection. Subsequently, the attachment function of the suckers is lost and they are of little or no importance in maintaining a permanent and firm adhesion.

Subsequent to the primary attachment involving the oral sucker, in many of the specimens examined after 18 hours of development, the lappets also adhered to a portion of host tissue. At this stage, the adhesion is still temporary, since the parasites have not yet reached the preferential site of attachment. Most of this primary attachment occurs within the small intestine. There exists some uncertainty as to the actual sequence of attachment which culminates in this

histological picture (Pl. 6, fig. 2; Pl. 9, fig. 3); i.e., which organ produces the primary attachment? Is the oral sucker the first to adhere to the host tissue followed by lappet attachment or is the sequence reversed with the lappets clasping onto a portion of the intestine so that the intestinal tissue is proximal to the oral sucker which in turn attaches? The answer to this sequence is not immediately evident from the illustrations. A solution to this problem may be derived from the observations previously described for the creeping movements of excysted metacercariae on a glass surface. Movement was observed to occur as the result of actions involving the oral sucker. These results suggest that the oral sucker is perhaps the primary organ involved in establishing parasite attachment and the lappets adhere only after this oral sucker attachment has been completed. Sections showed that the adherence of the lappets was by means of a mechanical action involving the muscular system previously described. This is in agreement with the results of Szidat (1929), Rees (1955) and Ohman (1965) who have also suggested that the muscles in the lappets are used for attachment. Van Haitsma (1931a) believed that in Cotylurus flabelliformis, the lappets did not function as adhesive organs at the early stages of development, but in the adult they were clearly organs of attachment. In contrast, the results of the present study would indicate that the parasites do use the lappets as they begin to develop. They

are operational before the parasites reach their definitive attachment site, although they are used in only a mechanical sense.

Many of the parasites recovered from the large intestine during the hours of 18 to 48 post infection were also found to adhere to the intestinal mucosa by a muscular pinching action of the lappets. After 48 hours, however, these organs became deep pocket-like cavities, and in addition to adhesion resulting from the mechanical action, it became apparent that other processes were involved in strengthening the attachment.

All previous workers have considered the lappets as organs of attachment even if the adhesion, according to various authors, is accomplished in different ways. In addition to the mechanical action suggested by Szidat (1929), Ress (1955) and Ohman (1965); Brandes (1890), Krause (1914), Von Linstow (1877) and Van Haitsma (1931a) found globules in the secreting surface of the lappets of Diplostomum flexicandum. Baer (1933) working with Harvardia sandgroundi stated that there were small refringent granules between the muscle fibers of lappets and that these were expelled by local muscular contractions. Pearson (1956, 1959) described the forebody glands in Alaria canis, A. arisaemoides and Strigea elegans as numerous unicellular glands which open to the exterior through the lappets by means of fine ducts. He suggested that these

glands possibly secrete mucin. In all reports, the authors suggested that the lappets were attached by means of the contents of these globules or granules which appeared to be mucilaginous. They also suggested that the parasites were attached by this substance rather than by the mechanical actions of the lappets.

In recent years a number of studies using histochemical methods have been undertaken to determine the nature of parasite secretions. Ohman (1965) studied the adult stage of Diplostomum spacthaceum. The metacercariae and adult stages of Diplostomum phoxini were investigated by Lee (1962). Ohman (1966a) reported her results of histochemical studies on the adult of Apatemon gracilis minor. In all the species studied, no acid mucopolysaccharide, which would promote adhesion by means of a mucilaginous substance, was secreted by the lappets of the parasites. However, these investigators have found a number of enzymes present, believed to be extracorporeally secreted through the lappet tegument into the proximally located host tissues. Among the enzymes detected were varying amounts of acid and alkaline phosphatase, leucine amino peptidase and non-specific esterase. Depending on the particular species investigated, these enzymes were found in varying concentrations; while in others species some or all of these enzymes were absent altogether.

Erasmus (1969a) has provided morphological features which he suspects are associated with the production and secretion of the enzymes associated with the lappets. My ultrastructural observations revealed similar structures to those described by Erasmus for Apatemon gracilis minor. The major enzyme activity of the lappets of A. gracilis minor has been shown by Ohman (1966a) to consist of a non-specific esterase. Since only one type of secretory body has been observed in the lappet gland cells, Erasmus (1969a) believes that "these relatively large membrane bound secretory bodies are vehicles of this activity." Erasmus and Ohman have both found this material to be associated with the host tissue plug. The presence of similar bodies within the cytoplasm of the lappet cells suggests that these are the sites for this enzyme synthesis. Ohman (1966a) has found a strong reaction for RNA and protein indicating to her that the enzyme formation takes place within these lappet cells. Lee (1962) found that the cuticle covering the lappets of D. phoxini did not contain cysteine. In contrast Monne (1959), Lee (1962), Bjorkman et al. (1963) have demonstrated that the remainder of the epidermis contains large amounts of cysteine. The lack of cysteine in the lappets suggested to Lee that the tegument is not toughened in these areas, or hardened as the remainder of the tegument. This is understandable if the enzyme is to be secreted through the tegument.

In the present study, I have not performed any histochemical tests; however, the secretory bodies described by Erasmus are morphologically similar to the ones shown in Pl. 17, fig. 1. It seems reasonable to conclude that lappets of Cotylurus variegatus are involved in enzyme secretion, and that the enzyme is likely a non-specific esterase.

Sections of adult parasites attached to the gull's intestine have shown that the normal columnar epithelium lining the villi is disintegrated in the region of the lappets; and that there is an intimate contact between the parasite's lappet,and the underlying lamina propria of the host tissue. A similar arrangement has been described by a number of other workers, e.g., Van Haitsma (1931a,b), Ohman (1965, 1966a), Erasmus (1969a). Van Haitsma (1931b) was of the opinion that the columnar epithelium had presumably been removed owing to post mortem digestive action. However, the presence of parasite secreted enzymes suggests that the regions of the villus which are in contact with the lappets are denuded of their epithelial layer by the enzymes. The removal of the epithelial layer occurs during the formation of adhesion. In the adults no tissue debris was found so that no post-mortem change, as described by Van Haitsma,could occur. This is not only possible for the parasites previously described,but is also possible for C. variegatus.

Bogitsh (1966) summarized the function of the parasite secretions: "these esterases partially digest the hosts' tissues, thus strengthening the adherence of the parasite to the villi of the digestive tract." Before this statement can be considered to be a valid conclusion, several basic underlying questions must be answered.

(1) Previous studies have indicated that enzymes, in particular non-specific esterases, are secreted from the parasite onto the host tissue. The result of these secretions has been described as a destruction of the epithelial layer of the villus. If a secretion is present, would it not also attack the underlying lamina propria and so ultimately destroy the entire villar structure? This does not occur. In contrast the lamina propria appears to actually increase in size (Pl. 1, fig. 6). What prevents the parasite secretions from continued host destruction?

(2) Is the material described in the lappet gland cells of C. variegatus and A. gracilis minor really a morphological representation of a non-specific esterase, or are these secretion bodies some other "chemical", produced by the parasite, which cannot be detected by standard histochemical tests?

(3) How would the destruction of host tissues result in strengthening the adherence of the parasite to its host?

In past reports, the questions put forward here have not been discussed by any of the many workers exploring

the attachment process. From the present study, no direct answer to all of these questions can be given; however, this study has provided a basis by which a number of general considerations may be put forward to provide an introduction for future investigators.

Based on the morphology of the lappets, it seems quite certain that the gland cells do secrete a substance onto the surface of the tegument. While is is possible that this substance (as suggested by Erasmus, 1969a) could be a non-specific esterase, it is equally probable that it is not. The techniques used for the identification of parasite secretions are essentially those used in the study of vertebrate tissues. Bueding (1952, 1962), Lee (1962), and Ohman (1966b) have frequently stated that the present day methods may not be sufficient with regards to the complete identification of parasite secretions.

If the secretion bodies do represent a non-specific esterase, the biological role of these substances is, in general, far from being solved (Ohman, 1966b). A few suggestions have been considered by a number of workers and the function of these enzymes apparently depends on their location within the parasite. Ohman (1966b) states that "those secreted to the exterior may be associated with the transport of metabolites through the cuticle and/or have a histolytic action. They may attack the lipoid layers in the plasma membrane and thus cause the breakdown of cell membranes

occurring in the host tissues in the vicinity of the attached parasites." Does this action, therefore, lead directly to a strengthening of the adhesion of the parasite to its host? The logic is not clear!

The fact that the host tissue is not completely destroyed requires consideration. If the secretions are a non-specific esterase having hystolytic action (as suggested by the aforementioned authors), it is difficult to understand why destruction does not proceed in the lamina propria as well as the epithelial mucosa. A number of suggestions are possible: (a) the secretion may not be an esterase, but a substance that attacks specific cells e.g., epithelial and mucosal cells, (b) the nature of the underlying tissue is somehow different, and thus serves as a barrier to complete chemical digestion; (c) after a certain stage of parasite development, the lappet glands no longer secrete the substance.

The first suggestion (a) is not likely, because the epithelial mucosa is sloughed off in intact sheets of cells which do not show extensive cellular damage. The second suggestion (b) is not likely, because the secreted material apparently penetrates a short distance into the lamina propria, and alters the internal layers comparable to the superficial layer. The third possibility (c) carries a greater probability. In the sections examined of the lappets from a 6 day old parasite, no lappet glands were seen. This

may indicate that the process of adhesion by the lappets is completed. However, such a process would propose activity of the glands for a precise length of time sufficient to remove the epithelium mucosa, followed by an abrupt cessation of activity.

If the secretion produced by the parasite is not a non-specific esterase, then the unidentified substance may in fact not result in the death or exfoliation of the lamina propria tissues; rather this substance produces the observed alteration of the "connective tissue" matrix of the villus to such an extent that their composition forms the thickened, amorphous layer, which under light microscopy appears as a "thickened basement membrane". This may also cause the release of the layer of mucous and epithelial cells. This altered, thickened tissue provides a firm basis not only for the attachment by the lappet muscles, but also for the long tegumentary projections of the lappet which are embedded in this tissue.

Earlier in the discussion, it was pointed out that the adhesion of the parasite to its host is very firm. It has been shown that neither sucker is involved in maintaining a permanent attachment. The adhesive organ is not involved in attachment until a firm adhesion is produced by the lappets, and in the subsequent discussion the role of the adhesive organ as a structure of attachment is questioned. It seems then, that the permanent attachment results from the adhesion

of the lappets. The lappet muscular systems would be adequate in providing a means for this attachment, and it also appears that the lappets have additional specializations, e.g. production and secretion of a substance that thickens and strengthens the host tissue to provide a firm basis of attachment for the lappet tegumentary projections and muscles. This aspect certainly warrants further investigations.

The fourth attachment organ investigated was the adhesive organ. The study describing the sequence of attachment has shown that prior to 48 hours, the adhesive organ was not involved in the initial stages of securing attachment. The specimens of metacercariae attached to the small intestine revealed that this organ was not associated with the host tissue and was therefore, not believed to be associated with the formation of a primary attachment. The adhesive organ lobes were always contracted with their distal ends folded inwardly. A similar situation has been described for worms recovered from large intestine. The growth studies have indicated however, that this adhesive organ has grown rapidly, and has increased in size by 238% in length and 207% in width. After the lappets have succeeded in establishing a firm adherence, the lobes of the adhesive organ evert and begin to wrap around the villus stretched between the lappets. Eventually, a contact is established between the entire inner walls of the lobes and host tissue. The lobes themselves can be seen to extend beyond the level

of the forebody cup in adult parasites.

In as much as the forebody wall apparently cooperates with the adhesive organ in functioning as an organ of attachment, it is necessary to discuss the mechanisms which enable it to function. As outlined earlier, the forebody wall has been described to contain many intercommunicating spaces. These spaces are in communication not only with large excretory spaces in the hindbody but also those within the adhesive organ. In addition, the forebody is provided with subetgumentary muscles, and strong muscle fibers which extend from one subtegumentary surface to another. La Rue (1932), Van Haitsma (1931a), Erasmus (1962), Ohman (1965, 1966a) and others have suggested that the muscles of the fore and hindbodies, and of the adhesive organ form one part of a pressure system, and the spaces filled with liquid another.

From a consideration of the structures described, it is apparent that a contraction of the muscles of the wall of the hindbody would force liquid from the spaces within the hindbody into the connecting ones in the forebody and adhesive organ. If the muscles of the forebody remained in the same state of contraction, the expansion of the volume of the adhesive organ would put increased pressure upon the host tissues held between the two inner surfaces of the adhesive organ lobes.

The flexibility of the forebody may provide a means whereby the primary attachment involving the suckers and lappets is accomplished. The dorsal part of the forebody cup with the oral sucker and lappets is very flexible and can be stretched out until the forebody is more spoon- than cup-shaped. The oral sucker and lappets are pushed in as far as the crypts of Lieberkuhn, whence the sucker and lappets attach themselves to a villus. After attachment, and during contraction of the circular muscles of the forebody, some of the liquid within the intercommunicating forebody spaces is pressed into those spaces found in the hindbody. This results in the hindbody becoming turgid. The large muscles, extending from the bottom and sides of the cup-like forebody to the organs and dorsal wall of the hindbody, are believed to contract forcibly to bend the hindbody dorsally against the forebody. This action serves to deepen the cup of the forebody, which results in the free end of the villus coming to lie within the cup-shaped forebody in close proximity to adhesive organ lobes. The intimacy of the association has been described for parasites after 72 hours of development (e.g. Pl. 16, fig. 1).

Sections, through parasites adhering to host tissue, showed that wherever the stratified epithelium came in contact with the inner surface of the adhesive organ, the mucosal epithelium was completely erroded. While pressure might be a contributing factor, it seems that it alone cannot

cause the loss of epithelium. In the intestine, regeneration of epithelium is very rapid; however, Ohman (1966a) found that in the region of the adhesive organ of Apatemon gracilis minor the epithelium was completely denuded. She states that, "this indicates that disintegration is caused by the parasite and that this is not due to natural shedding of epithelium."

An histolytic disintegration of the host tissues has been considered, by a number of workers, to have resulted from secreted enzymes produced by the glands located in the adhesive organ lobes (Brandes, 1890, Muehling, 1896; La Rue, 1927,1932; Szidat, 1929; Van Haitsma, 1931a; Baer, 1933; Lee, 1962; Erasmus, 1962; Erasmus and Ohman, 1963; Ohman, 1965, 1966a,b).

In recent years, attention has been focused on determining (by histochemical techniques) the nature of the parasite secretions. A few examples of the results are given: Alkaline phosphatase has been demonstrated in the adhesive organ tegument of Diplostomum phoxini metacercariae (Arvy, 1954; Lee, 1962); tegument lining adhesive organ of adult Cyathocotyle bushiensis (Erasmus and Ohman, 1963); Holostephamus luhei (Ohman, 1966b) and Apatemon gracilis minor (Ohman, 1966a). Parts of the tegument of adhesive organ lobes of Diplostomum spathaceum (Ohman, 1965) contain acid phosphatase, as do H. luhei (Ohman, 1966b), A. gracilis minor (Ohman, 1966a). Other enzymes detected have been amino acid peptidase in adhesive organ of C. bushiensis (Erasmus and Ohman, 1963)

and A. gracilis minor (Ohman, 1966a); non-specific esterases in D. phoxini (Lee, 1962), C. bushiensis (Erasmus and Ohman, 1963), A. gracilis minor (Ohman, 1966a) and Posthodiplostomum minimum (Bogitsh, 1966). Other enzymes may be secreted, but as pointed out by Lee (1962), the present histochemical techniques may not be adequate to allow complete demonstration of all the enzymes present.

A number of secretion bodies have been described in the tegument, lining the inner lobes of the adhesive organs, of Cotylurus variegatus (present study), A. gracilis minor (Erasmus, 1969b) and C. bushiensis (Erasmus and Ohman, 1965). It appears that these bodies (referred to as α-bodies by Erasmus and Ohman, 1965) may represent a non-specific esterase or a phosphatase. The similarity of these secretion bodies to the ones described in the lappet glands may indicate that the enzyme present in the present species is a non-specific esterase, or, as previously suggested, an unidentified substance.

Prior to the ultrastructural studies, the adhesive organ of C. variegatus was considered to function solely as an organ of attachment. In describing the ultrastructure of the inner surfaces of the lobes, a number of modified surface specializations were found. However, the types of modifications are not those that would be associated with attachment function. The inner surfaces of the lobes are simply oppressed against the host tissues, and it appears that

the surface specializations are involved in the process of parasite nourishment. The nature of this interface existing between the lobes of the adhesive organ and host tissue will be discussed in a subsequent section.

The role of the spines located on the outer surfaces of the adhesive organ lobes where they are not in contact with host tissue is not understood (Pl. 22, fig. 2). A suggestion may be that these spines provide the rigidity to maintain the continued oppression of the lobes against the host tissues. The adhesive organ lobes are not in themselves rigid, and require hydrostatic pressure to remain extented. When the pressure is withdrawn, there is a tendency for the forebody cup to retract, and the lobes to involute. However, the presence of spines and their resultant apposition to the inner walls of the forebody cup would tend to maintain the adhesive organ lobes in an extended condition, in constant contact with the host tissue.

4. Parasite Nourishment

Erasmus (1967b) has defined the host parasite interface as "representing that surface of the parasite which is in intimate contact with the fluids and tissues of the host and through which interchange of materials of physiological and immunological importance takes place." In the digenetic trematodes, Erasmus recognizes three main interfaces

defined as:

(i) the general body tegument,

(ii) parasite alimentary tract,

(iii) surface of the attachment organs.

(i) The general body tegument

In the past few years many studies have described the parasite body tegument. In the present study, a limited description of the body tegument of C. variegatus has been included, mainly for the purpose of providing a basis for comparisons with the surfaces of the attachment organs. However, a few general features have been considered pertinent to this section of the discussion and are outlined below:

The tegument is characterized by a plasma membrane on the outer surface bounding a syncytial layer containing a few mitochondria, and having cytoplasmic extensions uniting it with the subtegumentary cells. The outer cytoplasmic layer does not contain any nuclei nor is it subdivided by cell membranes, thus verifying its true syncytial nature. In its basic form the tegument of C. variegatus resembles that described by Threadgold (1963a,b) and Bjorkman and Thorsell (1964) for Fasciola; Burton (1964,1966) for Haematoloechus and Gorgoderina; Senft et al. (1961) and Lee (1966) for Schistosoma mansoni; Bils and Martin (1966) for a number of echinostomes as well as Parorchis acanthus and Acanthoparyphium spinulosum; Erasmus (1967b) for

Cyathocotyle bushiensis; and Belton and Harris (1967) for Acanthatrium oregonense.

The function of the outer coat of cestodes has been discussed by Lumsden (1966), and it seems likely that his hypotheses are applicable to the digenetic tegument (Erasmus, 1967b). The tegumentary cells possess the morphological characteristics of secretory cells, and it seems highly probable that the secretory bodies originate in these cells, as suggested by Lumsden (1966) for cestodes and by Burton (1966) for Gorgoderina. The role of the secretory bodies is not clear, but they may contribute to this granular coat, or, as suggested by Burton (1966) to the outer matrix zone.

The outer granular coat has been demonstrated to consist of a mucopolysaccharide (see review by Lee, 1966). Meyer (1945) has found that many mucopolysaccharides and mucoids show a high resistence to digestion by proteolytic enzymes. This has led Monné (1959), and Bjorkman et al. (1963), to conclude that the granular coat protects the trematode from the digestive enzymes of the host. Monné also believes that the main tegumentary layer produces neutral mucopolysaccharides, which are converted to acid mucopolysaccharides, and are secreted onto the surface of the worm.

Not only is the surface of the flukes, but also the surface of the host intestine, covered by an acid mucrpolysaccharide layer (Ito, 1965). For this reason, it

can be supposed that the intestines of the hosts are protected from self-digestion, and from the proteolytic effect of enzymes secreted by the parasite. The nature of the mucopolysaccharide may be of importance for the regulation of host-parasite relationships (Ackert, 1942; Becker, 1953).

A number of processes could be involved in the uptake of nutrients through the tegument of the parasite. Vesicles have been observed in the outer portion of the tegument (especially inside forebody cup) of C. variegatus (Pl. 6, fig. 1). These vesicles closely resemble the ones described by Bogitsh and Aldridge (1967) in Posthodiplostomum minimum. The nature of the membranes of the vesicles is similar to the multilayered external plasma membrane, and in many cases, the structures have been described to swell in diameter, which led Bogitsh and Aldridge to "surmize that these vesicles may represent specialized endocytolic vesicles bringing material into the worm from the exterior."

A number of enzymes have been demonstrated in the general teguments of many species of trematodes, e.g. alkaline phosphatase has been detected in Fasciola hepatica (Halton, 1963,1967a; Thorpe, 1967, 1968); Schistomsoma mansoni (Dusanic, 1959 (cited by Lee, 1966); Lewert and Dusanic, 1966 (cited by Lee, 1966)); and Cyathocotyle bushiensis (Erasmus, 1968). Acid phosphatase was found in F. hepatica (Halton, 1963,1967a; Thorpe, 1967, 1968), and C. bushiensis (Erasmus, 1968).

It is possible that similar enzymes may also be present in the tegument of C. variegatus. The function of phosphatase enzymes in tegument of trematodes is not clearly known. In vertebrates phosphatases are present in tissues, such as the intestinal mucosa, where active transport is known to occur, and a functional relationship has been suggested (see review by Lee, 1966). In any case, a number of substances are known to pass through the trematode tegument, e.g. glucose is taken up by some trematodes. Mansour (1959) and Burton (1962) have both shown that glucose crosses the tegument of F. hepatica and Haematoloechus medioplexus. Senft et al. (1961) found that headless S. mansoni lived for weeks in a nutrient medium, and the authors claimed that absorption of nutrient took place through the tegument. Kuralec and Ehrlich (1963) showed that there is a transfer of amino acids in both directions across the tegument of F. hepatica. Tapeworms are capable of active transport with regards to amino acids (Read et al., 1963; Hopkins and Callow, 1965; and Read, 1966). Lee (1966) also believes that the trematode's tegument may be involved in excretion or osmoregulation.

Ohman (1966b) has recently reviewed the relationship of phosphatases in the helminth cuticle to (a) carbohydrate metabolism, (b) absorption, (c) secretion, (d) formation of tegument, and concludes that "some or all of these processes are associated with phosphatase activity in the cuticle of strigeids studied."

A number of possibilities thus exist for the absorption of nutrients through the tegument of the present species of trematode. The importance of the tegument in transporting nutrients varies throughout the parasite kingdom. In the Acanthocephala (Rothman, 1967; Hammond, 1968), and the cestodes (Lumsden, 1966; Read, 1966; Braten, 1968a,b) the tegument is extremely important. These parasites lack any type of a digestive tract, and so absorption takes places solely through the tegument. In a number of trematode parasites, e.g. Haematoloechus medioplexus (Rothman, 1968), F. hepatica, S. mansoni, the parasites are continuously bathed in a nutrient rich medium. It is reasonable to suppose, that the acquisition of necessary nutrients through the tegument has resulted from specializations by these parasites.

