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Taxonomic studies on adult and larval ascidians from California

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TAXONOMIC STUDIES ON ADULT AND LARVAL ASCIDIANS FROM CALIFORNIA

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INTRODUCTION

The primary purpose of this research is to further morphological studies on larval ascidians and to encourage greater reliance on larval structures in ascidian taxonomy. This may necessitate some revisions in current classification, but it is hoped that such revisions will lead to a better understanding of the group. Some general considerations of ascidians are here included for the benefit of those who may not be familiar with them. All specimens used in this study were collected at the Pacific Marine Station area at Dillon Beach, California, during June, July, and August of 1957.

ACKNOWLEDGEMENTS

The writer wishes to express appreciation to Dr. Alden E. Noble, Chairman of the department of Zoology at the College of the Pacific, for his guidance and for his assistance in the collection and preparation of materials which made this report possible. Acknowledgement is extended to Mr. Edmund Smith for his photographic aid and also to the interlibrary loan staff of the College of the Pacific.

METHODS AND PROCEDURES

Collection

Ascidians were collected, when possible, from their natural environments during low tides. Most shore-cast colonies were found to be unhealthy and occasional deterioration prevented identification. Specimens were removed carefully, packed in fresh algae, and placed in plastic or canvas containers. Contracted zooids thus remained healthier than when placed in sea water which often became warm and contaminated before the colonies reached the laboratory. Colonies were placed in fresh, oxygenated sea water with a minimal loss of time. It was found that colonies lived longest when placed in at least 50 times their volume of sea water at temperatures approaching those of their natural habitats.

Larvae were obtained by either allowing them to emerge naturally from the adult or by rupturing the atrial cavity of the adult with probes. The second method was employed for most collections because it required less time and produced larvae of a wider range of development.

Colonies that were to be preserved were first relaxed in a solution of 4 grams of magnesium chloride per 500 ml of sea water. About 12 hours later they were transferred to 5%

formalin. Because of the large percentage of water in the test, the formalin was replaced at least once after 24 hours and larger colonies were slit open to allow a more thorough penetration.

Microtechniques

Staining of Bouin-fixed zooids and larvae was necessary to reveal many of the structures. Most biological dyes have strong affinities for yolk material which is abundant in developing ascidians. Celestin Blue B dye, when used as outlined below, however, avoids coloring the yolk and hence clearly differentiates structures which would otherwise be obscured. The following procedure was found to be most effective:

1. Specimens were removed from fixative and washed in 6 to 8 changes of distilled water.
2. They were stained in Celestin Blue B for 6 to 24 hours or over night. The staining solution was made by bringing to a boil 4 grams of iron alum, 100 ml. of distilled water and 2 ml. of sulfuric acid. While the solution was boiling, 1 gram of Celestin Blue B was added. It was then cooled and 10 ml. each of 100% ethyl alcohol and concentrated glycerine were added.

3. The specimens were washed in distilled water for several changes until the water remained clear.
4. Partial dehydration was accomplished by successive immersions in 35%, 50%, and 70% alcohol for about 10 minutes each.
5. Destaining in acid alcohol was carried out in the following manner:

   Specimens were placed in a syracuse watchglass and a mixture of 20 ml. of 70% alcohol and one drop of concentrated hydrochloric acid was added. If the solution became deeply colored by extracted dye, it was replaced with a fresh solution, but the acid content was not increased unless adequate differentiation could not be obtained in one hour's time. The strength of the acid in alcohol sometimes was doubled at equally long intervals of time in stubborn cases. Frequent observations under 100 magnifications were necessary to determine when the destaining was complete.
6. When the specimen was properly destained, dehydration was completed by first washing out the acid in 2 or 3 changes of 80% alcohol. If the specimen was to be stored, only neutral alcohol

was used. The specimen was then immersed in 95% and 100% alcohol for 10 minutes each. A counterstain of dilute Eosin was used to lightly tinge the test without obscuring internal details.

7. A second change of 100 % alcohol was used for about 30 minutes to insure complete dehydration.

8. Immersion of the specimen in a 50-50 mixture of 100% alcohol and 100% xylol for 20 minutes was done to start replacement of the alcohol with xylol.

9. The specimens were cleared in 100% xylol for at least one hour. At this point the results of the destaining were evaluated and, if necessary, the specimen was returned to 70% alcohol and further destained.

10. Replacement of the xylol with balsam was accomplished by immersing the specimen in a mixture of one part thick balsam to nine parts of 100% xylol and allowing the xylol to evaporate without the aid of heat until the balsam was of mounting consistency.

The finished slides revealed most of the structures in light blue outline with a light pink background.