In C. variegatus and a number of other Strigeids inhabiting the lower portions of the host's digestive tract, a question exists as to the importance of the tegument as an interface involved in nutrient uptake. It has been suggested that the nutrients may be incorporated by (1) active transport or (2) pinocytosis. The interpretation of detectable phosphatase activity, as an indicator of sugar absorption by active transport, is considered incorrect by Read (1966). The vesicles described in the ventral body tegument (Pl. 6, fig. 1) are suggestive of the pinocytotic process (Bogitsh and Aldridge, 1967) in action. However, this is

highly conjectural and morphology alone cannot support this hypothesis.

If nutrients were absorbed through the tegument, then only by diffusion from cell to cell would these materials eventually reach the active body areas in the adults, e.g. the reproductive organs, which are involved in producing thousands of eggs per day. Studies on the rates of diffusion are lacking for the strigeid trematodes, but microscopic observations indicate that this distance would be in the range of millimeters. This leads one to suspect, that in the sexually mature parasites, the diffusion rates would be inefficient to supply the nutrient requirements. That diffusion occurs within the juvenile parasites (where the ratio of surface area to body volume is large) cannot be overlooked at this time.

(ii) Parasite Alimentary Tract

Strigeid trematodes possess a mouth (surrounded by oral sucker), pharynx, oesophagus and two caeca. Material, taken in through the mouth, is digested within the caeca. The surfaces of the caeca have been shown to consist of a number of protoplasmic projections resembling the intestinal microvilli of vertebrates. A number of vesicles have been found, which are believed to be associated with digestive secretory activity (Wotton and Sogandares-Bernal, 1963; Davis et al., 1963; Dike, 1967). Similar observations have

been made concerning the nematode intestine, e.g. Ascaris suum, Sheffield (1964); Nippostrongylus brasiliensis, Jamuar (1966). The intestine has therefore been considered to have a dual role - acting in secretion as well as food absorption. Thorsell and Bjorkman (1965) have found that secretions, produced by the intestinal epithelial cells of F. hepatica, result in lysis of ingested material, and that the microvilli are involved in the absorption of amino acids. Ohman (1966b) recently reviewed the histolytic nature of parasite secretions, and the occurrence of extracorporeal digestion and absorption through the caecal walls of a number of strigeid trematodes.

In C. variegatus, the developing metacercariae were first observed to feed 18 - 20 hours after infection. This feeding occurs just after primary attachment has been established. Within the digestive tract a small number of host erythrocytes were associated with a large amount of amorphous, eosinophilic material. The feeding on epidermal tissues and mucous secretions probably involves the mechanical action of the oral sucker. Halton and Jennings (1965) and Halton (1967b) have found that in a number of monogenetic and digenetic trematodes, feeding is accomplished by means of mechanical actions involving the oral sucker.

In addition, it is quite probable that the enzymes loosened host epithelial and mucous tissues, in the vicinity of the lappets, and perhaps the adhesive organ, are taken in

by the oral sucker. This supports the earlier ideas of Brandes (1890), La Rue (1927), Van Haitsma (1931a) and Baer (1933).

Prior to the attainment of parasite sexual maturity, primary feeding through the sucker seems to represent an adequate means for the nourishment of body cells. After three days, rapid growth of the hindbody occurs, and a corresponding development of the reproductive apparatus takes place. Although ingested food has been observed in the caeca of sexually mature specimens, the diffusion rates of these materials may be inadequate to supply the reproductive organs (for similar reasons as previously outlined in conjection with diffusion of materials taken in through the general body tegument). It seems probable that another mechanism is at work in the adult parasites, and would possibly provide the active organs with their nutritional requirements.

(iii) Surface of the Adhesive Organ

After attachment to the mucosa of the host by parasite, a portion of the tissue lies between the lobes of the adhesive organ. In the area of contact, the mucosal epithelium of intestinal villus is completely erroded and in this manner the host's lamina propria is in intimate contact with the inner surfaces of the adhesive organ lobes. This portion of host tissue is richly vascularized, and many small blood vessels lie directly in contact with the parasite's

tegument, with only a thin layer of endothelium separating the blood stream from the surface of the adhesive organ. Szidat (1929) thought histamine was released by the parasite into the intestinal tissues and resulted in the dilation of capillaries. Ohman (1966a) has not detected histamine in A. gracilis minor, and she has suggested that the swelling is probably due to a host reaction (involving histamine secretions) to the tissue damage caused by the parasite.

The large area of contact morphologically resembles a placenta (La Rue, 1932). The occurrence of a similar arrangement has also been described for a number of other strigeid trematodes (La Rue, 1927; Szidat, 1929; Van Haitsma, 1931a; Baer, 1933, etc.) and it was suggested that nutritive material from the host was absorbed through the tegument of the adhesive organ. No direct experimental evidence was available to confirm this suggestion.

Until recently the only experimental basis for a placental function came from histochemical studies on the tegument of adhesive organ. The presence of alkaline phosphatase activity was taken to represent an association of the transport of metabolites through helminth tegument (Erasmus and Ohman, 1963; Lee, 1966). However, if Read's (1966) suggestion is correct, then the association of alkaline phosphatase with absorption is no longer tenable.

In describing the ultrastructural morphology of the inner surfaces of the adhesive organ tegument for C. variegatus,

the surface modifications further suggest a placental arrangement. As previously described, the external plasma membrane is elevated into numerous microvilli. The microvilli greatly increases the surface area of the inner surfaces of the lobes, which are in direct contact with the host tissues. A similar arrangement has been described for H. luhei (Ohman, 1966b), C. bushiensis (Erasmus, 1967b, 1968; Erasmus and Ohman, 1963, 1965) and A. gracilis minor (Erasmus, 1969b). These authors have suggested possible mechanisms by which the secretions of enzymes, produced by the gland cells of adhesive organ, pass through to the exterior of parasite into the host tissues.

A question which follows from this is - what is the vehicle for the transportation of host nutrients once they have travelled through the adhesive organ tegument? Only two routes appear possible by which this could be accomplished: (a) diffusion from cell to cell, and (b) conveyance by the liquid contents of the excretory lacunae and ducts. In the past, it has been suggested that distribution was by the liquid contents of the excretory lacunae (La Rue, 1932; Van Haitsma, 1931a; Ohman, 1966a,b; Erasmus, 1967a, 1969b).

Prior to the present investigation no work had been reported concerning the morphology of the epithelial linings of adhesive organ lacunae. However, during the course of this investigation, Erasmus (1969b) published a paper on the ultrastructure of the adhesive organ in A. gracilis minor.

Erasmus (1967a) reported that external plasma membrane of lacuna epithelium was elevated to form stacks of lamellae lying on the surface. Histochemically these lamellae were shown to contain high levels of phosphatase activity, and he suggested that these characteristics may be related to a different function other than lipid excretion. In both C. variegatus and A. gracilis minor, the morphology of the lacunae epithelium suggests involvement in a secretory function, and in addition the lamellae have been described to be very close to the outer surfaces of the adhesive organ lobes (Pl. 21, fig. 1; Pl. 22, fig. 1).

In the living specimens, then, there is close proximity between the host tissues and the lacunae. The lacunae are filled with a liquid that is in immediate contact with the stacks of lamellae. Within the large voluminous spaces of the reserve bladder system the liquid surges from one part of the body to another (as a result of body muscular contractions). If the liquid contains nutrients, these substances would continuously bathe the organs of the hindbody and thus provide these cells with a supply of materials.

Not only do the lappets, sucker, etc. but also the reserve bladder system make their appearance during the development of metacercariae within the second intermediate host. They have also been demonstrated to be completely

functional in developed metacercariae (see review by Erasmus, 1967a). It may be that, in addition to functioning in providing rigidity and flexibility of the parasite against which muscles may act, this system may also be developed to act as a type of circulatory system used, for the distribution of nutrients when they become available.

It would seem then, that in light of the results discussed here, that the "adhesive organ" does not function as an attachment organ. It is equipped with the necessary mechanisms to provide a close juxtaposition to host tissues. The end result of this contact does not appear to be for the primary purpose of attachment, but rather for the provision of an intimate host-parasite interface - a "placenta" through which nutrients may pass. It seems that the name previously given to this organ is a misnomer and should perhaps be changed to the "accessory feeding organ". In order to establish the feeding function of this organ, research of the following types would have to be performed: (a) determining the chemical nature of the liquid found within the reserve bladder system, in particular detecting the presence of host materials, (b) a series of labelling experiments in conjunction with autoradiographic studies: (i) Label host materials and trace their paths as they migrate through the parasite tegument and finally into the lacunae. (ii) Label the excretory liquid and determine if the reproductive organs take up these materials.

SUMMARY AND CONCLUSIONS

1. In describing the growth of the parasites, it was found that during the first 4 - 8 hours after entry into the final host, the body dimensions decreased in size. Explanations have been suggested for this decrease by postulating an adaptive phase to a new environment, the lack of feeding during the interim, etc. After 8 hours, growth proceeded rapidly.

2. In developing parasites, growth of the attachment organs has been correlated with their attachment functions. No constant proportional increase in growth was maintained with time, when comparing one organ to another, or one organ to body size.

3. Eighteen hours after infection, primary attachment involving the oral sucker and lappets was immediately followed by parasite ingestion of host tissue.

4. Permanent attachment occurs within the large intestine and involves the following sequences:
- Primary attachment resulting from adhesion by the oral sucker and lappets; and, to a limited extent, the ventral sucker may also participate.

- The lappets invaginated into deep pocket-like cavities containing adherent host tissue.
- The adhesive organ everts, so that the inner surfaces of the lobes become closely appressed against host tissue.
- The ventral lip of the forebody extends up to the anterior body margin completing the suctorial cup.

5. During the growth and eversion of the adhesive organ lobes, both the oral and ventral suckers are displaced from the vicinity of host tissue. Thus, the suckers are of no importance in the maintenance of a permanent adhesion.

6. Permanent adherence of the parasite to its host, is the result of the lappet organs. Ultrastructural studies have demonstrated that the lappet organs are specialized for the purpose of maintaining a permanent adherence. Host tissue, in the area of the lappet contact, shows considerable alteration of the connective tissue. It is suggested that this altered host tissue is caused by lappet gland secretions. This altered tissue forms a firm basis for attachment by the finger-like projections of the lappet tegument, as well as the lappet's muscular system.

7. Evidently the adhesive organ is not involved in the formation of attachment, rather, the ultrastructural morphology is suggestive of a placental arrangement through

which nutrients are absorbed. It is possible that an interchange of materials may also take place through this area. Although the evidence is inferential, the terminology "adhesive organ" is questioned. In light of the present study, it is suggested that this organ be labelled as the "accessory feeding organ".

8. Additional support is given to the suggestion that the reserve bladder system functions as a primitive circulatory system.

DIAGRAMS 1 TO 3

Diagram 1

Illustrates the development of the characteristic body form and the adhesive organ in developing Cotylurus variegatus metacercariae. (All drawn to the same sizes for basis of comparison).

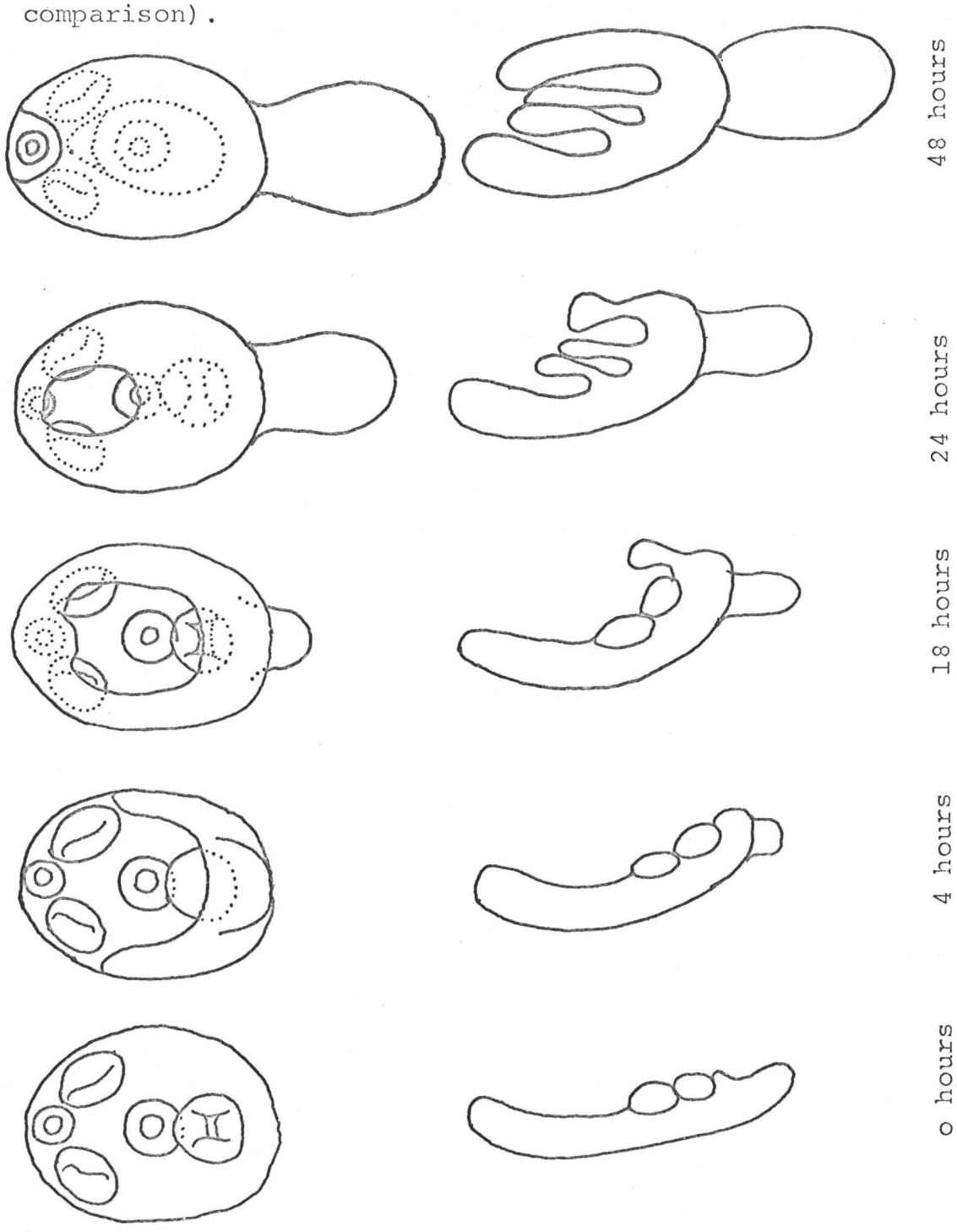


Diagram 2

Adult of Cotylurus variegatus showing the orientation of the attachment organs

1, caecum; 2, dorsal lobe of adhesive organ; 3, eosophagus; 4, lateral lappet; 5, lateral wall of suctorial pocket; 6, oral sucker; 7, pharynx; 8, anterio-ventral lip of suctorial pocket; 9, ventral sucker; 10, ventral lobe of adhesive organ

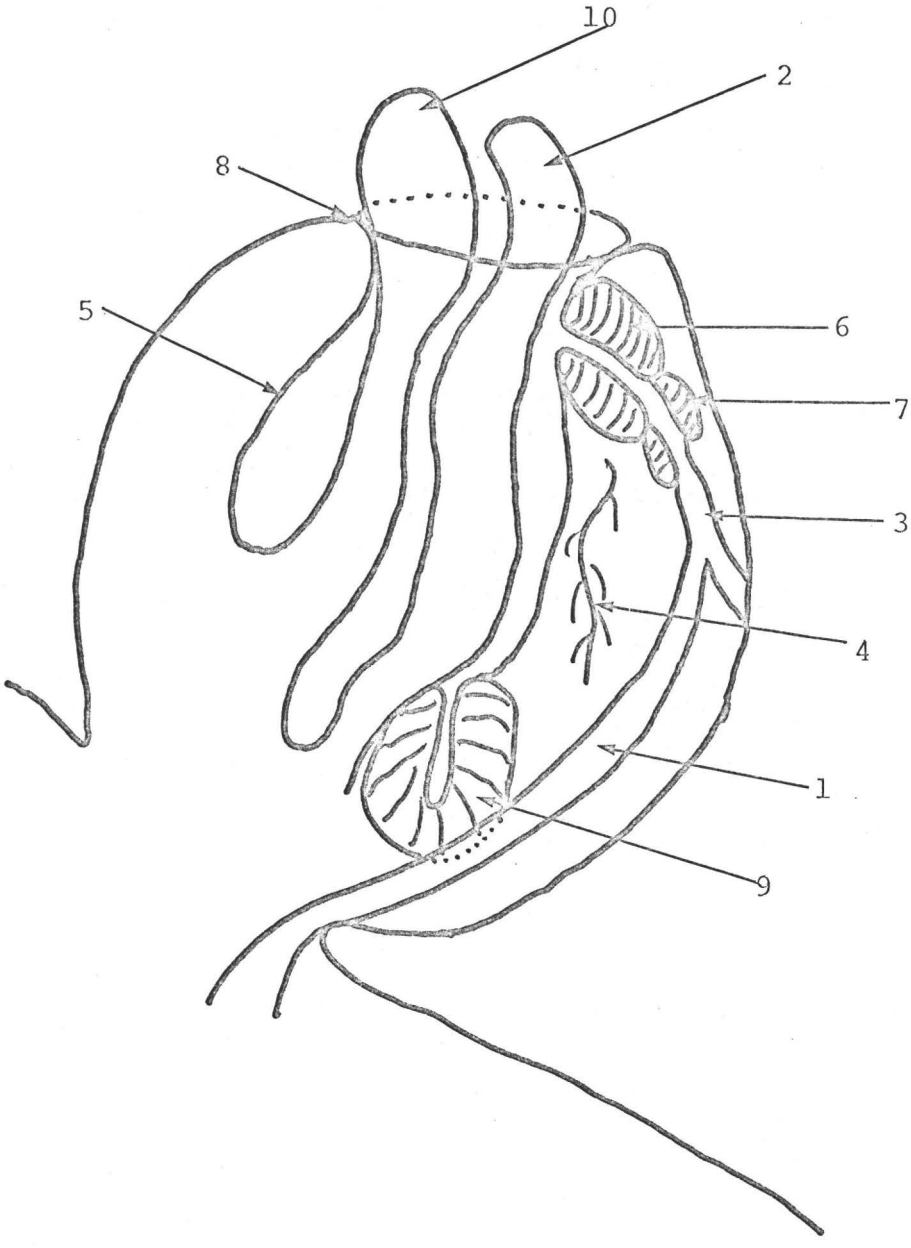
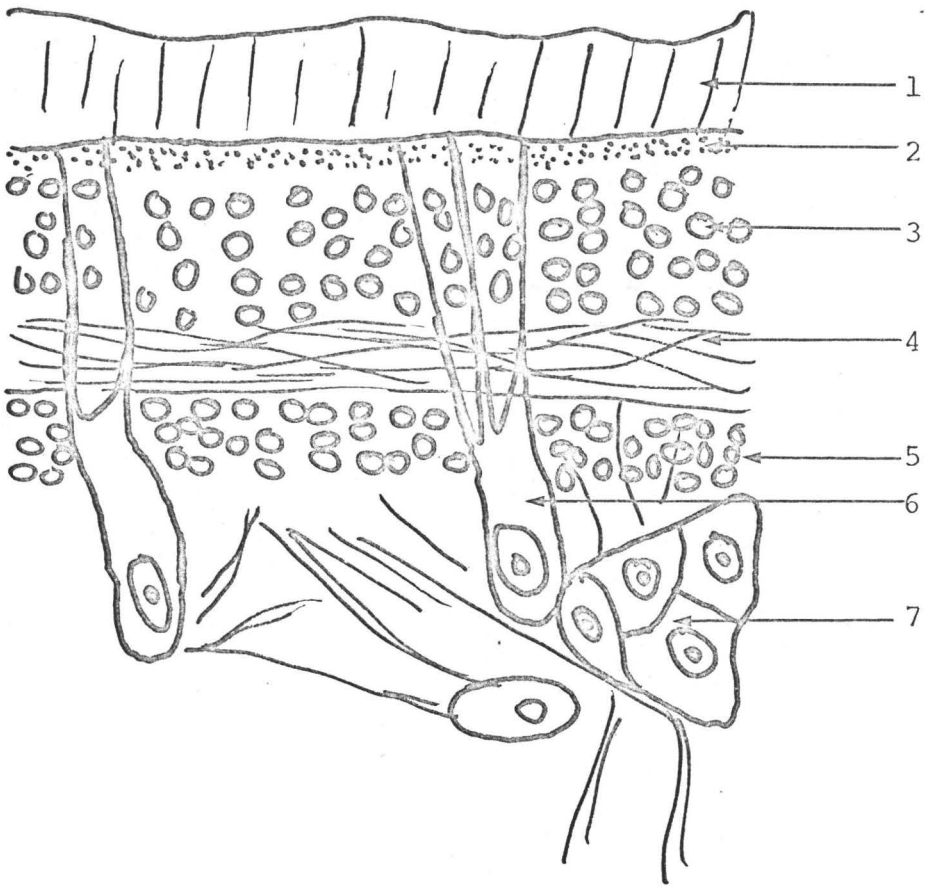


Diagram 3

Section of the Body Wall from Cotylurus variegatus

1, tegument; 2, basement membrane; 3, circular muscles; 4, longitudinal muscles; 5, oblique muscles; 6, subtegumentary cells; 7, parenchyma



PLATES 1 TO 22

LEGEND

AO	Adhesive organ
AOS	Adhesive organ surface
AP	Apical process
BL	Basement layer
C	Caeca
CLS	Cytoplasm lining lacunae system
DAL	Dorsal adhesive organ lobe
ER	Endoplasmic reticulum
GA	Golgi apparatus
HT	Host tissue
LU	Lumen
L LU	Lumen of lacunae
LAC	Lacunae
LAP	Lappet
Lap L	Lappet lip
Lap ST	Lappet subtegument
Lap T	Lappet tegument
Lap Tu	Lappet glandular tissue
M	Mitochondria
MC	Mucoid coat
MT	Microtubules
MU	Muscular bundles
MV	Microvilli
NU	Nucleus

OS	Oral sucker
P	Pharynx
PC	Parenchymal tissue
PM	Plasma membrane
S	Spine
SB	Secretion bodies
SC	Subtegumentary cells
SE	Setae
SLA	Stacks of lamellae
T	Tegument
TM	Tegument matrix
V	Vesicles
VAL	Ventral adhesive organ lobe
VBL	Ventral body lip
VBT	Ventral body tegument
VS	Ventral sucker

Plate 1

Fig. 1

Photograph of a gull nest taken on Mohawk Island near Dunnville, Ontario showing a newly hatched chick beside incubating eggs. Note the egg at the right hand side, it was at this stage just before hatching, that eggs were gathered for laboratory rearing. Mag 0.30 X.

Fig. 2

Photograph showing the opened abdominal cavity of an infected ring-bill gull. Note the enlarged portion of the large intestine. Mag 0.25 X.

Fig. 3

Photograph showing excised large intestine from an infected gull. A positive parasite infection is indicated by the enlargement of the large intestine just anterior to the caecal diverticula. Note the engorged blood vessels along this infected area. Mag 0.40 X.