GENERAL CONSIDERATIONS

Adult morphology

Ascidians are members of the phylum Chordata, sub-
~m Urochordata (Tunicata), class Ascidiacea. The adult
t conspicuously display chordate characteristics
e free-swimming larva (tadpole) clearly demonstrates
hree basic features of chordates: a hollow dorsal
e cord, branchial gill slits, notochord.

The first of the three main adult body forms recog-
zed and used for classification purposes is the holoso-
atous type (Plate I, fig. 1). The zooid is more or less flattened and globular with the branchial siphon at the anterior end and the atrial siphon midway on the dorsal side. This type is sometimes called simple or solitary because of the lack of budding and consequent absence of morphological connections. Other holosomatous ascidians are connected by stolons and are called social. This condition is the result of budding, with the maintenance of individual tests.

The second is the merosomatous type and consists of a thorax and a posterior abdomen (Plate I, fig. 2). The branchial siphon position is the same as in the holosomatous type but the atrial siphon is more anterior. The abdomen usually contains the stomach, oesophagus, heart, and gonads.

These zooids are found growing in colonies within a common test matrix and are referred to as compound ascidians.

The third group, also merosomatous, is termed the synoicid type and the zooids resemble the second group but have a post-abdomen (Plate I, fig. 3). This post-abdomen usually contains the gonads and, at the posterior tip, the heart. The branchial and atrial siphons are found close together at the anterior end and, indeed, they are sometimes difficult to distinguish. Like the merosomatous type, these animals are also embedded in a common matrix.

The most conspicuous structures of adult ascidians are indicated below.

Atrial siphon: Exit for sea water, fecal pellets, gametes, larvae, metabolic wastes.

Bladder cells: Scattered spicule forming cells embedded in the test.

Brain: Residual ganglion from larval metamorphosis.

Branchial sac (pharyngial pouch): Large respiratory and food concentrating sac connecting the branchial siphon with the digestive system. It is perforated by numerous pores which permit the flow of water into the atrium.

Branchial siphon: Entry for sea water and food.

Digestive system: Oesophagus, stomach, intestine, rectum, and anus.

Endostyle: An elongated, grooved, food-concentrating device on the ventral floor of the branchial sac. It adds strength to the sac and produces a mucus blanket which catches food particles. This blanket then is drawn into the oesophagus and digested along with the food.

Gonads: Ovaries and testes, the animals being hermaphroditic.

Mantle: Thin membrane enclosing the entire body of the animal.

Stigmata: The perforations of the branchial sac.

Subneural gland (dorsal tubercle): This is thought to be exocrine in function.

Tentacles: Sensory devices which aid in regulating the entry and flow of sea water.

Test (tunic): Protective body covering secreted by the mantle. The main component of the test is tunicin, a compound closely related to cellulose. In compound ascidians this test forms the matrix in which the zooids are embedded.

Larval morphology

Most ascidian life cycles involve a free-swimming larval stage called a tadpole, which sooner or later attaches itself to the substrate and metamorphoses into the sessile adult. The larva is a dual organism made up of larval structures and adult structures in various stages of early development (Plate I, fig. 4), but it is thought that the larval system functions only until the concentration of metabolic wastes triggers metamorphosis. The most conspicuous larval structures are listed below:

Adhesive papillae (suckers): Structures that concentrate the adhesive substance at the anterior end of the larva for proper attachment. They are absorbed during the latter part of metamorphosis.

Ampullae (adhesive glands): Mucus-producing enlargements of the anterior portion of the mantle which supply the adhesive material for attachment.

Dorsal nerve cord: Hollow nerve cord which lies dorsal to the notochord and, except for the ganglion which persists in the adult, is absorbed during metamorphosis.

Eyespot: A light-sensitive structure in the larva that becomes a nerve ganglion in the adult.

Notochord: A structure of about forty cells which extends from inside the animal body down the tail. It is withdrawn into the test during metamorphosis by muscle bands and absorbed, probably by phagocytosis (Grave, 1935).

Statolith (otolith): A balancing organ which degenerates and combines with the eyespot to form a ganglion between the siphons of the adult.

Tail muscle bands: Longitudinal muscles which provide for the swimming action of the larval tail and then contract to pull the notochord into the test during metamorphosis. They are absorbed with the tail.

Yolk: Nutrients obtained from the egg.