Fig. 4

Large intestine of gull (Fig. 2) opened *in situ* showing the massive infection of adult parasites attached to the intestinal wall. Mag 0.20 X.

Fig. 5

Opened large intestine (Fig. 3) to show the cotylurid infection. In the anterior portion of the infection note the compacted faecal material surrounding the parasites. Mag 0.50 X.

Fig. 6

Opened large intestine of a gull previously infected with trematodes and by post mortem autolysis and freezing lost the infection. This photograph shows the enlarged flaps of villar tissue to which the strigeids had been attached. Mag 1 X.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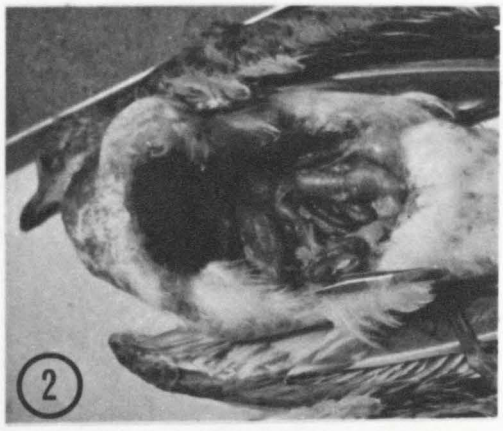
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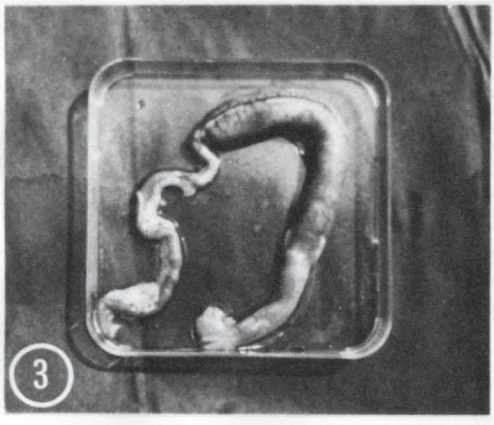
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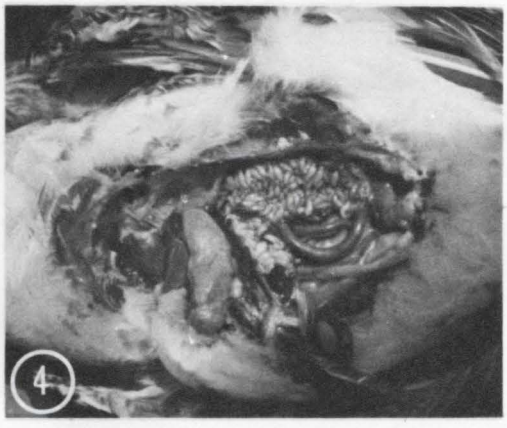
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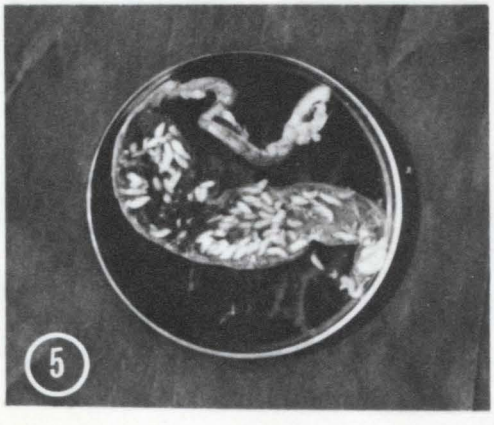
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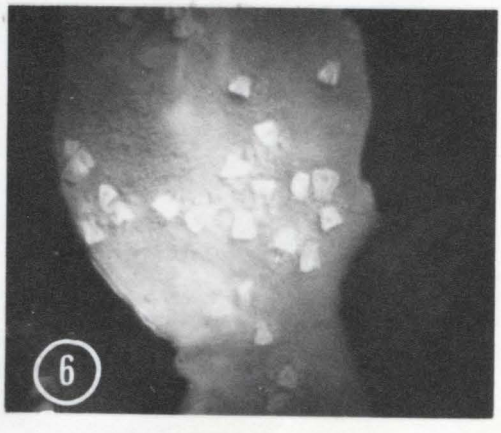
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Plate 2

Fig. 1

Longitudinal section of normal gull large intestine showing the general morphology including the villi, gland cells, and muscular layers. Formalin - H & E. Mag 250 X.

Fig. 2

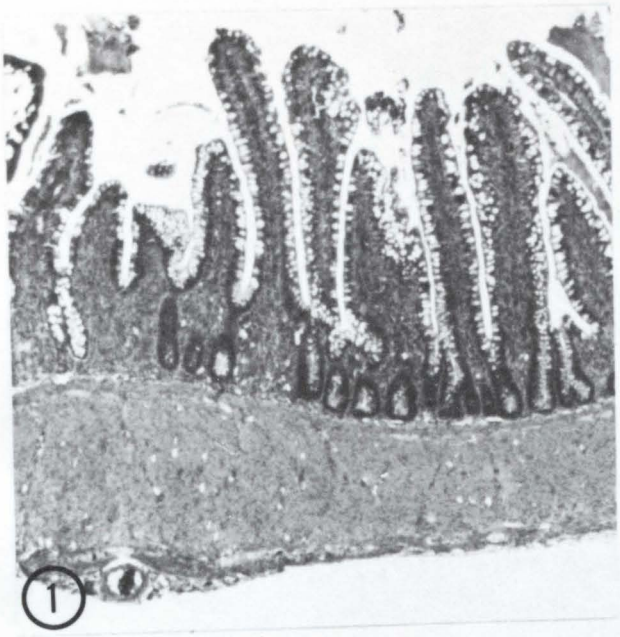
Longitudinal section of a villus showing the outer epithelial mucosa consisting of columnar epithelium and mucous cells. Beneath this layer of cells lies the lamina propria. Arrow indicates the striated border of the epithelial cells. Formalin - H & E. Mag 2,080 X.

Fig. 3

Electron micrograph of villar epithelium. Note the mucous cells. Also present are the long microvilli which give the epithelium its striated border appearance under the light microscope. Uranyl acetate - lead citrate. Mag 4,140 X.

Fig. 4

Whole mount of an excysted metacercaria showing the positions of the attachment organs as seen from the ventral surface. Formalin - aceto carmine. Mag 420 X.



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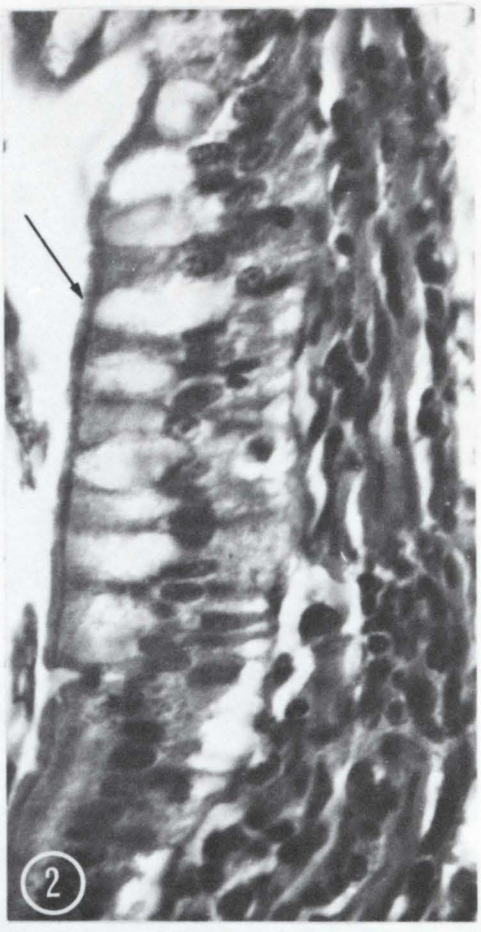

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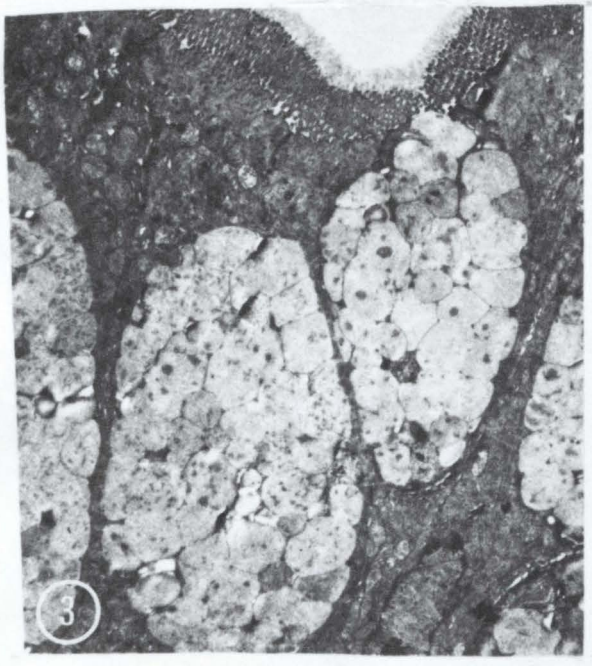

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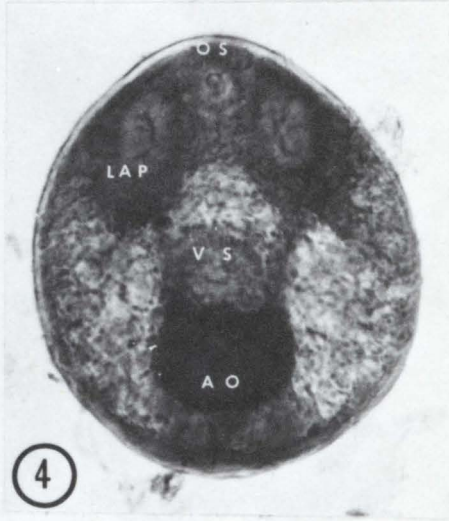

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Plate 3

Fig. 1

Whole mount of a metacercaria two hours post infection. Ventral view, ventral body lip not apparent. Mag 420 X.

Fig. 2

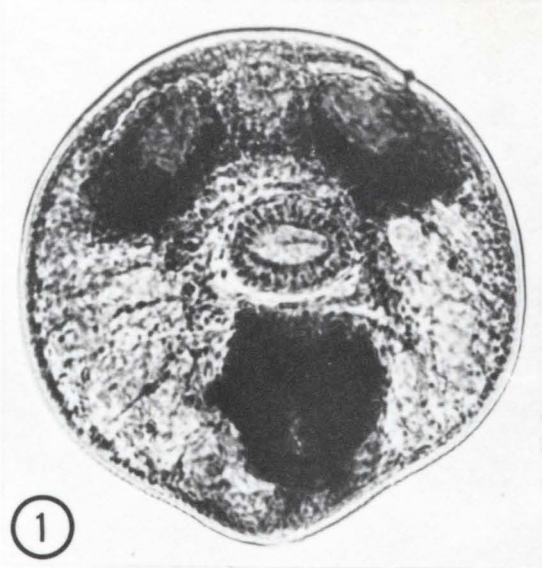
Metacercaria 20 hours post infection, ventral view. Note the development in comparison with 0 hour metacercariae (Plate 2, Fig. 4), of the posterior body. The position of the ventral lip is indicated by the arrow. Zenker - aceto carmine. Mag 470 X.

Fig. 3

24 hours after infection, whole mount, unattached metacercaria. Note the enlargement of the adhesive organ and posterior body. The arrow indicates the position of the ventral body lip as it extends across the surface of the adhesive organ towards the level of the lappets. Zenkers - aceto carmine. Mag 420 X.

Fig. 4

Whole mount of a 48 hour specimen as seen from the ventral surface showing increased development of the hindbody (equals the length of the forebody). Note position of ventral body lip (arrow) giving the parasite its characteristic cup-shaped forebody containing ventral sucker, adhesive organ, oral sucker, and lappets. The oral sucker opens posterioral and lappet's lips are facing inwards (compare their present position with that of the 0 hour metacercariae). Zenker - aceto carmine. Mag 400 X.



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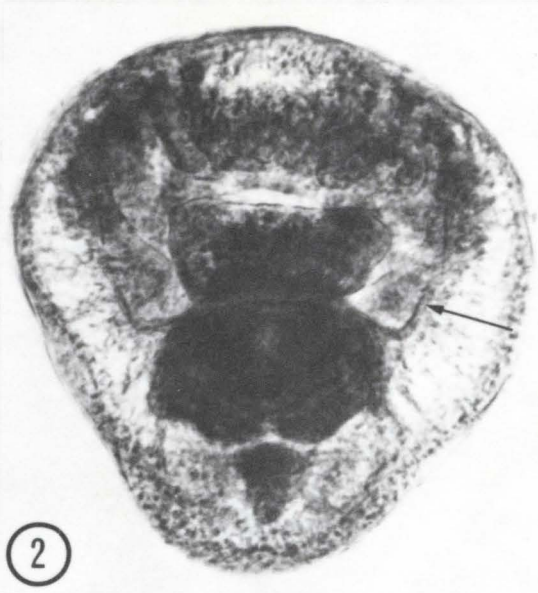

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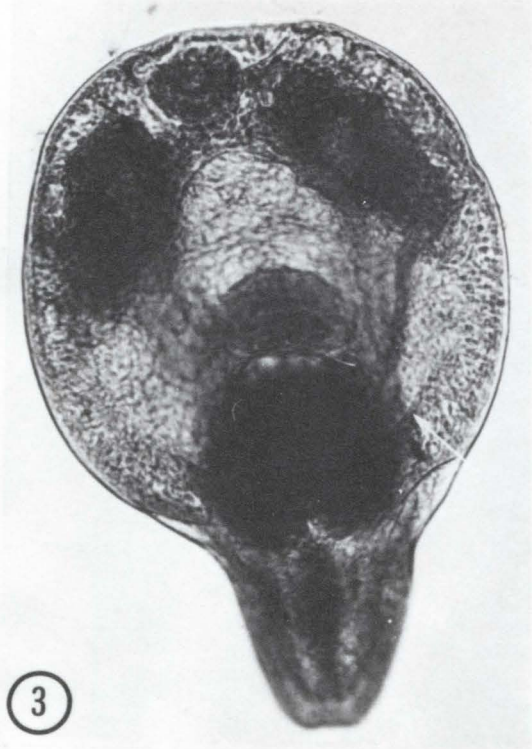

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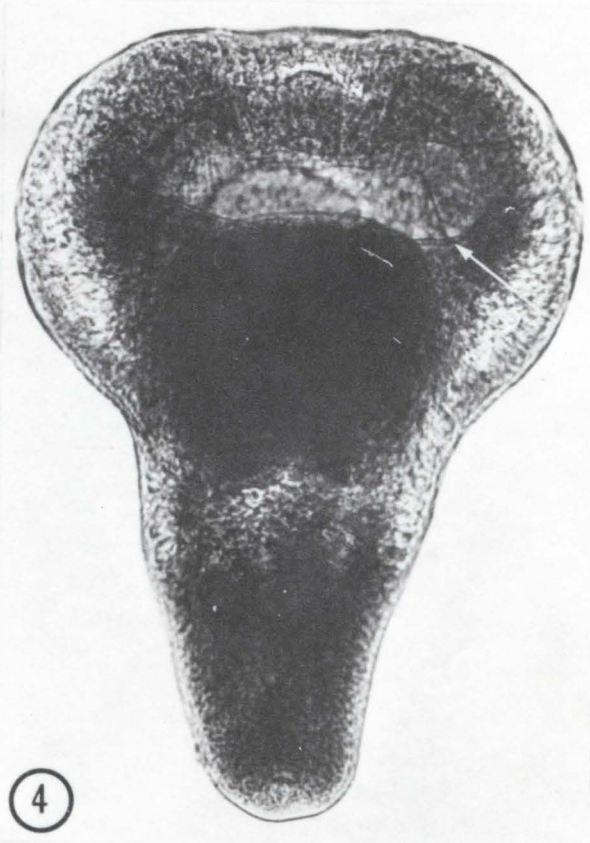

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Plate 4

Fig. 1

Electron micrograph of the general unspined tegument of a newly excysted metacercaria showing the morphology of the tegument and underlying subtegumentary layers. Arrows indicate the numerous tubular invaginations of the basal plasma membrane (not illus) as they project into the tegumentary matrix. Uranyl acetate - lead citrate. Mag 19,800 X.

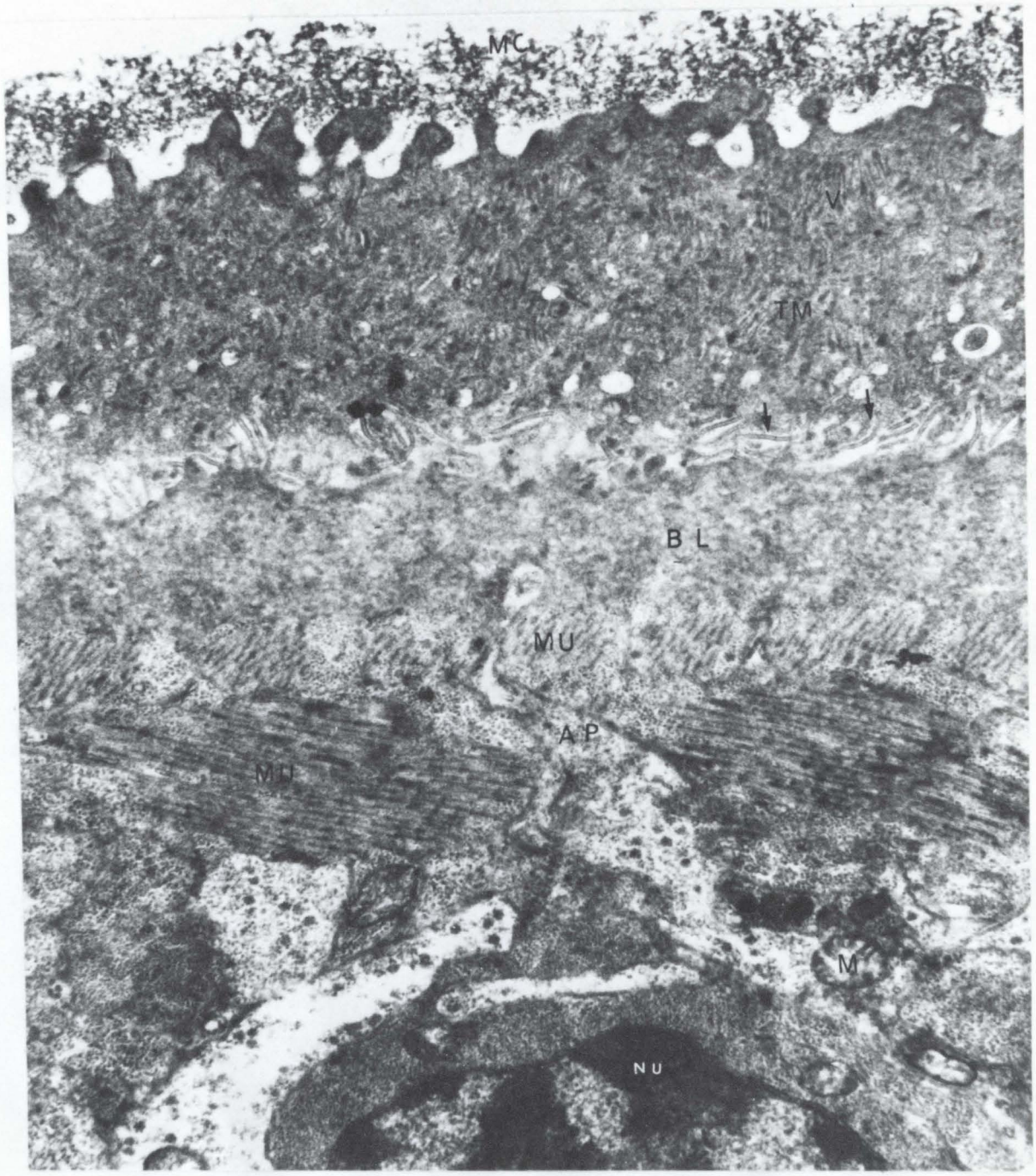
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Plate 5

Fig. 1

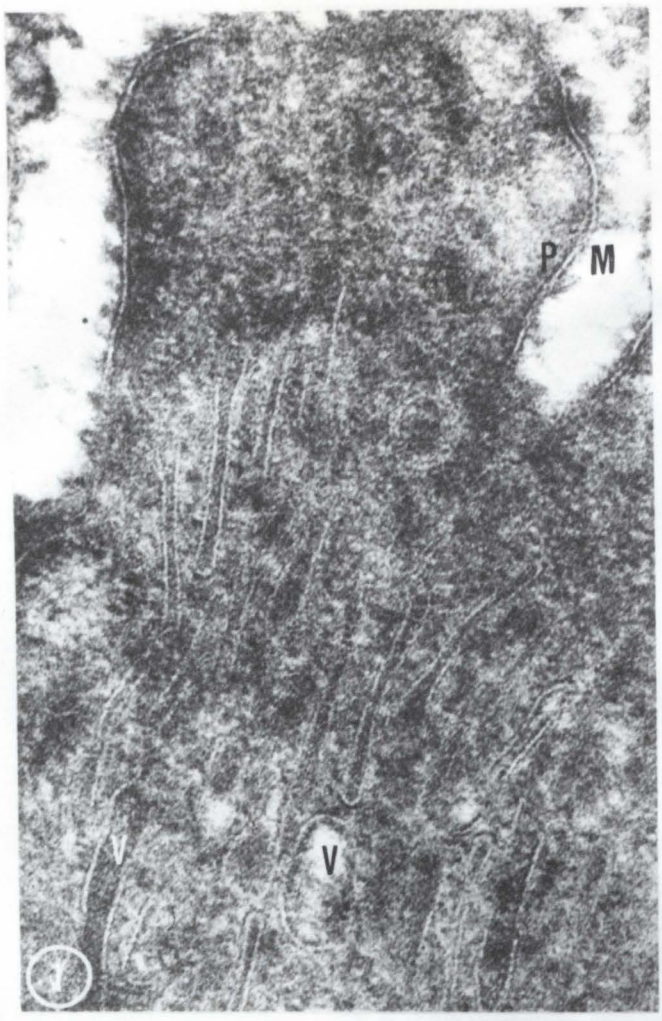
Section through outer body tegument showing the numerous membrane bound vesicles embedded in the matrix of the tegument. The external plasma membrane limits the cytoplasm of the tegument. Uranyl acetate - lead citrate. Mag 135,000 X.

Fig. 2

Section through a portion of the subtegument. The numerous nuclei are located within the cytoplasm of the membrane bound subtegumentary cells. Also present in their cytoplasm are numerous mitochondria and vesicles. Uranyl acetate - lead citrate. Mag 7,200 X.

Fig. 3

Detail of subtegumentary cell cytoplasm showing aggregations of vesicles. Note the Golgi complex and abundant endoplasmic retraction. Uranyl acetate - lead citrate. Mag 30,000 X.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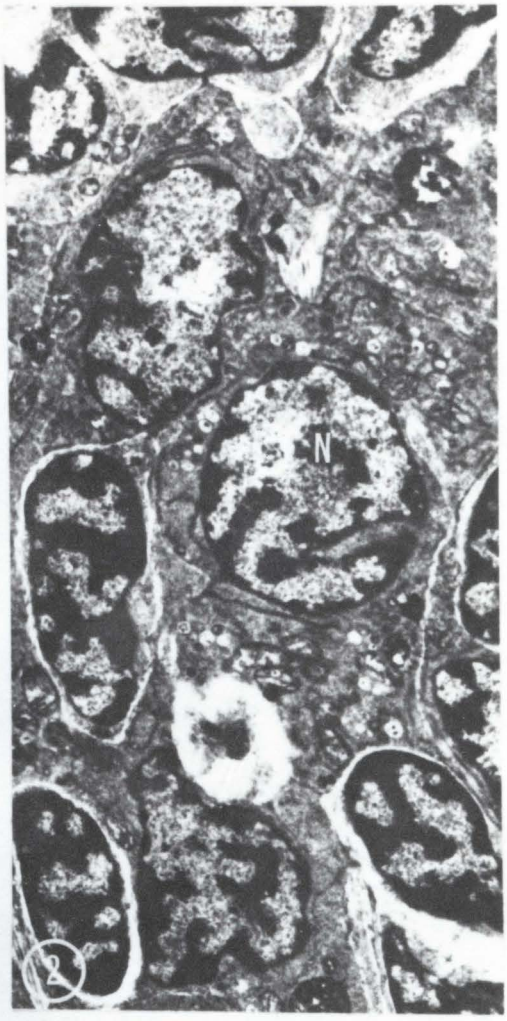
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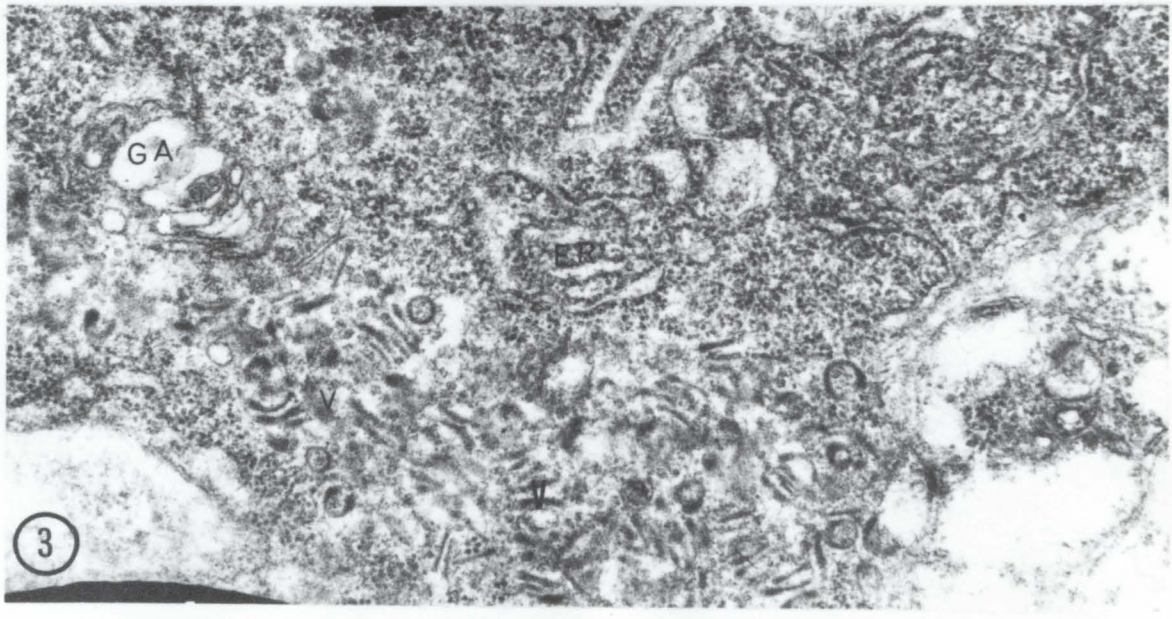
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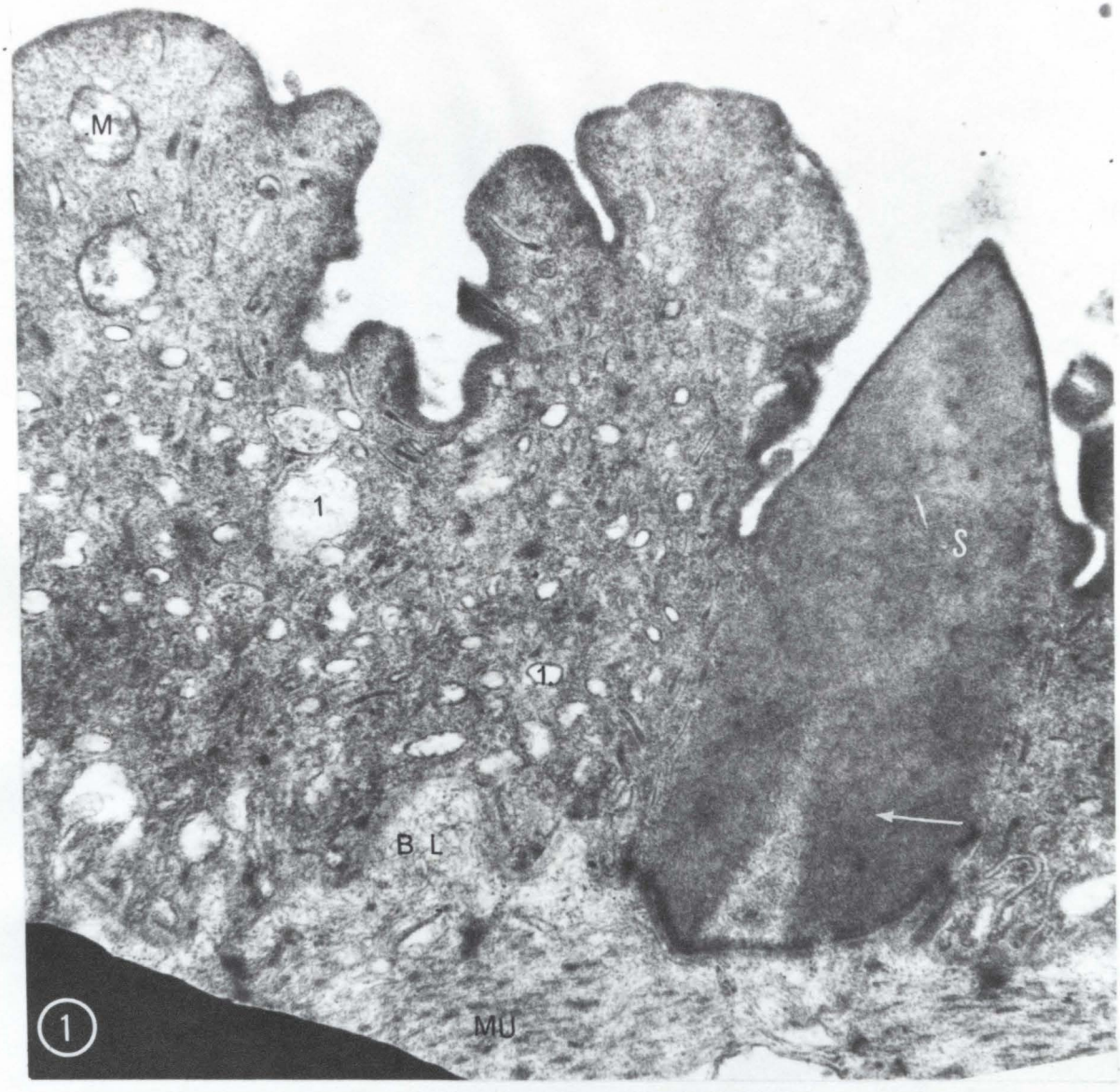
Plate 6

Fig. 1

Section through the spined, ventral body tegument showing the distal cytoplasm and spine. Arrow indicates the distinct lattice structure of the spine. Note the numerous mitochondria in the cytoplasm of the tegument. In addition to the rod and spherical vesicles a third type of vesicle (l) is present. These latter vesicles are considered to function in pinocytosis. Uranyl acetate - lead citrate. Mag 33,000 X.

Fig. 2

Transverse section through a metacercaria 20 hours post infection showing that the contour of host tissue is similar in outline to the attachment organs suggesting previous attachment. Formalin - H & E. Mag 500 X.


M
1
1
S
B L
MU
1

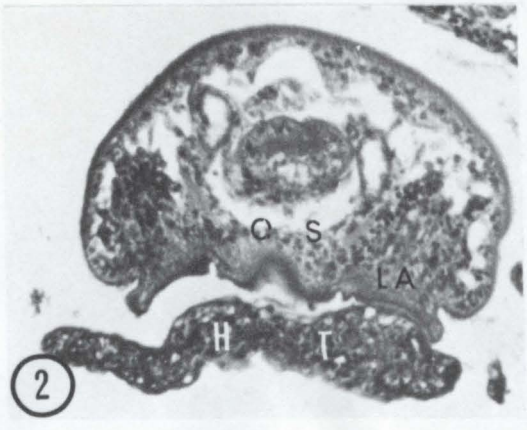

O S
LA
H
T
2

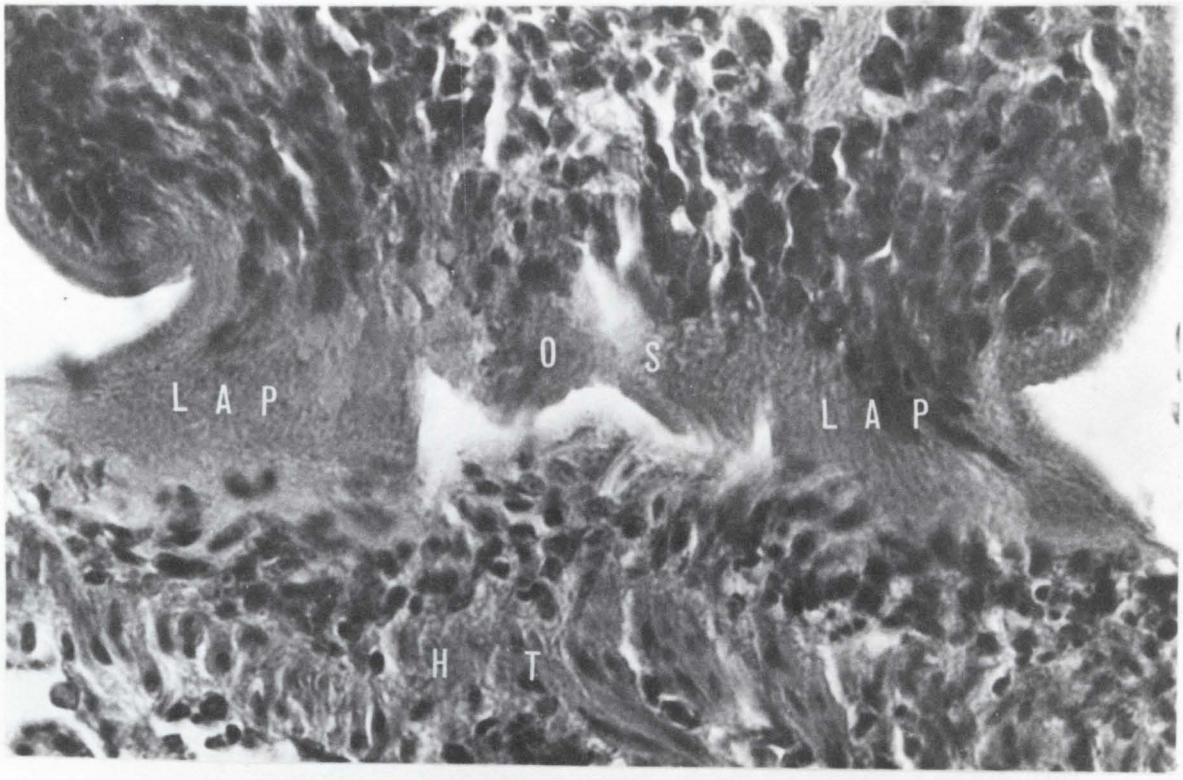
Plate 7

Fig. 1

Cross section of a metacercaria 24 hours post infection attached to the large intestinal villar tissue by means of the lappets and oral sucker. Formalin - H & E. Mag 2,000 X.

Fig. 2

Cross section of 72 hour metacercaria at the level of the oral sucker. Note portion of dorsal adhesive organ lobe within the cavity of the sucker and the host tissue between the two lobes of the adhesive organ. Formalin - H & E. Mag 500 X.


LAP
OS
LAP
HT

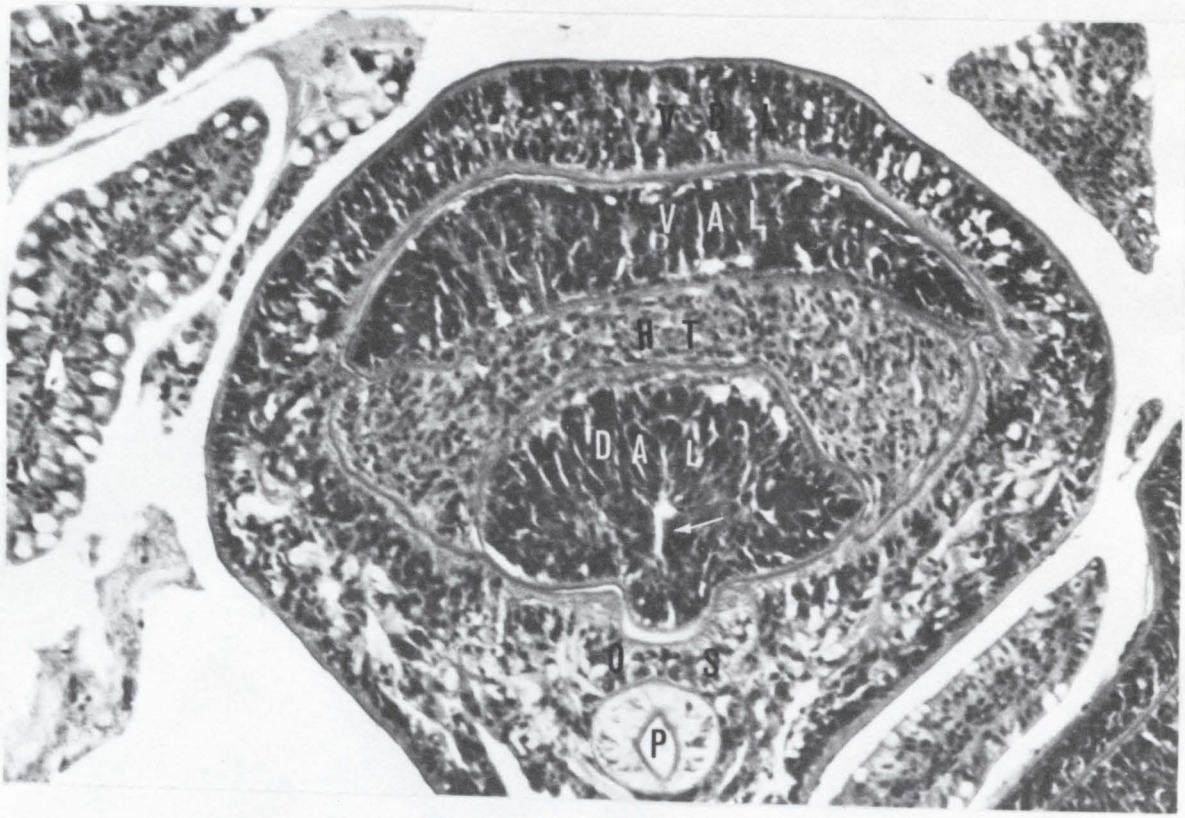

VAL
VAL
HT
DAL
OS
P

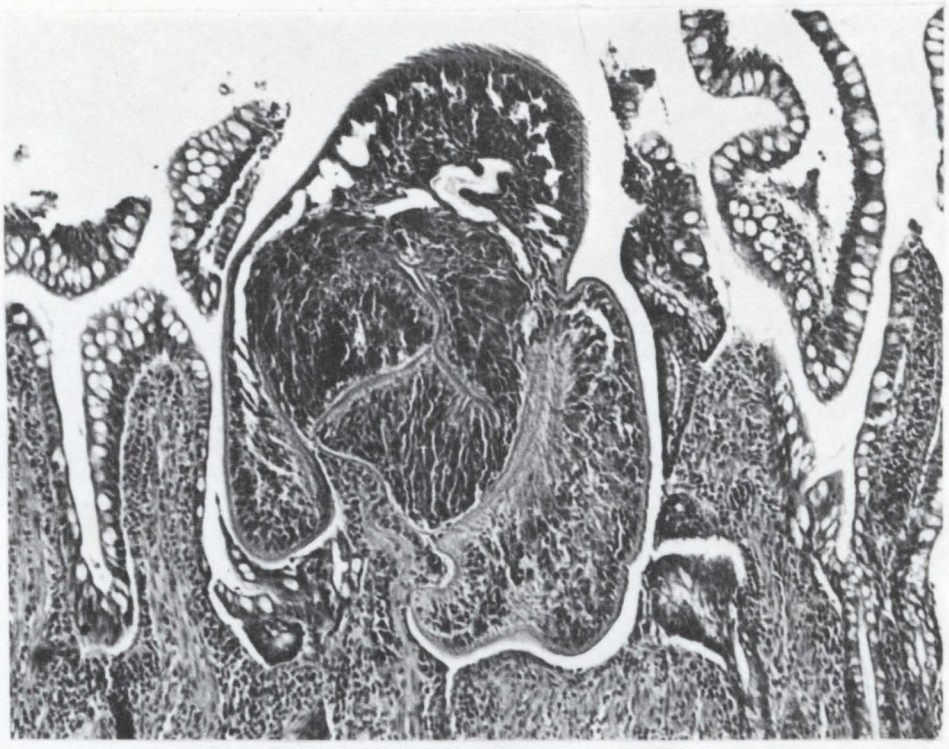
Plate 8

Fig. 1

Cross section of a 48 hour metacercaria recovered from the large intestine showing the orientation of the parasite to the villus. Formalin H & E. Mag 400 X.

Fig. 2

Enlarged photomicrograph of the 48 hour metacercaria (Fig. 1) showing the host tissue within the cavity of the oral sucker. Note that the epithelial layer adjacent to the adhesive organ has undergone very little morphological alteration at this time. Formalin - H & E. Mag 1,600 X.



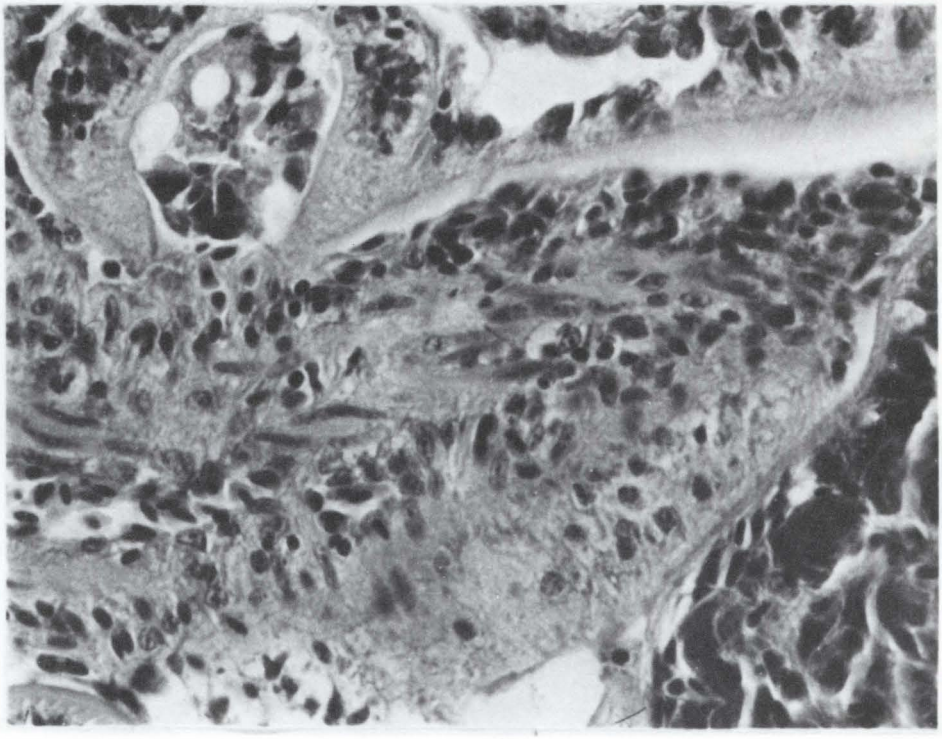


Plate 9

Fig. 1

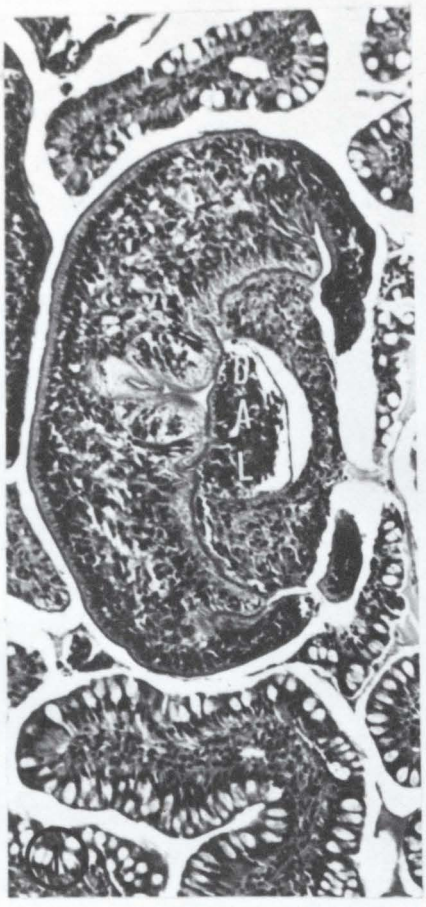
Cross section of a 72 hour metacercaria attached to a villus within gull's large intestine. The anterior portion of the dorsal adhesive organ lobe is in the proximity of the oral sucker separating it from the host tissue. Formalin - H & E. Mag 380 X.