Metamorphosis

Caswell Grave (1935) wrote:

> The ascidian larva is a dual organism, the action system of the larva being quite separate from the action system of the ascidiozooid, the former exercising an inhibiting effect on the growth and differentiation of the latter throughout the free-swimming period, the inhibition ceasing upon the occurrence of the disruptive phase of metamorphosis.
>
> Metamorphosis advances by three stages: (a) changes in the adaptive responses of the larva to light and gravity; (b) the attachment of the larva to the surface of some foreign object; (c) the disruptive phase during which the entire larval action system is destroyed.
>
> Swimming activity causes the production and concentration of some metabolic product in the larval tissues that is essential to the induction of metamorphosis.

This involves absorption of tail structures, papillae, ampullae, and most of the yolk. Conversely, it involves growth and differentiation of the branchial sac and related organs. During metamorphosis the test thickens and a general body rotation of about 90 degrees of arc results (Trason, 1957). The branchial siphon moves from perpendicular to the main larval axis to the anterior end of the main adult axis.

In the laboratory, mature larvae removed from the adult were seen to swim at once and rise to the surface in short spurts. Surface contact seemed to initiate a short rest period in which the larva slowly began to sink. This pattern was repeated several times followed by much longer swimming periods of 30 to 90 seconds. The usual pathway during these longer periods was near the surface but some larvae suddenly attached themselves to the side or bottom of the container. Others would rest for successively longer periods and finally start metamorphosis without attachment. Free-swimming time before attachment ranged from 30 seconds up to several hours. The time required for metamorphosis showed similar variation, with limits of 30 minutes to about 60 hours, depending on the species and environmental variations. Partial metamorphosis of those larvae that did not attach seemed to require less time, but no controlled experiments were conducted to test this phase.

SPECIES DESCRIPTIONS

For a general description of the adults and their distribution one is referred to Van Name (1945): North and South American Ascidians. The following data on eleven species are confined to the writer's observations which either modify or add to previous reports.

Amaroucium californicum Ritter and Forsyth, 1917

Adults up to five mm. in length and enclosed in a reddish brown test were collected. The larvae display well developed, in-line, adhesive papillae and globular ampullae. Most of the ampullae have a short stalk connecting to the mantle, but those found between the papillae are on longer stalks and there are usually two in number. The concave endostyle is about one-half its length away from the branchial siphon. Two branchial sacs, each with six primary gill slits per row, are present but these open into one dorsal branchial siphon. The average notochord length is 0.98 mm. (0.96 mm. to 1.0 mm.).

Amaroucium solidum Ritter and Forsyth, 1917

The zooid thorax is 3.0 mm. in length (2.8 mm. to 3.1 mm.) and the abdomen 1.5 mm. (1.47 mm. to 1.59 mm.). The test is greyish white to dull yellow. The adhesive papillae are in a row and undifferentiated, with ampullae similar to *A. californicum* but more numerous. The concave endostyle, branchial sacs, and branchial siphon are similar to *A. californicum* but there are ten primary gill slits per row and the average notochord is only 0.69 mm. (0.61 mm. to 0.72 mm.) long.

Amaroucium propinquum Van Name, 1945

The zooids collected are not as long as those described by Van Name. The adhesive papillae are very similar to A. solidum but the ampullae are club-shaped and number from 30 to 36. The areas between the papillae each have two ampullae which are much longer. The concave endostyle extends to the single branchial siphon and there are four primary gill slits per row. The average notochord length is 0.85 mm. (0.78 mm. to 0.92 mm.).

Amaroucium arenatum Van Name, 1945

The zooid thorax is 0.7 mm. long (0.6 mm. to 0.75 mm.), the abdomen is 0.9 mm. long (0.79 mm. to 0.95 mm.), and the post-abdomen of various lengths. The stomach has one or two more ridges than those described by Van Name. As expected, the adhesive papillae and ampullae resemble those of *A. solidum*. The endostyle touches the single branchial siphon but is convex and not concave. There are six primary gill slits per row and an average notochord length of 1.06 mm. (0.98 mm. to 1.11 mm.).

Synoicium par-fustis (Ritter and Forsyth), 1917

Colonies up to 80 mm. in length and 25 mm. in diameter were collected. The ovary is situated in the anterior portion of the post-abdomen while the testes are located in the posterior end. Van Name states that the ovary is embedded in the testes which fill the post-abdomen, but the writer was unable to verify the statement. Possibly these are younger zooids and the testes have not yet grown to full size. Ritter and Forsyth (1917) show an oval mass at the anterior end of the post-abdomen which contains both ovary and testes.

The adhesive papillae are well developed as in *Amaroucium californicum*, but the ampullae are almost all round and borne at the end of stalks. There are four primary gill slits in each row and two branchial siphons. The right siphon is in line with the atrial siphon and the otolith, but the left siphon is off to the side. The notochord averages 1.2 mm. (1.1 mm. to 1.28 mm.) in length.