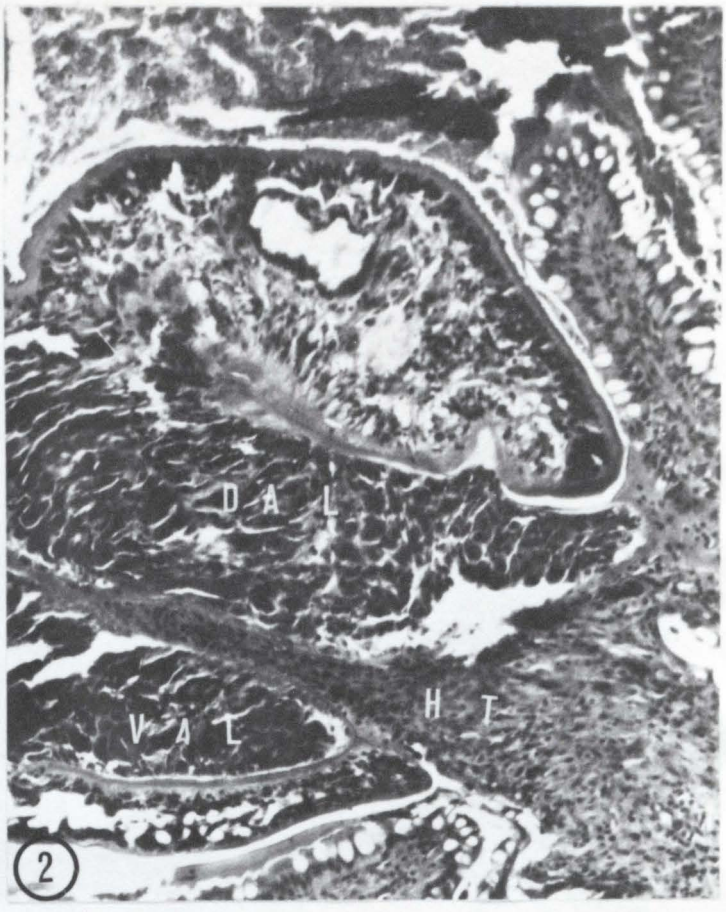
Fig. 2

Longitudinal section of a 72 hour specimen showing the oral sucker adhering to a portion of the dorsal adhesive organ lobe. Note that the host villar tissue lies between the two adhesive organ lobes. Formalin - H & E. Mag 400 X.

Fig. 3

Cross section through an 18 hour metacercaria demonstrating the histology of the oral sucker. Formalin - H & E. Mag 1,520 X.


D
A
L


D A L
V A L
H T
2

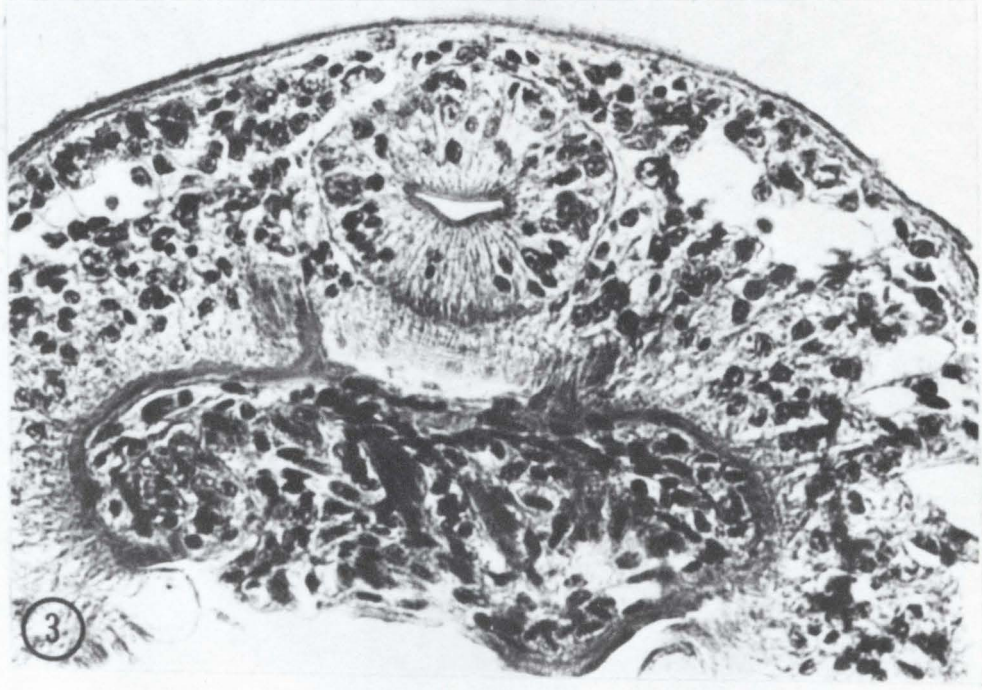

3

Plate 10

Fig. 1

Electron micrograph showing in cross section the morphology of the oral sucker, including the tegument, muscular systems and subtegumentary cells. Uranyl acetate - lead citrate. Mag 8,300 X.


L U
T
M U
S C
M U

Plate 11

Fig. 1

Electron micrograph showing the structure of the tegument and underlying basement layer and muscular bundles of the oral sucker. The arrows indicate the tubular invaginations of the basal plasma membrane as they project into the tegumentary matrix. Uranyl acetate - lead citrate. Mag 81,000 X.

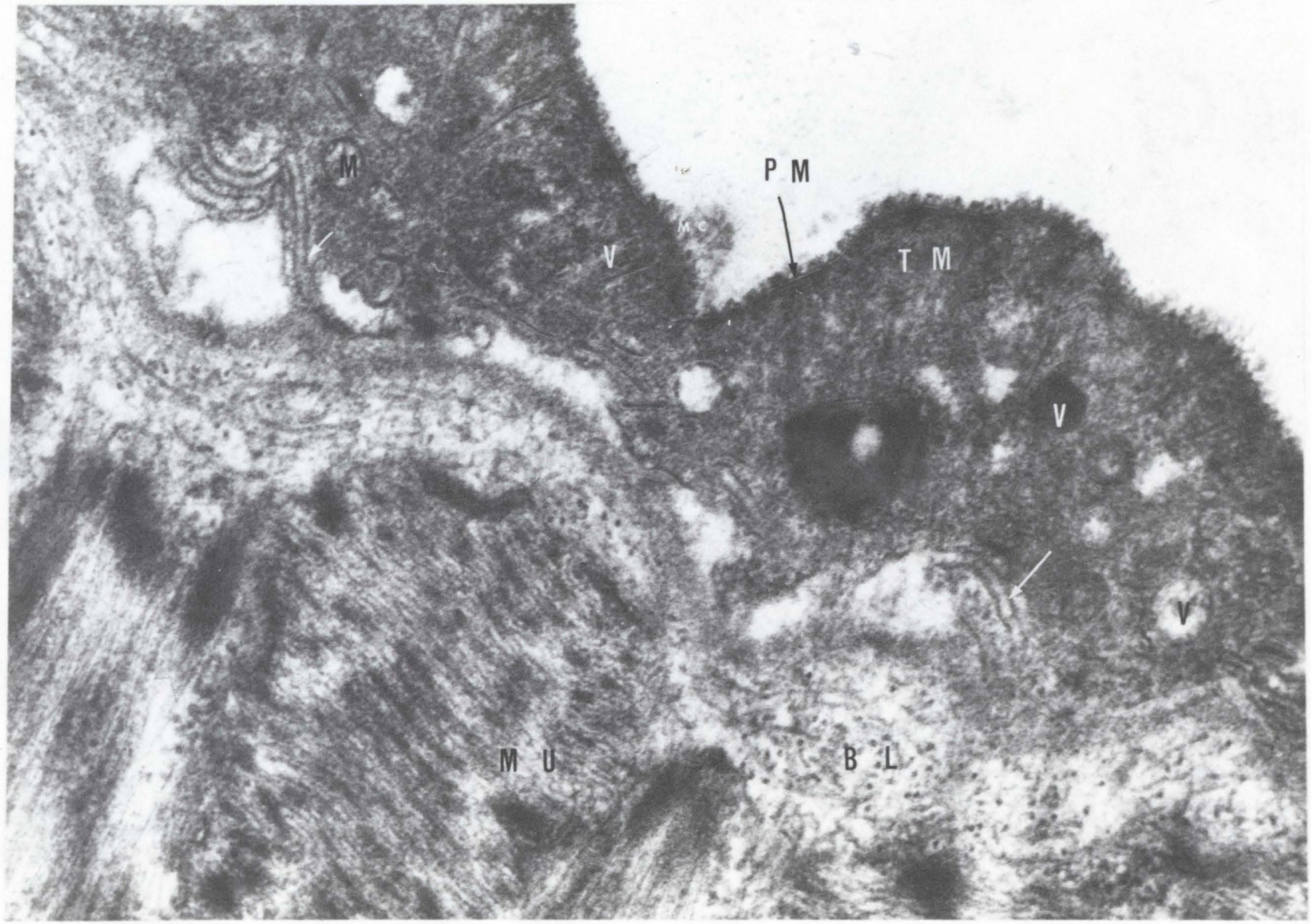

M
V
P M
T M
V
V
M U
B L

Plate 12

Fig. 1

Electron micrograph of the proximal wall of the oral sucker showing the detail of the base region including the limiting membrane, numerous muscular bundles with associated membranous structures (arrow heads) and numerous subtegumentary cells. The arrow indicates the membranous wall of an underlying cell.
Uranyl acetate - lead citrate. Mag 33,000 X.

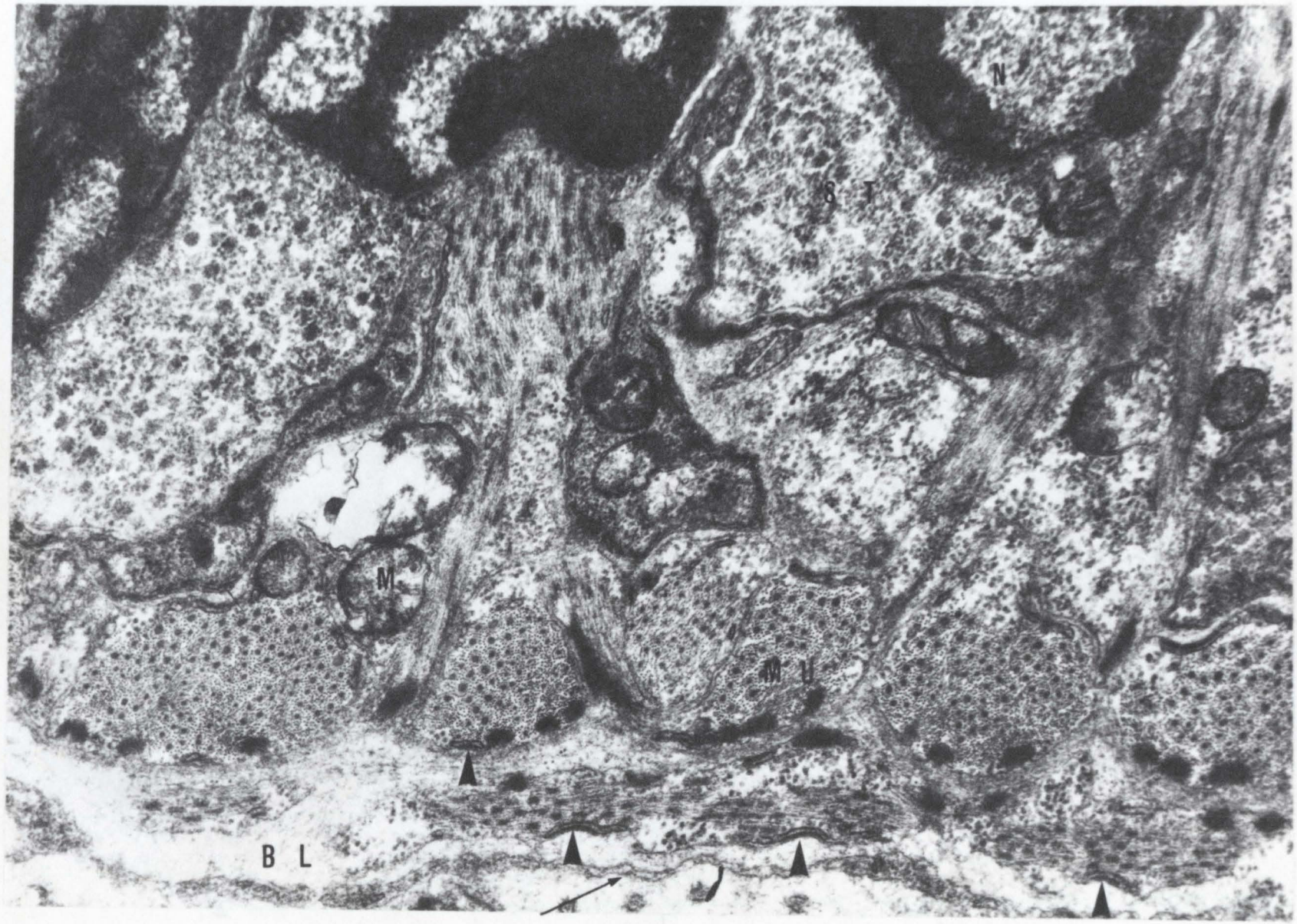

N
S
T
M
M
B L

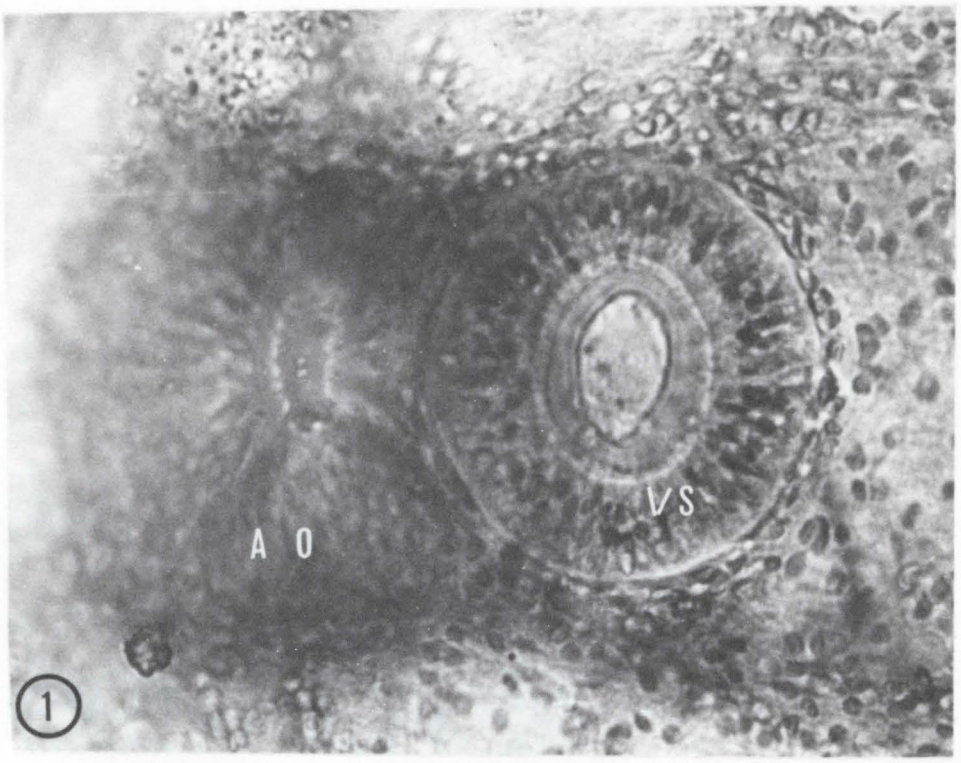
Plate 13

Fig. 1

Whole mount of newly emerged metacercaria, ventral surface, showing the position and histology of the ventral sucker. Formalin - aceto carmine. Mag 1,880 X.

Fig. 2

Cross section of a 72 hour metacercaria attached to a villus of the gull's intestine. Note the position of the ventral sucker lying in the junction between anterior body wall and dorsal adhesive organ lobe. Formalin - H & E. Mag 380 X.


A O
V S
1

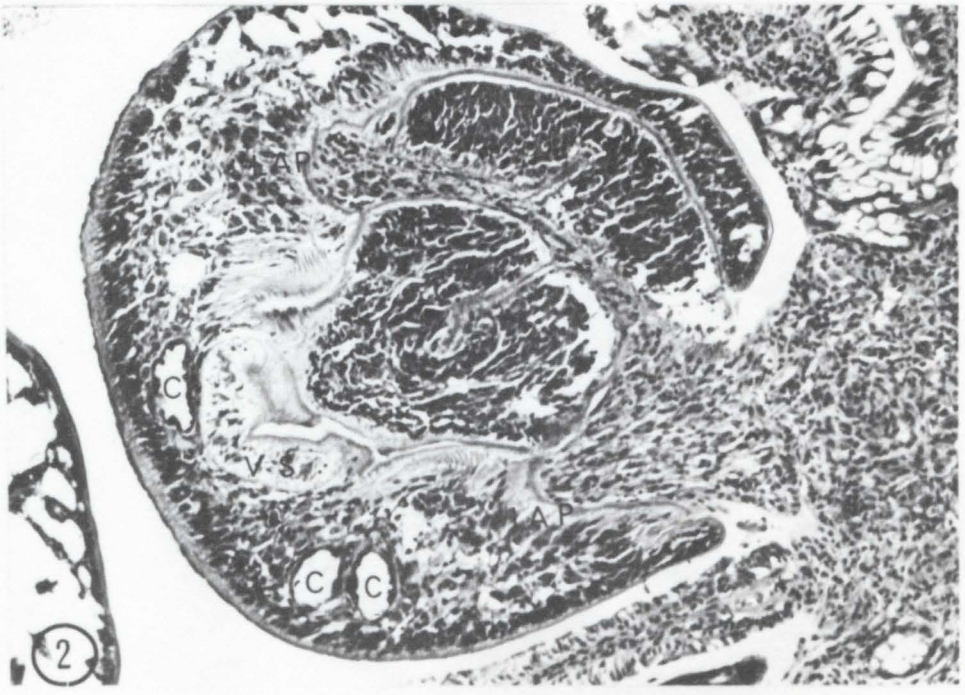

A P
C
V S
A P
C
C
2

Plate 14

Fig. 1

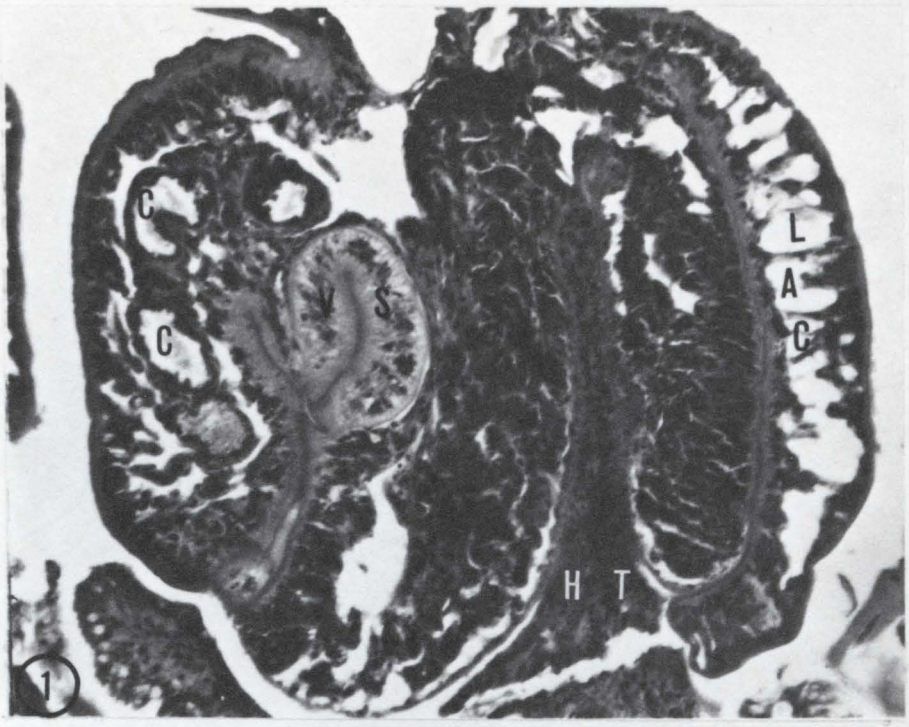
Cross section of 72 hour specimen showing the ventral sucker lying in the cephalic face of the dorsal adhesive organ lobe at some distance from host tissue. Formalin - H & E. Mag 400 X.

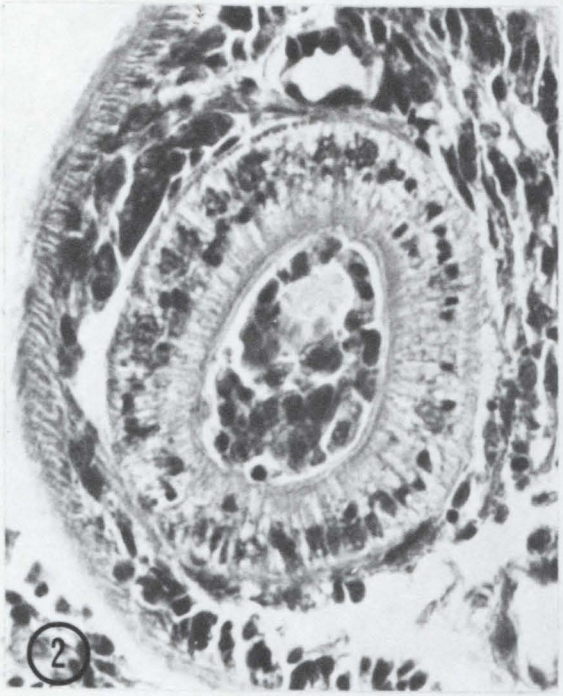
Fig. 2

24 hour metacercaria showing in cross section the cavity of the ventral sucker filled with host tissue. Formalin - H & E. Mag 1,680 X.

Fig. 3

Cross section of 24 hour metacercaria showing the general histology of the ventral sucker and the host tissue debris located within the sucker's cavity. Formalin - H & E. Mag 1,600 X.


C
C
V
S
L
A
C
H T
1


2

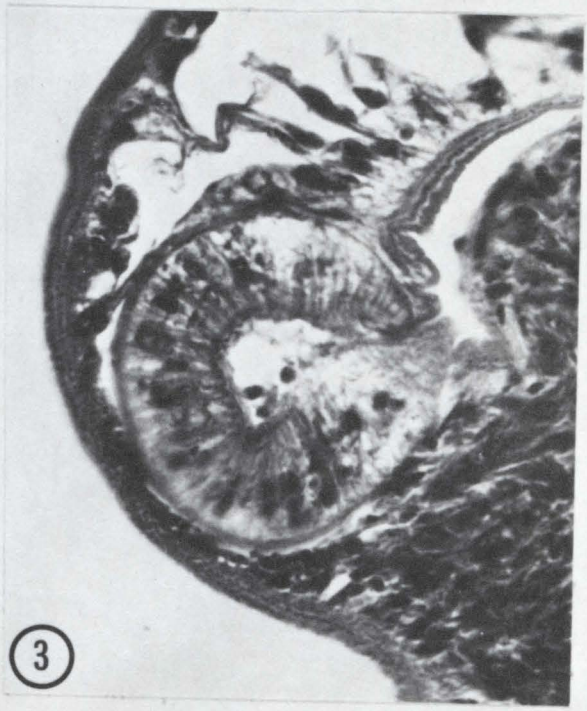

3

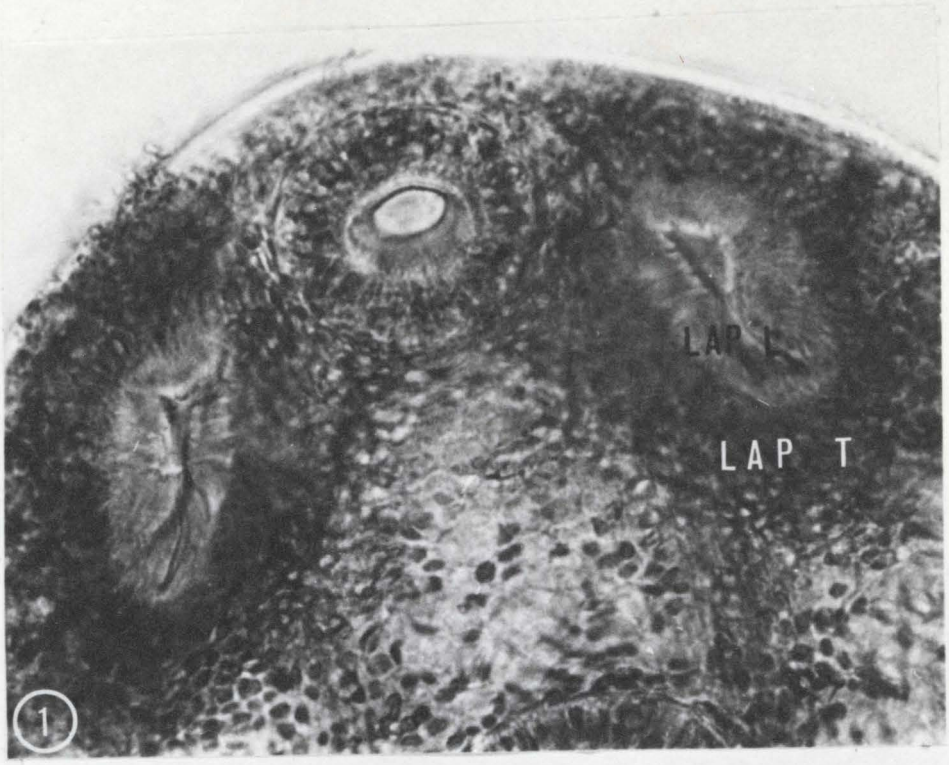
Plate 15

Fig. 1

Photomicrograph of the ventral surface of a whole mounted, newly excysted metacercaria showing the orientation and morphology of the inverted lappets. Formalin - aceto carmine. Mag 1,560 X.

Fig. 2

Whole mount of 18 hour metacercariae showing the lappets in the everted phase as seen from the ventral surface. Zenker - aceto carmine. Mag 1,560 X.


LAP L
LAP T
1

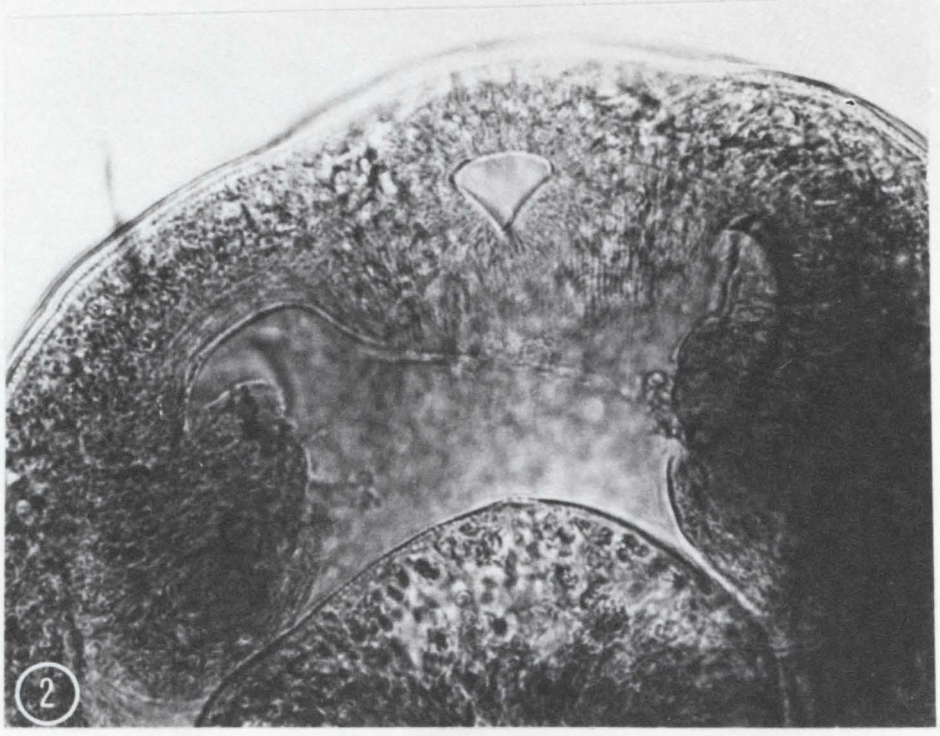

2

Plate 16

Fig. 1

Cross section of a 72 hour parasite to show the invaginated lappets (arrows) with host tissue within their cavities. Arrow head shows the ventral body lip closely applied to ventral adhesive organ lobe. Altered host tissue is intimately associated between the two adhesive organ lobes. Formalin - H & E. Mag 500 X.

Fig. 2

Cross section of a parasite 48 hours post infection showing the histology of the lappet organ. Zenkers - H & E. Mag 1,560 X.


P

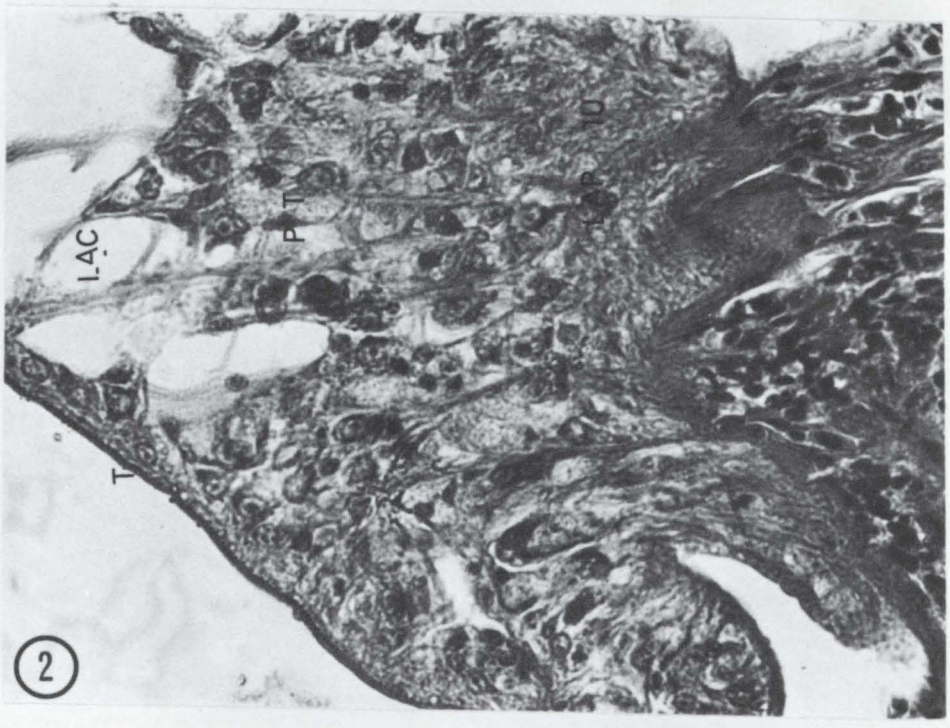

LAC
PT
T
2

Plate 17

Fig. 1

Electron micrograph of the lappet tegument, subtegument and gland cells of a newly excysted metacercaria. Arrows point to the probable route in releasing the secretory products. Arrow heads show the lattice like structure of the setae. Uranyl acetate - lead citrate. Mag 26,400 X.

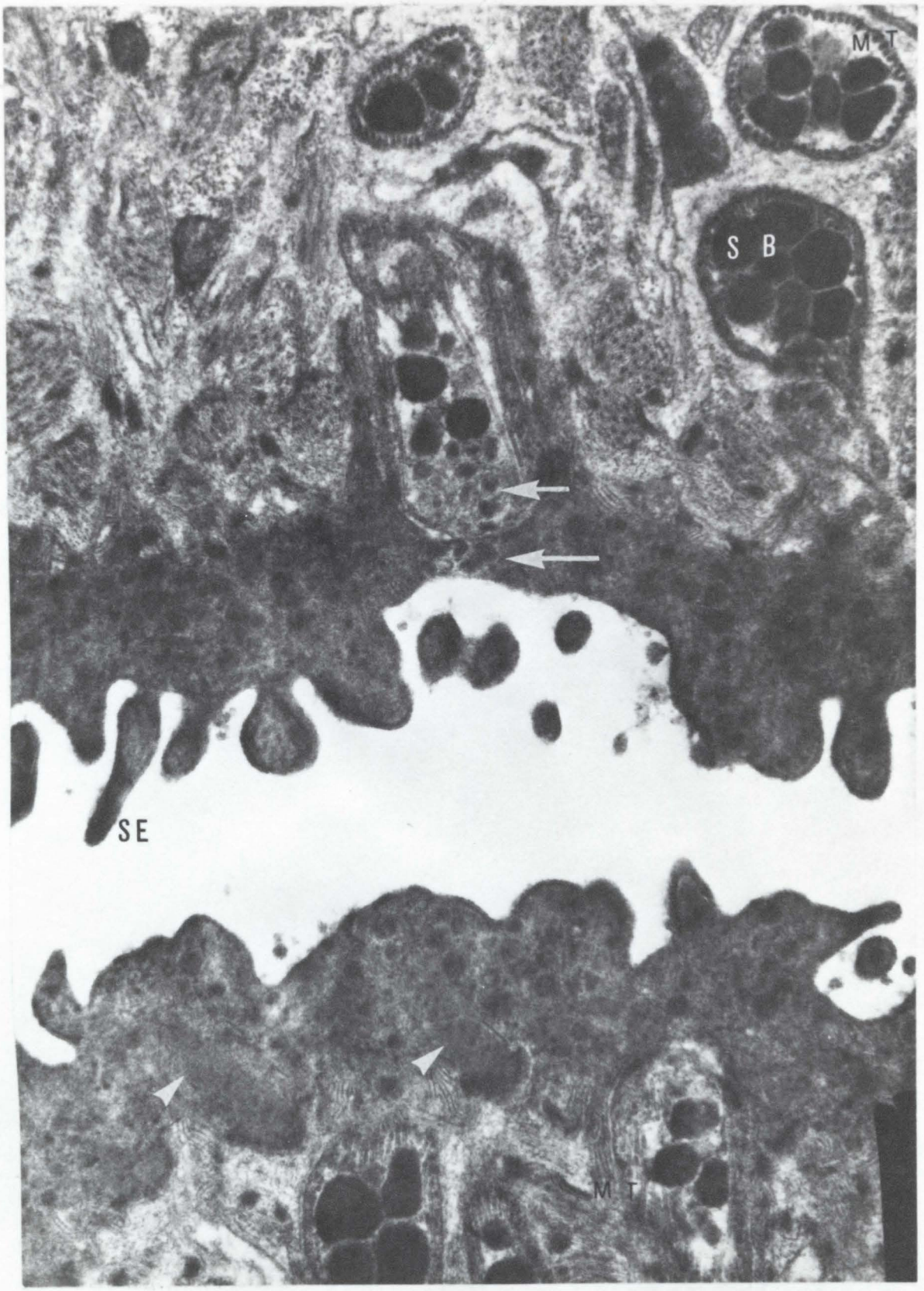

M T
S B
SE
M T

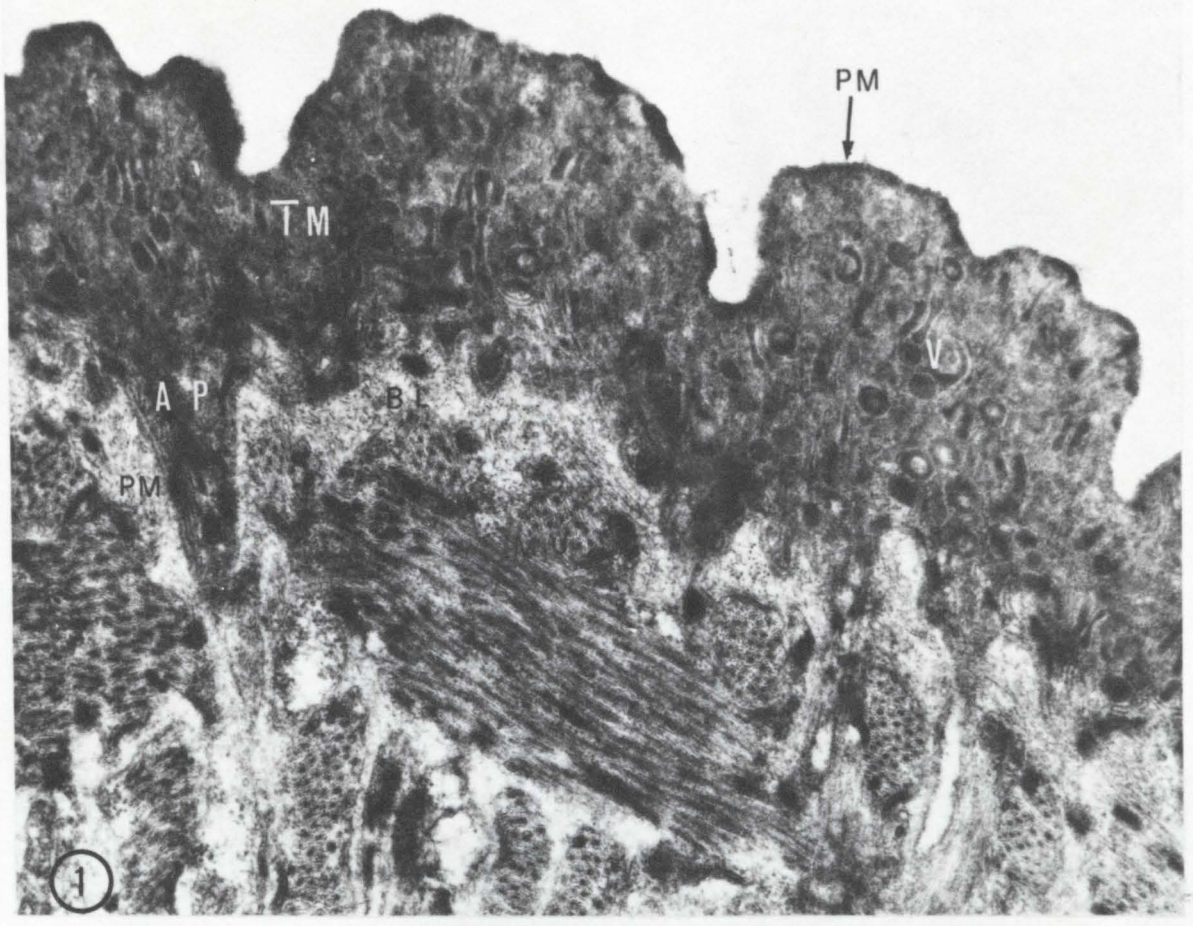
Plate 18

Fig. 1

Electron micrograph showing a portion of the lappet lip of a newly excysted metacercaria. Note the morphology of the tegument and subtegument areas. Uranyl acetate - lead citrate. Mag 24,000 X.

Fig. 2

Cross section of the lappet subtegument showing the process of a subtegument cell whose cytoplasm is filled with numerous vesicles. Also present are the processes of two lappet gland cells filled with electron dense secretion bodies. Note that at this level the microtubules of the gland cells are absent. Uranyl acetate - lead citrate. Mag 41,400 X.


PM
TM
V
A P
B I
PM
1

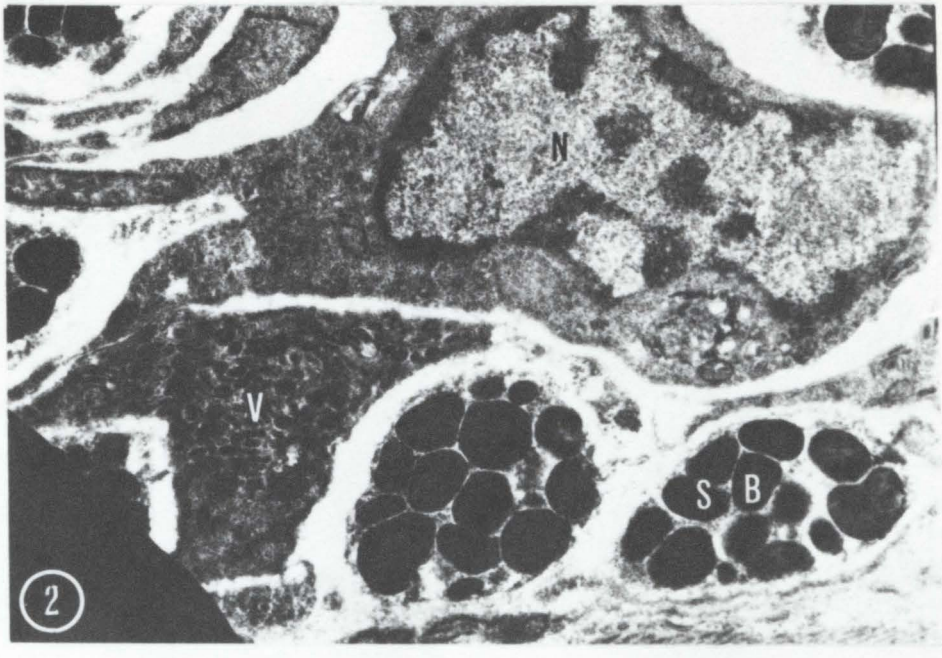

N
V
S B
2

Plate 19

Fig. 1

The outer region of the lappet tegument of a 6-day old specimen showing the adhesion to the proximal host tissue. Arrows indicate the strands of altered host tissue penetrating the main mass of the host tissue. Uranyl acetate - lead citrate. Mag 4,100 X.

Fig. 2

Electron micrograph of labelled area in Fig. 1 showing the altered lappet tegument. The arrows indicate the particulate material which lines the outer lappet projections. Uranyl acetate - lead citrate. Mag 13,800 X.


LAP
LAP T
A O
1

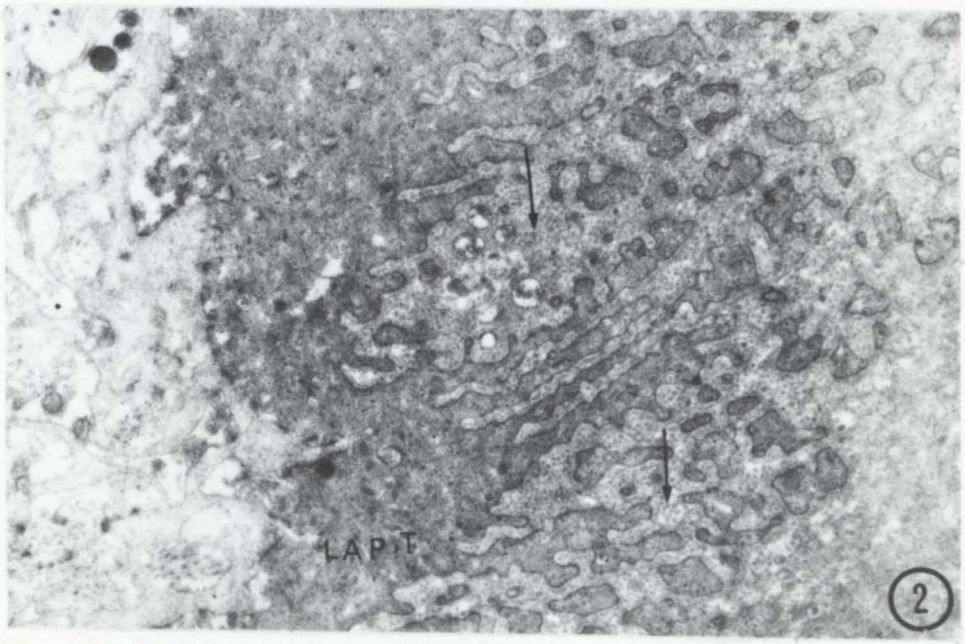

LAP T
2

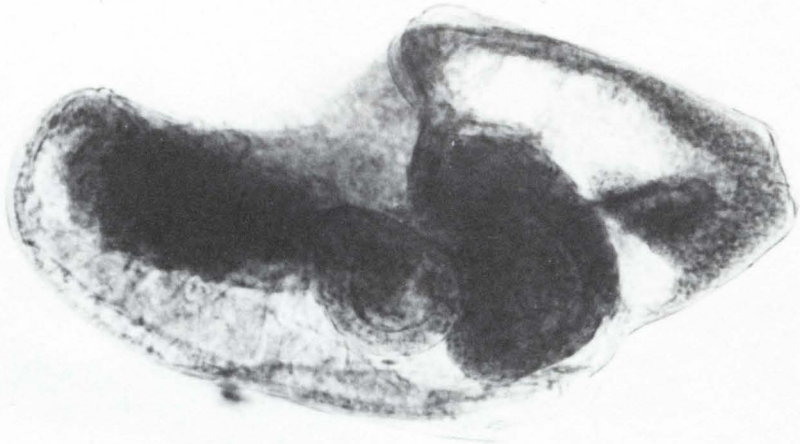
Plate 20

Fig. 1

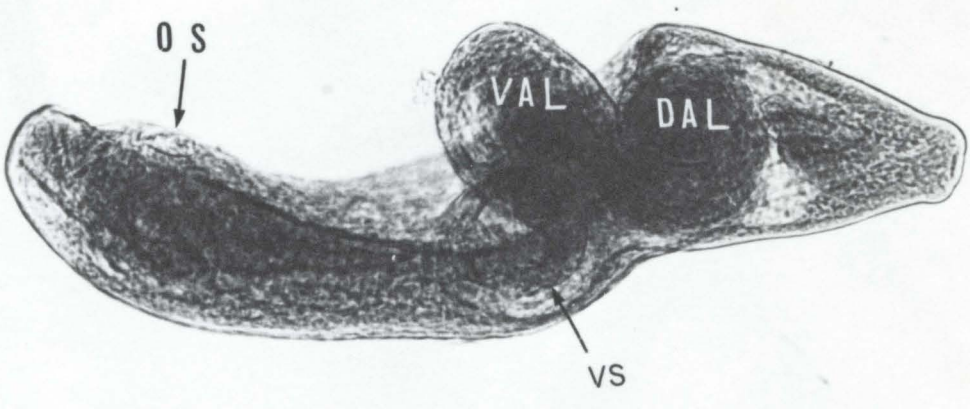
Whole mount of an 18 hour specimen, lateral view, showing the lobes of the adhesive organ in the contracted or involuted position. Zenkers - aceto carmine. Mag 500 X.

Fig. 2

Whole mount of an 18 hour specimen, lateral view, showing the ventral adhesive organ lobe in a state of complete eversion. Zenkers - aceto carmine. Mag 500 X.