Sigillinaria pulchra (Ritter), 1901

The adults fit the description given by Van Name except that the testes extend to the posterior tip of the post-abdomen and leave little room for the heart. The colonies are not of "inversely conical or capitate (in) form" as are those described and shown by Ritter (1901) but are short, fat and club-shaped. Ritter's form may have been due to crowding of the colonies. The in-line adhesive papillae have a large, clear anterior end with a few large granules in the stalk. The areas between the papillae each contain one thick club-shaped ampulla and two or three large, spherical ampullae. There are one or two club-shaped, several large spherical, and six or eight small stalked ampullae outside of the areas. The endostyle is concave and extends to the branchial siphon. There are four primary gill slits per row and the notochord has a length of 0.90 mm. (0.77 mm. to 0.95 mm).

Didemnum carnulentum Ritter and Forsyth, 1917

The colonies and zooids collected conform to Ritter and Forsyth's description, but the spicule size is from 0.010 mm. to 0.032 mm. with an average of 0.021 mm. The writer agrees with Van Name that Ritter and Forsyth may have misplaced a decimal point. The adhesive papillae of the larvae are almost mushroom shaped and are in a row. Eighteen or twenty capitate ampullae attach to the mantle extension which produces the papillae. No atrial siphon is evident and there is only one immature branchial siphon. The eye-spot and otolith are well formed, but other adult structures are undifferentiated. There are four primary gill slits in each of the three rows on one side of the branchial sac. The average notochord length is 1.17 mm. (0.92 mm. to 1.30 mm).

Cystodytes lobatus (Ritter), 1900

The colonies and zooids closely conform to the description given by Van Name, even to the point that some colonies were found growing on bivalves shells.

The most striking feature of the tadpoles is the ring-shaped ampulla that encloses the adhesive papillae stalks. This ring is connected to the mantle by several stalks (Plate II, fig. 8). Muci-carmine (Mayer) was used on these larvae, but no differential staining was shown. The endostyle is dorso-ventrally placed and does not quite reach the single branchial siphon. Four rows of nine primary gill slits are found on each side of the branchial sac. Average notochord length is 1.74 mm. (1.36 mm. to 1.82 mm.).

<u>Distaplia</u> <u>occidentalis</u> Bancroft, 1899

The colonies are all flat and encrusting with no evidence of heads forming. There are two rows of thin club-shaped ampullae, one on each side of the papillae stalks and extending dorso-ventrally. Between the in-line papillae there is found a clump of three to six larger clubs. The slightly convex endostyle extends to the branchial siphon and lies almost parallel to the larval longitudinal axis. There are six primary gill slits per row and the notochord average length is 0.99 mm. (0.9 mm. to 1.11 mm.).

<u>Ascidia</u> <u>ceratodes</u> (Huntsman), 1912

The zooids conform to Van Name's description except for the measurements. Some individuals measured 120 mm. from the branchial siphon end of the test to the test base, while expanded zooids removed from the test measured up to 100 mm. in length. The writer is not sure whether Van Name measured the test or the zooid but in either case the specimens collected at Dillon Beach were much larger. Although commonly collected from various substrates, almost all individuals taken by the writer were from holdfasts of the kelp *Macrocystis*.

Two instead of one dorsal tubercle aperture are present and each is in the shape of a letter C. The open parts of the C's are lateral, not anterio-posterior. The larval body test averaged 0.138 mm. (0.132 mm. to 0.140 mm) in length and the notochord averaged 0.39 mm. (0.36 mm. to 0.41 mm). The adult of this species is by far the largest included in the study, but the larvae are much the smallest. The papillae and ampullae are combined into one lump found on the anterior end of the mantle. There is one branchial siphon and about five gill slits in the single row per side of the branchial sac. No endostyle, heart, intestine, or eyespot are discernible, but a stomach is evident. The notochord extends to the otolith cavity.

Undescribed species

An undescribed species of the family Diazonidae was encountered in large numbers. No effort is made here to name the species because it was learned that Dr. Donald P. Abbott of the Hopkins Marine Station was preparing a manuscript on the subject.

The colonies consist of numerous clubs connected at their bases by stolons. Each club contains 25 to 75 zooids. All colonies are orange and free of encrusting sand except for an occasional sand cap which may cover the most anterior portion of the club. The club length averages about 35 mm. but some reach 60 mm.

The merosomatous zooids lie in systems perpendicular to the longitudinal axis of the club with the atrial siphons entering a common cloaca. The atrial siphons of some are open on the left side of the siphon, some on the right side, and some open only to the front (Plate V, fig. 4). The position of the zooid relative to the common cloaca determines which side of the siphon is open. The gonads are on the left side of the uncrossed intestinal loop. The branchial sac has four rows of stigmata and twelve simple branchial tentacles.