①



②

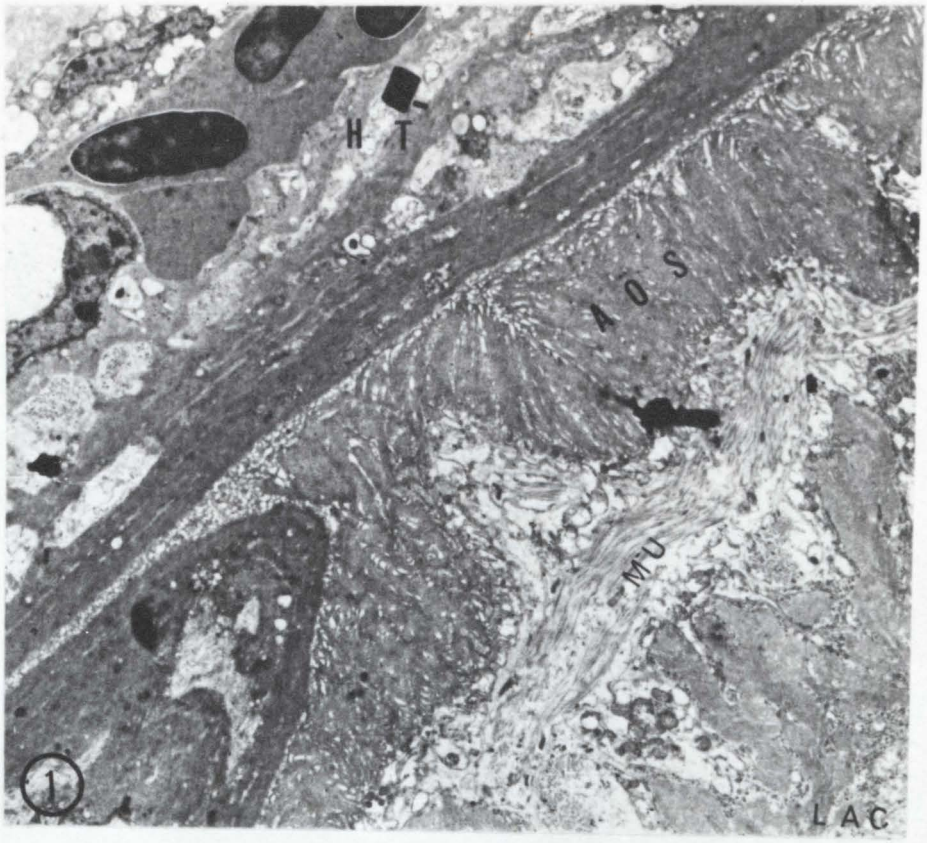
Plate 21

Fig. 1

Electron micrograph of a 6-day old specimen showing the surface of the adhesive organ and associated host tissue. Note the proximity of the excretory lacunae to the surface of the adhesive organ. Uranyl acetate - lead citrate. Mag 4,100 X.

Fig. 2

Electron micrograph showing the detail of the adhesive organ surface. Arrow indicates position of two secretory bodies. Uranyl acetate - lead citrate. Mag 13,800 X.


H T
A O S
MU
LAC
1

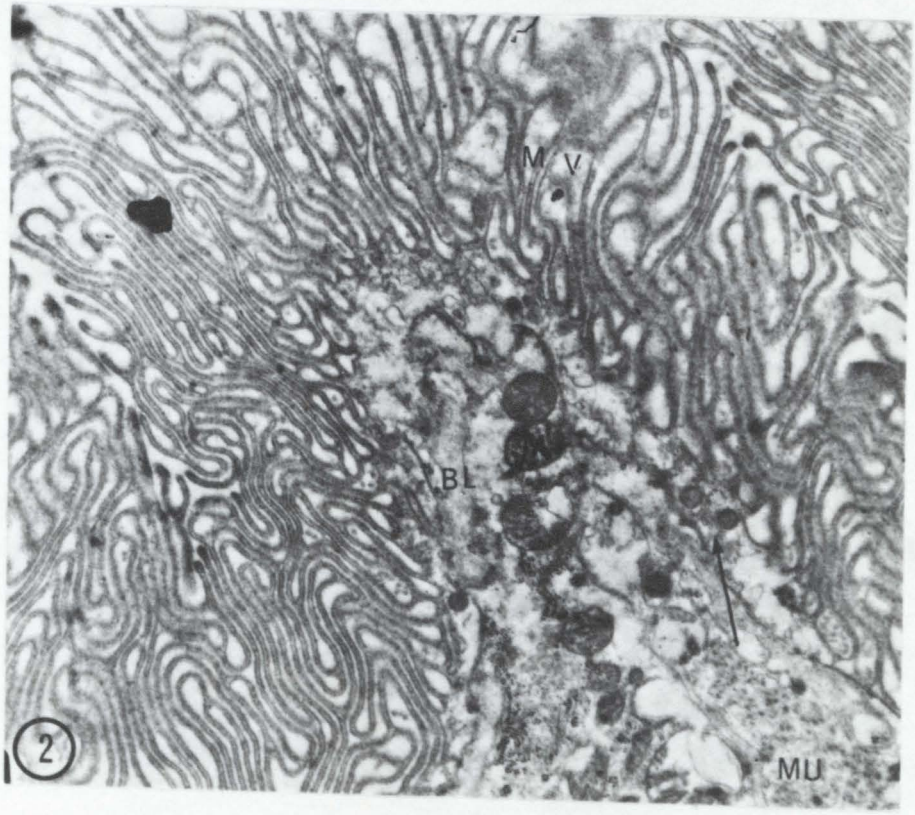

M V
BL
MU
2

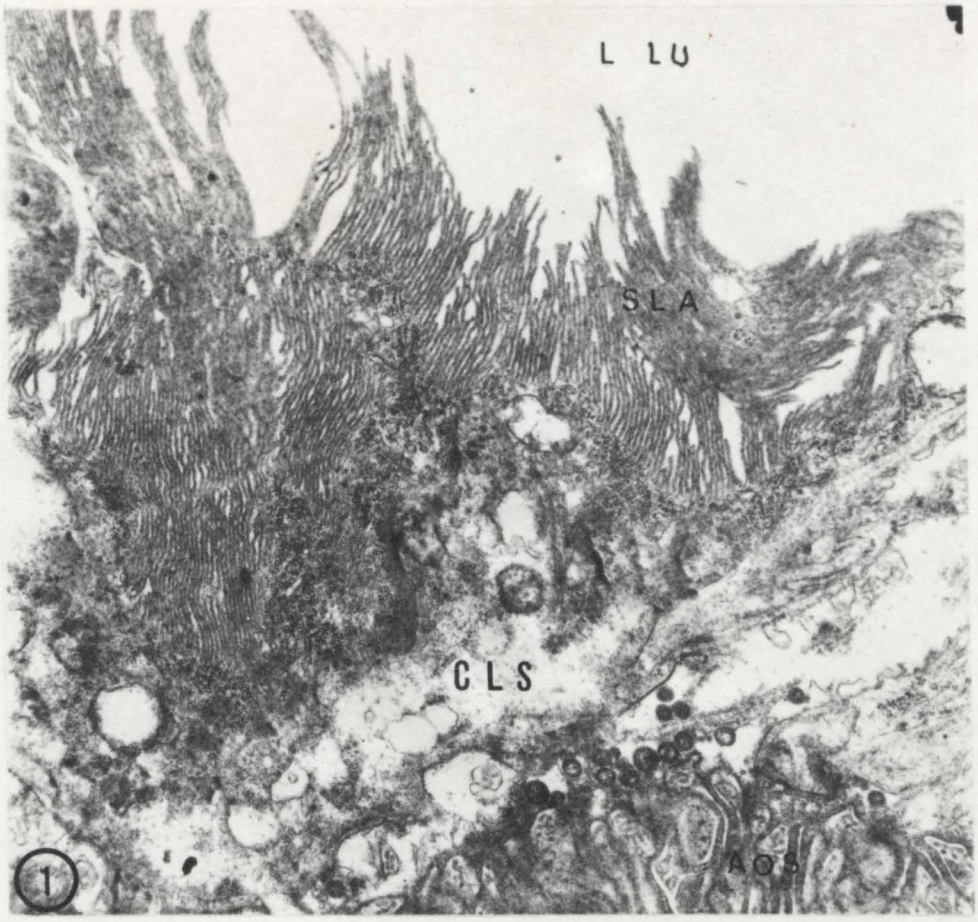
Plate 22

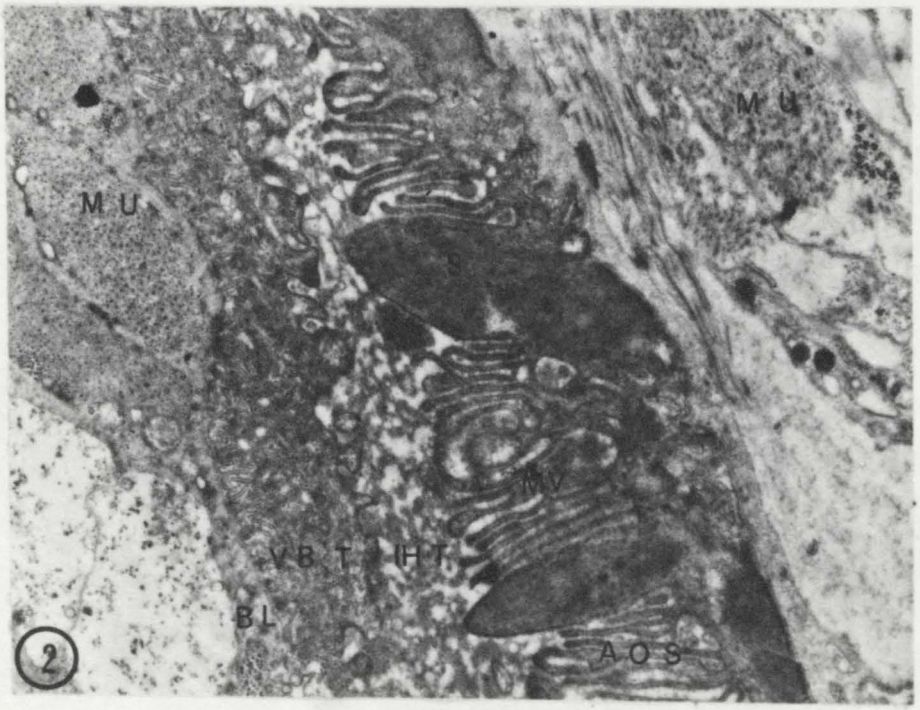
Fig. 1

Electron micrograph showing the detail of the epithelial cells lining the excretory lacunae. Note the proximity of the adhesive organ surface. Uranyl acetate - lead citrate. Mag 13,800 X.

Fig. 2

Electron micrograph showing the transition zone between the inner and outer adhesive organ lobe surfaces. Also present is a portion of the ventral body tegument showing the detail of the tegument. Uranyl acetate - lead citrate. Mag 13,800 X.


L LU
SLA
CLS
AOS
1


MU
MU
MV
VBT IHT
BL
AOS
2

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APPENDICES I TO VII

APPENDIX I

Fixatives

1. 10% Buffered Formalin

Commercially prepared by Fisher Scientific Company as certified 10% formalin phosphate buffered, pH 7.0. Tissues were fixed for 24 hours, followed by a change to fresh fixative for an additional 24 hours.

2. Zenker's Fluid

Stock Solution:

Mercuric Chloride	50 gm
Potassium Dichromate	25 gm
Sodium Sulfate	10 gm
Distilled Water	1000 ml

Working Solution:

Zenker's Stock Solution . . .	95 ml
Glacial Acetic Acid	5 ml

The acetic acid was added before the fixative was used. Tissues were fixed in this solution for 24 hours, washed (in running tapwater) overnight. To remove all residues of insoluble mecuric chloride prior to staining, the tissue was washed in 0.5% aqueous iodine for 5 minutes followed by 5% aqueous sodium thiosulphate for

5 minutes.

3. Bouin's Fluid

Stock Solution:

Saturated aqueous picric acid (prepared by heating 20 gms Picric acid in 1000 ml distilled water)	750 ml
Formaldehyde Solution (40%)	250 ml

Fixative Solution:

Stock solution	95 cc
Glacial acetic acid	5 cc

The acetic acid was added prior to use. Tissues were fixed in this solution for 24 hours. Following this they were transferred to 30% alcohol, 50% alcohol and finally 70% alcohol with several changes of each to remove excess picric acid.

APPENDIX II

Harris's Haematoxylin and Eosin

Reference: R. Dury and E. Wallington (1967), Carleton's Histological Technique.

Fixation: 10% formalin, Zenker's or Bouin's Fluids.

Technique: Paraffin sections cut at 6 μ

Solutions:

Stock Harris's Haematoxylin

5.0 gm haematoxylin crystals were dissolved in 50.0 ml of 95% alcohol. 100.0 gm of aluminum potassium sulfate was dissolved in 1000.0 ml distilled water by the aid of heat. These two solutions were combined and then brought to a boil. Next, the solution was removed from the heat and 2.5 gm mercuric oxide were added. The mixture was reheated (until turned dark purple); promptly removed from the flume and cooled in ice-cold water.

Alcoholic Eosin Solution

Eosin Y, water soluble	2 gm
Distilled Water	160 ml
95% Alcohol	640 ml

The deeper shade was obtained by adding a drop of glacial acetic acid per 100 ml solution.

Acid Solution

Distilled Water	1000 ml
Concentrated HCl	10 ml

Saturated Lithium Carbonate

Lithium Carbonate	1 gm
Distilled Water	100 ml

Sodium Thiosulfate Solution

Sodium Thiosulfate	5 gm
Distilled Water	100 ml

Iodine Solution

1 gm iodine crystals were partially dissolved with 25 ml of 95% alcohol and made to total volume of 100 ml with addition of distilled water.

Staining Procedure:

1. Sections were deparaffinized through two changes of xylene, absolute and 95% alcohol, remaining in each solution for less than 2 minutes.
2. Sections brought to distilled water quickly passing through 80%, 70%, 50%, 30% alcohol.
3. Materials previously fixed in Zenker's Fluid were
   (a) treated with iodine solution for 10-15 minutes
   (b) washed in tap water, 5 minutes
   (c) sodium thiosulfate, 5 minutes
   (d) washed in tap water, 15 minutes
4. Harris's haematoxylin , 6 minutes

5. Distilled water - remove excess stain
6. Differentiated in acid solution - 3-10 quick dips
7. Distilled water - remove excess acid. Dipped slowly until water ran off slides in form of drops.
8. Dipped in saturated lithium carbonate until sections turned bright blue.
9. Dipped into distilled water.
10. Quickly dehydrated to 80% alcohol, passing through 3 quick dips of 30%, 50% and 70% alcohol.
11. Stained with eosin - 1-2 minutes
12. 3 changes 95% alcohol, 2 changes absolute and
14. Cleared in xylene - 2 changes
15. Mounted in Permount

Results:

Nuclei - blue

Cytoplasm - pink

APPENDIX III

Sörensen's Phosphate Buffer

Stock Solution A:

7.1 gm Na_2HPO_4 (m.w.: 142) in 500 ml distilled water

Stock Solution B:

6.8 gm KH_2PO_4 (m.w.: 136) in 500 ml distilled water.

Working Buffer Solution:

7 parts solution A and 3% solution B gives a 0.1 M solution with pH 7.2 - 7.4.

3.5% Buffered Glutaraldehyde Solution

86 ml of phosphate buffer

14 ml of glutaraldehyde* (25% in water)

Prepared daily prior to use.

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* Eastman Organic Chemicals

APPENDIX IV

Modified Palade's Buffered Osmium Tetroxide Fixative

Veronal Buffer Solution (Halander, 1962)

Sodium Veronal (barbital) . . . . . 1.5 gm

Sodium Acetate (crystal reagent) . . . 1.0 gm

Dissolve in Double Distilled Water . . 50 ml

This buffer is stable and keeps for some months at 4°C.

2% Buffered Osmic Acid

(a) Fill 30 ml double distilled water into a clean (acid-cleaned) stoppered bottle.

(b) Add 10 ml buffer solution.

(c) Add 10 ml of 0.1 N HCl (i.e., until pH 7.3-7.5)

(d) Cut the ampoule of osmic acid* with a score, break and place into the solution. (Capsule previously washed with soap and rinsed in distilled water.)

(e) Let the osmic acid dissolve at room temperature for 24 hours.

(f) Add sucrose (Ito, 1961) 2.25 gm in 50 ml solution or 0.045 gm of sucrose per ml of solution.

(g) Solution keeps for some weeks at 4°C if stored in dark container. Discard when solution discolours (brownish).

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* British Drug Houses

APPENDIX V

Preparation of Epon (Luft, 1961)

Stock Mixture:

Solution A:	
Epitoke 812 Resin (Epon 812*)	62 ml
DDSA (Dodecenyl succinic anhydride)** . .	100 ml
Solution B:	
Epon 812	100 ml
NMA (Nadic methyl anhydride)**.	89 ml

Mixture for Embedding:

Solution A	60%
Solution B	40%

Accelerator [DMP - 2,4,6-Tri (dimethyl aminomethyl) phenol]*** - 2% of volume.

After accelerator is added, mixed thoroughly for 5 minutes.

Infiltration Procedure:

(a) 1 part Epon mixture:2 parts propylene oxide****. Spin for 1 hour.

(b) 2 parts Epon mixture:1 part propylene oxide. Spin for 2 hours.

(c) Pure Epon. Spin for 1/2 - 1 hour.

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* Shell Oil Company Canada Limited
** E. V. Roberts and Associates, Inc.
*** Fisher Scientific Company Limited
**** Eastman Organic Chemicals

APPENDIX VI

Toluidine Blue Stain:

Distilled Water	100 ml
Borax	1 gm
Toluidine Blue	0.5 gm

Mixed and stored in the refrigerator.

Staining Procedure:

(a) After slides are cool place a drop of the stain over the sections.

(b) Heat slide for a second.

(c) Leave stain on for 30 seconds

(d) Remove excess stain by dipping into water (2 changes)

(e) Blot dry.

(f) Mount, e.g., in Permount.

APPENDIX VII

Factors Influencing the Magnitude and Relationships of the Measurements

A number of conditions may have influenced the aforementioned developmental patterns. Factors such as (a) the effects of fixation, (b) the effects of crowding and (c) the effects of the host's age have been studied in order to ascertain their roles in influencing the results.

(i) Effects of Fixation

Tables 2, 6, 8 and 9 compare the results obtained from samples fixed in either 10% buffered formal or Zenker's fixatives. Each sample has been taken from the same host. The procedures before and after fixation were exactly the same, so that changes in size result only from the effects of fixation. The results are plotted on histograms (Figs. 13 and 14).

After 2 and 18 hours of development within the host, the TBL and TBW of metacercariae fixed in Zenker's are larger than those parasites fixed in formalin. On first examination it appeared that the type of fixative used influenced the previously mentioned decrease in body

size which reached a minimum at 8 hours. Included in Table 8, are the measurements recorded for material fixed in Zenker's at 8 hours of development. A similar body decrease as reported for the formalin material can be seen. It appears that the decrease in body size did not result from a fixation phenomenon but was the result of a true growth phenomenon.

The results for ABL, ABW, PBL and PBW show in greater detail the influences of fixation. Although the material fixed in Zenker's is usually somewhat larger than that of the formalin material, the effects are inconsistent. One can only say that different types of fixatives may alter the body sizes at any given period of development; but that their effects over the entire series are apparently random and do not influence the overall picture of comparative body development.

When one considers the developing attachment organs, a similar random effect is seen. In most cases the Zenker's fixed material may be somewhat larger; however, definite generalizations cannot be made. A correlation might exist between the effects of the two fixatives. This could be determined if a statistical analysis was applied which took into account the variability within each sample and the difference between two samples as a result of fixation. Time did not permit such an analysis.

TABLE 8

Measurements of the body components of developing (Cotylurus variegatus) metacercariae from 0 - 48 hours (Zenker's Fixative)

		Period of Development (Hours)				
		2	8	18	24	48
ABL	Average	0.3196	0.2911	0.3549	0.4054	0.3491
	Sample Size	15	4	21	18	10
	Standard Error	0.0472	0.1045	0.0977	0.0691	0.0375
	Standard Deviation	0.0122	0.0523	0.0213	0.0163	0.0119
ABW	Average	0.2741	0.2677	0.2982	0.3607	0.4205
	Sample Size	15	4	21	18	10
	Standard Error	0.0313	0.0358	0.0567	0.0295	0.0511
	Standard Deviation	0.0081	0.0179	0.0124	0.0070	0.0162
PBL	Average	0.0953	0.1031	0.1564	0.1494	0.2718
	Sample Size	11	4	20	18	10
	Standard Error	0.0297	0.0259	0.0400	0.0219	0.0691
	Standard Deviation	0.0089	0.0129	0.0089	0.0052	0.0218
PBW	Average	0.1306	0.1177	0.1624	0.1531	0.2372
	Sample Size	11	4	20	18	10
	Standard Error	0.0176	0.0190	0.0205	0.0200	0.0232
	Standard Deviation	0.0053	0.0095	0.0046	0.0047	0.0073

Continued on Page 181

		Period of Development (Hours)				
		2	8	18	24	48
TBL	Average	0.3181	0.3423	0.4355	0.4744	0.6018
	Sample Size	15	4	21	18	10
	Standard Error	0.0456	0.0730	0.0802	0.0495	0.0968
	Standard Deviation	0.0118	0.0365	0.0175	0.0117	0.0306
TBW	Average	0.2741	0.2677	0.2982	0.3607	0.4205
	Sample Size	15	4	21	18	10
	Standard Error	0.0313	0.0358	0.0567	0.0295	0.0511
	Standard Deviation	0.0081	0.0179	0.0124	0.0070	0.0162

TABLE 9

Measurements of the attachment organs of developing (Cotylurus variegatus) metacercariae from 0 - 48 hours (Zenker's Fixative)

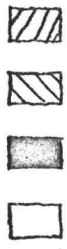
		Period of Development (Hours)				
		2	8	18	24	48
OSL	Average	0.0508	0.0512	0.0597	0.0627	0.0742
	Sample Size	15	4	20	18	9
	Standard Error	0.0063	0.0045	0.0077	0.0045	0.0084
	Standard Deviation	0.0016	0.0022	0.0017	0.0011	0.0029
OSW	Average	0.0543	0.0608	0.0715	0.0763	0.0881
	Sample Size	15	4	20	18	9
	Standard Error	0.0055	0.0032	0.0105	0.0063	0.0077
	Standard Deviation	0.0014	0.0016	0.0023	0.0015	0.0026
VSL	Average	0.0567	0.0758	0.0782	0.0647	0.1043
	Sample Size	15	4	20	18	5
	Standard Error	0.0089	0.0055	0.0152	0.0105	0.0063
	Standard Deviation	0.0023	0.0027	0.0034	0.0025	0.0028
VSW	Average	0.0741	0.0845	0.0934	0.1037	0.1298
	Sample Size	15	4	20	18	5
	Standard Error	0.0084	0.0063	0.0095	0.0045	0.0161
	Standard Deviation	0.0022	0.0032	0.0021	0.0011	0.0072

Continued on Page 183

		Period of Development (Hours)				
		2	8	18	24	48
Lap L	Average	0.0483	0.0422	0.0764	0.0900	0.1002
	Sample Size	14	4	19	17	9
	Standard Error	0.0152	0.0077	0.0170	0.0077	0.0205
	Standard Deviation	0.0041	0.0039	0.0039	0.0019	0.0068
Lap W	Average	0.0610	0.1359	0.0444	0.0442	0.0612
	Sample Size	14	4	19	18	9
	Standard Error	0.0703	0.1395	0.0126	0.0118	0.0152
	Standard Deviation	0.0188	0.0697	0.0029	0.0028	0.0051
AOL	Average	0.0989	0.0951	0.1406	0.1326	0.2385
	Sample Size	15	4	20	18	10
	Standard Error	0.0336	0.0241	0.0409	0.0230	0.0358
	Standard Deviation	0.0087	0.0120	0.0091	0.0054	0.0113
AOW	Average	0.1014	0.0978	0.1402	0.1512	0.2243
	Sample Size	15	4	21	18	10
	Standard Error	0.0122	0.0045	0.0202	0.0118	0.0315
	Standard Deviation	0.0032	0.0022	0.0044	0.0028	0.0099

Fig. 13

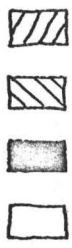
Effect of fixatives on measurements of developing Cotylurus variegatus metacercariae.
Left bar of each group represents formalin-fixed specimens; right bar, Zenker fixed worms.



2 hours post infection
18 hours post infection
24 hours post infection
48 hours post infection

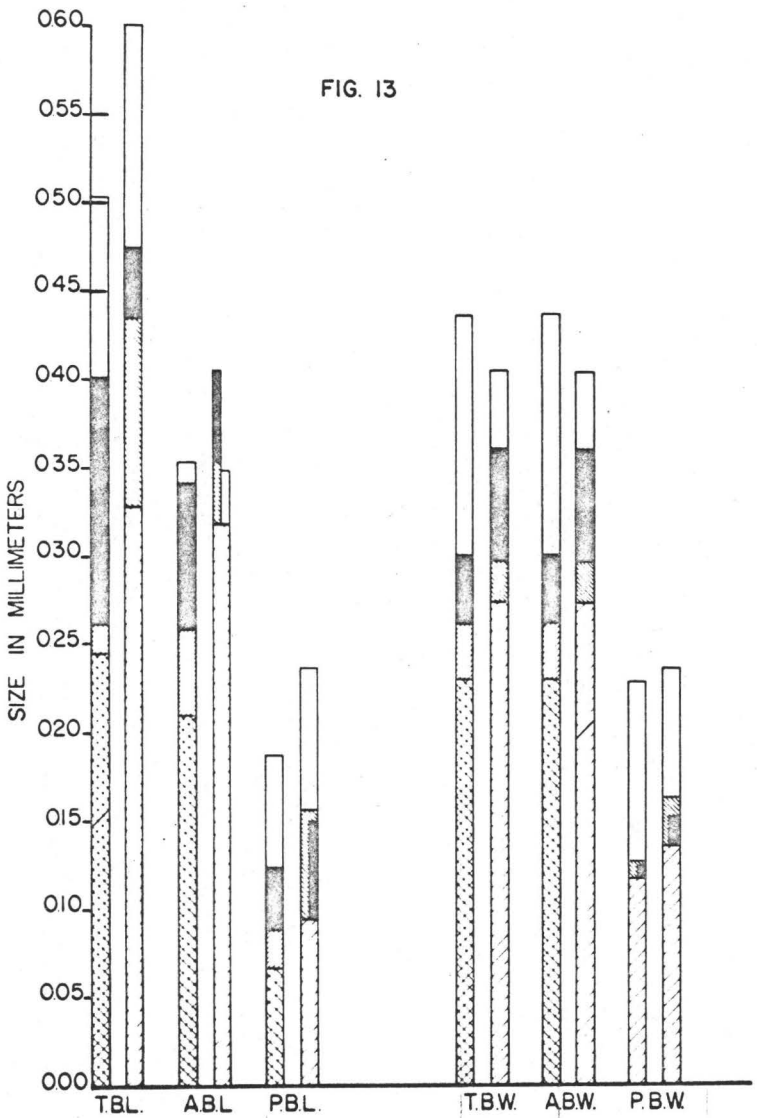
Fig. 14

Effect of fixatives on measurements of the attachment organs of developing Cotylurus variegatus metacercariae.
Left bar of each group represents formalin-fixed specimens; right bar, Zenker-fixed worms.



2 hours post infection
18 hours post infection
24 hours post infection
48 hours post infection

FIG. 13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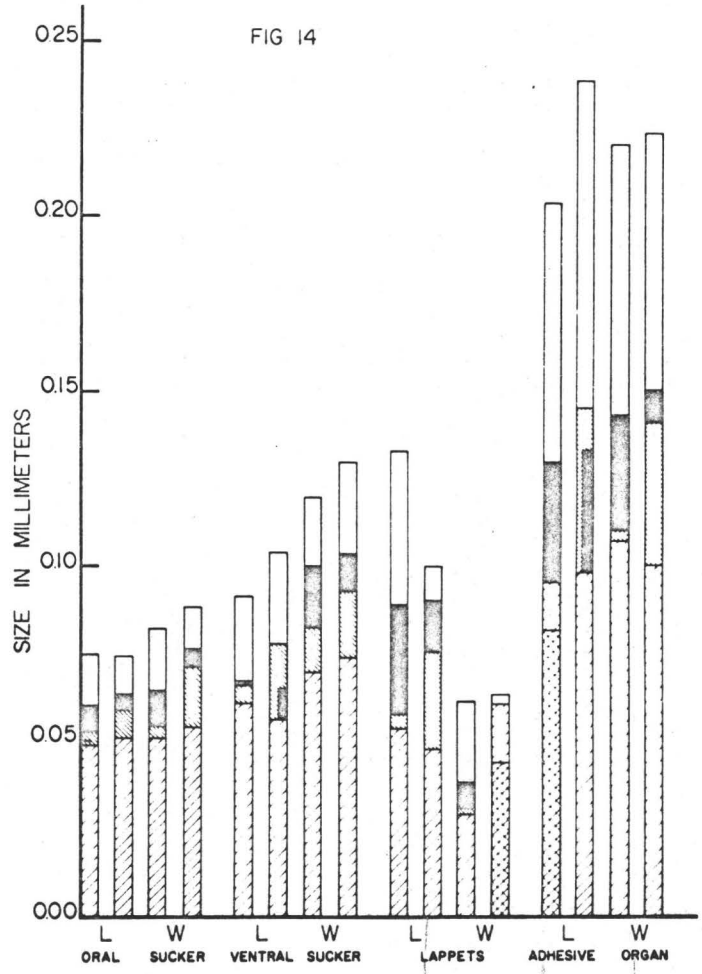
A.B.L
P.B.L.
T.B.W.
A.B.W.
P.B.W.

FIG 14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(ii) Effects of Crowding

Crowding has been shown by Chandler (1939), Read (1950a), Holmes (1961), Holmes (1962) and Roberts (1964), etc. to influence the growth and ultimate size achieved by parasites. This factor has not been extensively studied in this program, however, the following observations are included.

Many cysts were originally fed to the birds to ensure a good sample recovery. The number fed per bird ranged from a minimum of 250 to as high as 350-400. The numbers of recovered worms were always smaller than the original dosage size. Adequate techniques to recover 100% of the original dosage were unavailable so that a correlation of the number fed with the number recovered is unavailable.

Upon entry into the host, and up to the time of attachment, competition is probably at a minimum. The metacercariae are extremely small, and were usually observed throughout the small intestine. It does not seem likely then that these juveniles are competing for food or even protection within the host mucus.

It is in the large intestine that final attachment took place. In all the infections examined, the worms were localized in the portion of the gut surrounding the openings of the caeca. In examples of heavy infections, this preferential site of adherence was extremely crowded (Table 1, Expt. V, Bird 4). Flukes were closely juxtaposed

and in some cases were attached to the same villus. At the same time, some parasites were located just anterior and posterior to the preferential sites in what might be considered marginal habitats, and would account for the great difficulty in acquiring host materials. At this stage, competition amongst the individuals would probably be at a maximum.

(iii) Age of the Birds

Table 10 presents the results obtained from the measurements of samples taken from birds differing in their ages. One group of hosts was 6-8 weeks old, while the second consisted of a group of gulls one year old. The collected samples of parasites were treated exactly the same throughout and only the dimensions of TBL and TBW were used in this analysis. The interval analyzed extends from 24 to 144 hours post infection. Comparative samples were not available prior to 24 hours.

The results show a general trend emerges in which prior to 2 days the younger birds harboured the largest worms. Following 2 days, the situation is reversed, the helminths from the older birds are much larger. The following statistical approach was adopted to test the proposed hypothesis.

From theoretical considerations what is required to solve this statistical problem is a multivariate two-way

factorial fixed factor analysis (Richardson, personal communication). It is multivariate because there are several measured responses on each individual; two-2ay factorial because there are all possible combinations of two treatments, days post infection and age of the gull; and fixed for technical reasons. Unfortunately one cannot do this because (i) non-homogeneous variances exist, the bigger the worm, the bigger the variance, and (ii) in each category the number of worms is different. For an analysis this complex,the number of replications must be equal.

To overcome these difficulties the problem has been reduced from a multivariate one to a univariate one by multiplying TBL by TBW and using this as the measured response. This has been done for every individual and the means for each group have been included in Table 10. From these results Fig. 21 was plotted. The next step was to calculate simple linear regression lines for responses versus days post infection for each on the 6 week and 1 year groups (2 lines, to be fitted to the data plotted in Fig. 21). There are two problems with this procedure. One is the aforementioned non-homogeneity of variance. To overcome this the logarithms of TBL X TBW have been used rather than the raw value as the response. The second problem is that a straight line obviously does not fit the raw data however the graph of log response versus days seems a reasonable fit (Fig. 22 and 23).

The regression lines were calculated from the data of each individual worm response converted to the logarithmic value versus days post infection. The formula used was:

The equation of a straight line can be written as:

$y = a + bx$

x = the parameter of days post infection

y = the parameter of measured response, i.e. TBL x TBW

Calculation a, b, able to determine linear equation

$$a = \frac{\Sigma X_i^2 \, \Sigma Y_{im} - \Sigma X_i \, \Sigma (X_i \, Y_{im})}{n\Sigma X_i^2 - (\Sigma X_i)^2}$$

$$b = \frac{n\Sigma (X_i \, Y_{im}) - \Sigma X_i \, \Sigma Y_{im}}{n\Sigma X_i^2 - (\Sigma X_i)^2}$$

X_i = day post infection, e.g. 1, 2, 3 6

Y_{im} = (TBL x TBW) for each sample at a specific time post infection

n = total number of samples

The values obtained were:

	6 week group	1 year group
a	.5785	0.6129
b	.3693	0.4281

From these regression lines plotted on Figs. 22 and 23, slopes have been calculated as 0.369 for 6 week group and 0.428 for 1 year group. The original hypothesis was that the 6 week group start higher (i.e. harbour the larger parasites) and end lower and so they should have a lower slope than the

1 year group. From the calculations this can be seen to exist. Thus the aforementioned hypothesis has been verified.

The factors which influence and regulate parasite development are not very well understood. Nutritional, hormonal and immunity factors have received much recent attention. Read (1950b), Smyth (1966) and Von Brand (1953,1966) have summarized the results to date. In addition the host's age is considered to be an important factor in controlling parasite development. Dobson (1962a,b) summarized effect of host age on parasite development. In almost all examples, the older hosts harbour a smaller parasitemia. These results are quite confusing when one attempts to describe the age factor in greater detail. In addition to knowing the correlation of host's age and numbers of parasites, it would be useful to describe the effects of host's age on the remaining parasites. Information along this line is wanting. The present study, however, has investigated the development on those parasites which remain within their hosts. It appears that the development within older birds is retarded up to a particular point of their development, in this case 3 days, afterwards their development is greater than the worms from younger birds. It is interesting to note that the reversal, i.e., 6 week old hosts, harboured the largest worms up to end of 2 days; at 3 days the situation is reversed. This reversal in growth occurs at the same time, when the process of attachment was considered to be complete. Certainly, this particular aspect of parasite development warrants further investigation.

TABLE 10

Comparisons of body sizes of developing Cotylurus variegatus metacercariae recovered from host 6 weeks and 1 year old

Age of Bird		1 day		2 days		3 days	
		6 wks.	1 yr.	6 wks.	1 yr.	6 wks	1 yr.
TBL	Average	0.4111	0.3473	0.5030	0.5592	0.7330	1.6187
	Sample Size	8	18	16	12	17	20
	Standard Deviation	0.0313	0.0468	0.0612	0.0899	0.0741	0.02251
	Standard Error	0.0111	0.0110	0.0153	0.0260	0.0180	0.0503
TBW	Average	0.3031	0.2760	0.4374	0.3100	0.4697	0.7115
	Sample Size	8	18	16	12	17	20
	Standard Deviation	0.0268	0.0261	0.0778	0.0332	0.0399	0.0744
	Standard Error	0.0095	0.0061	0.0195	0.0096	0.0097	0.0166
Average TBL x TBW*		0.1250	0.0962	0.2212	0.1739	0.3459	1.1915
Log (Average TBL x TBW) x 10^{-2}*		1.0969	0.9833	1.3448	1.2303	1.5390	2.0763

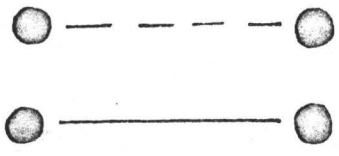
Continued on Page 191

TABLE 10 (continued)

Age of Bird			4 days		5 days		6 days	
			6 wks.	1 yr.	6 wks.	1 yr.	6 wks.	1 yr.
TBL	Average		2.8775	2.5449	4.4584	3.9534	4.9131	
	Sample Size	N	20	20	20	20	10	
	Standard Deviation	O	0.2895	0.3702	0.0508	0.3468	0.5861	
	Standard Error		0.0657	0.0828	0.1135	0.0775	0.1853	
TBW	Average	D	1.0713	1.2167	1.4061	1.5696	1.6882	
	Sample Size	A	20	20	20	20	10	
	Standard Deviation	T	0.1564	0.0894	0.1578	0.1270	0.1724	
	Standard Error	A	0.0350	0.0200	0.0353	0.0284	0.0545	
Average TBL x TBW*			3.1078	3.1039	6.3347	6.3116	7.2495	
Log (Average TBL x TBW) x 10^{-2}*			2.4925	2.4919	2.8018	2.8002	2.8018	

Fig. 21

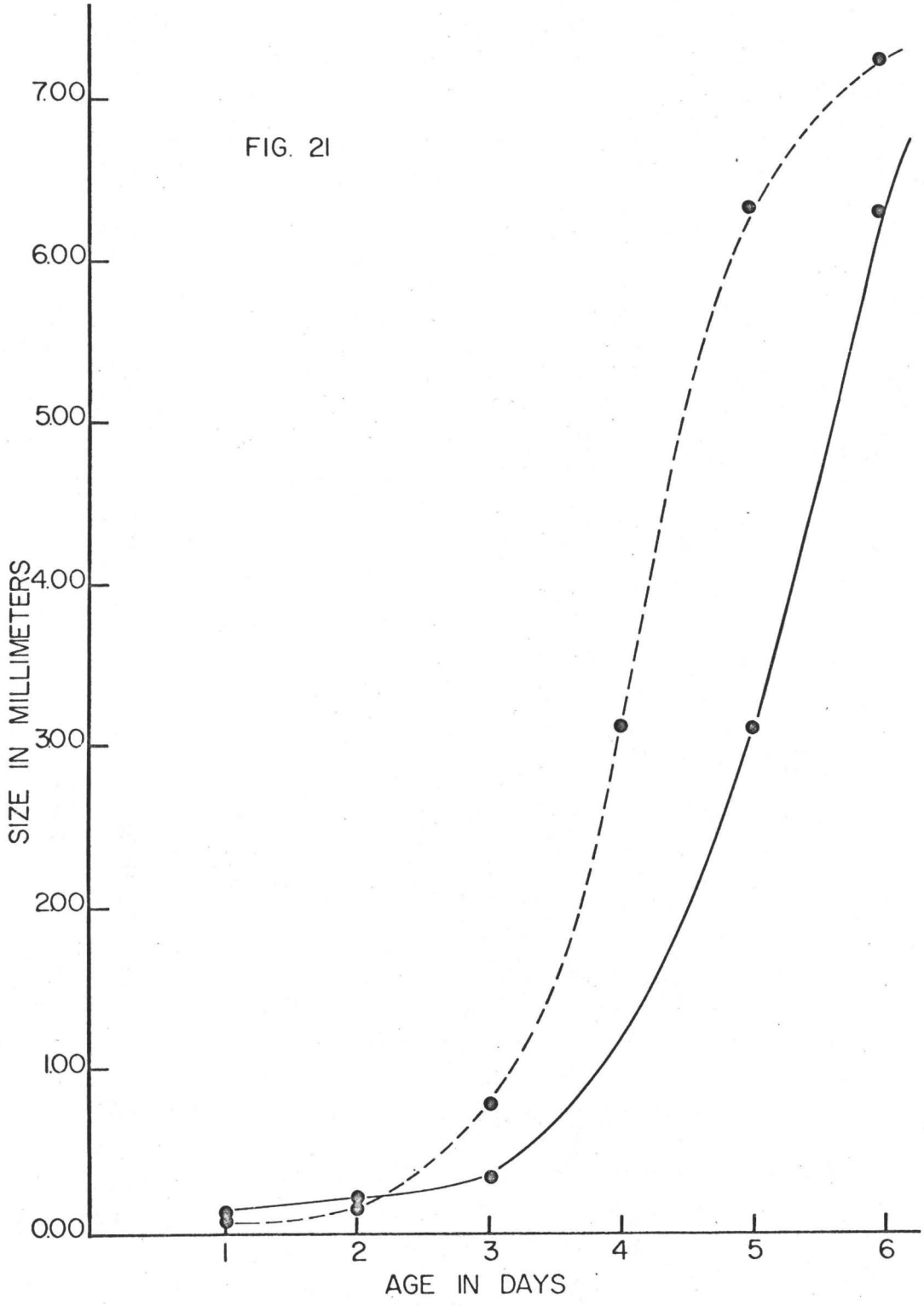
Relationship between the measurement of TBL x TBW and age of the developing metacercariae (1-6 days) of Cotylurus variegatus.



samples obtained from 1 year old host

samples obtained from 6 week old host

FIG. 21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AGE IN DAYS

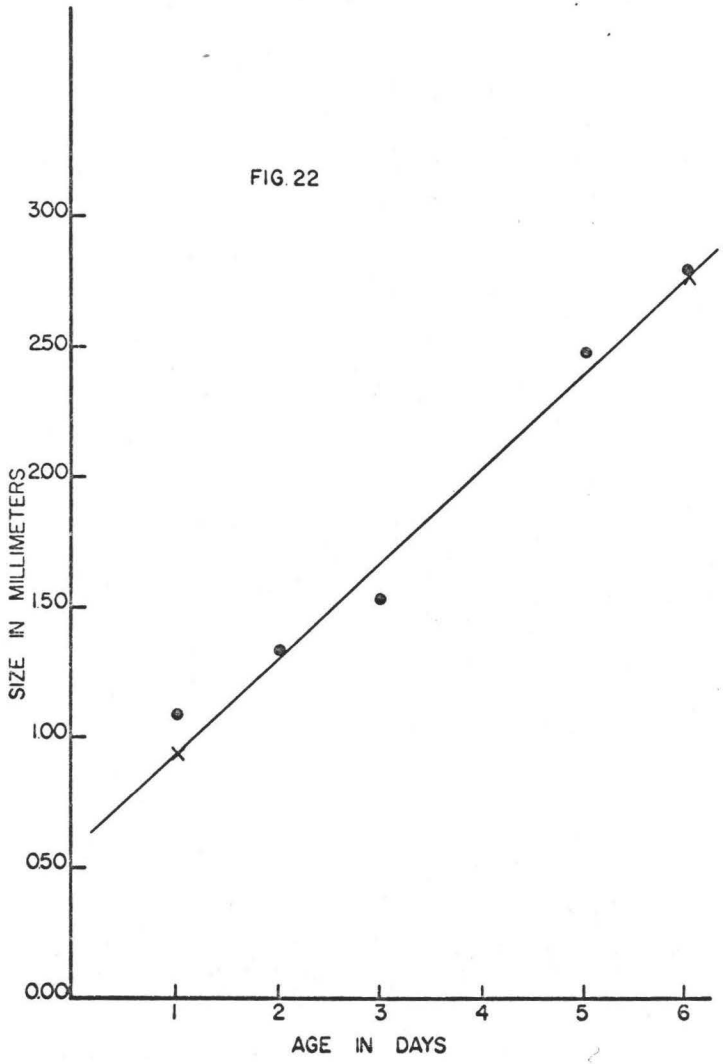
Fig. 22

Relationship between the measurement of log TBL x TBW and the age of the developing metacercariae (1-6 days) of Cotylurus variegatus recovered from hosts 6 weeks of age. The straight line was fitted by the method of least squares.

Fig. 23

Relationship between the measurement of log TBL x TBW and the age of the developing metacercariae (1-6 days) of Cotylurus variegatus recovered from hosts 1 year of age. The straight line was fitted by the method of least squares.

FIG. 22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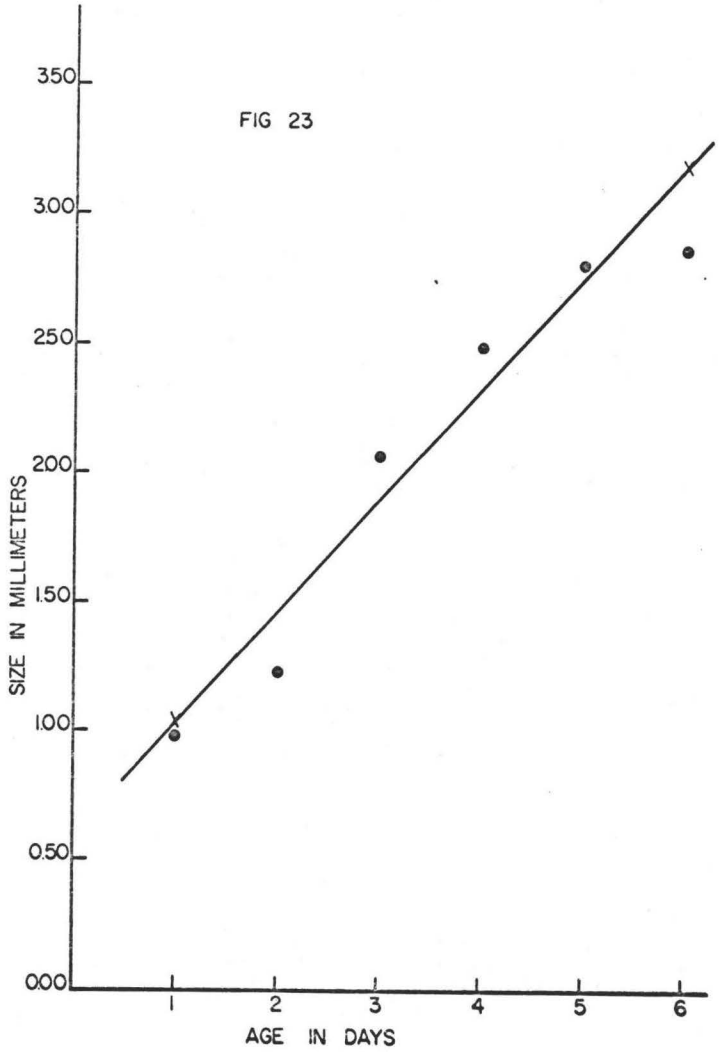
AGE IN DAYS

FIG 23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