The bodies of these large larvae average 1.26 mm. (0.92 mm. to 1.38 mm.) in length exclusive of the tail, and the notochord averages 1.2 mm. (1.0 mm. to 1.52 mm.) in length. The test has papillae pores through which the well developed papillae can be extended and retracted. The papillae are not in a row, but two are placed anterio-laterally and one ventro-medially. The triangular arrangement can, upon attachment, draw the larva's ventral side to the substrate and thus metamorphosis does not entail a full 90 degree rotation of the animal body. The ampullae are reduced to enlargements of the papillae stalk with two or three rounded projections at the stalk base. The large diameter of the stalk suggests the incorporation of ampullae tissue within it. The intestine crosses the oesophagus and has an elongated intestinal gland on the left side. The branchial sac has four rows of stigmata on each side with eleven primary gill slits per row, six branchial tentacles on the single branchial siphon, and three dorsal languets per side. The concave endostyle lies almost at right angles to the larval axis.

DISCUSSION AND CONCLUSIONS

The comparatively few species studied in this report are inadequate for sweeping generalizations, but they suggest one significant conclusion. Larval structures appear to be as distinct and taxonomically reliable as are those of the adult. Indeed, some larval features are less variable than adult ones. It would therefore seem advisable to incorporate larval structures in taxonomic arrangements which heretofore have been based almost exclusively on adult morphology.

Two of the most valuable larval structures are the adhesive papillae and the ampullae which, when observed in the actively swimming larva, are quite constant in size and shape. The papillae are either in a row or form a triangle and, before attachment, they are either well defined and developed as in *Amaroucium californicum* or glandular and undeveloped as in *Distaplia occidentalis*. Also during the free-swimming period the ampullae are quite characteristic and, as in *Cystodytes lobatus*, sometimes are very distinctive. The immature adult structures found in the larvae, with the exception of the gill slits and the number of branchial siphons, are not as characteristic and dependable.

It is the writer's opinion that adult morphology and the larval structures mentioned in the preceding paragraph, together with proportional measurements, are sufficient for classification to genus and, in most cases, to species. Such a unified system of classification would avoid the type of difficulty encountered in the double classification of some of the coelenterates and trematodes. It is hoped that these studies may lead to a complete correlation of larval and adult features in ascidian taxonomy.

SUMMARY

An account of the collection, preservation, and staining techniques for ascidians is presented. The three basic adult types and a generalized larva are described. General metamorphosis and behavior patterns during the free-swimming period are reported as they were observed in the laboratory. The adults and larvae of the eleven species studied, including a new species of the family Diazonidae, are described. This study indicates that larvae should be used as an integral part of ascidian taxonomy.

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EXPLANATION OF PLATES

All drawings except those of the composite figures on Plates I and II were made with the aid of a camera lucida. The larval tails are not figured in their entirety, but measurements of notochords from body tests to posterior ends are given; these measurements are taken from fixed animals.

a	anus	mnm	mantle muscle
amp	ampullae	mp	muscular process
at	atrium	ntc	notochord cell
ats	atrial siphon	oe	oesophagus
bl	blood vessel	oto	otolith
blc	branchial lobe	ov	ovary
brs	branchial siphon	ovd	oviduct
brsc	branchial sac	pam	periatrial muscle
brsf	branchial sac fold	pbm	peribuccal muscle
brt	branchial tentacle	ppc	papillae center piece
dlg	dorsal languet	pph	peripharyngeal ring
dlm	dorsal lamina with dorsal languets	ppr	papillae ring
		pps	papillae stalk
dtu	dorsal tubercle	ps	pigment spots
end	endostyle	r	rectum
eye	eyespot	rph	retropharyngeal band
fp	fecal pellet	sg	stigmata
gang	ganglion	sl	stolon
ht	heart	slb	stolon bud
in	intestine	spd	sperm duct
ingl	intestinal gland	st	stomach
k	kidney	stpl	stomach plication
l	liver	tlf	tail fin
lcb	longitudinal ciliated band	tlm	tail muscle cell
		trb	transverse bar
lg	languet	ts	testes
lnb	longitudinal bar	tt	test or tunic
mn		yk	yolk

PLATE I

1. Generalized holosomatous adult.
2. Generalized merosomatous adult.
3. Generalized synoicid adult.
4. Generalized larva.

PLATE II

1. Free-swimming larva
2. Early stage of metamorphosis
3. Late stage of metamorphosis
4. Completed adult.

PLATE III

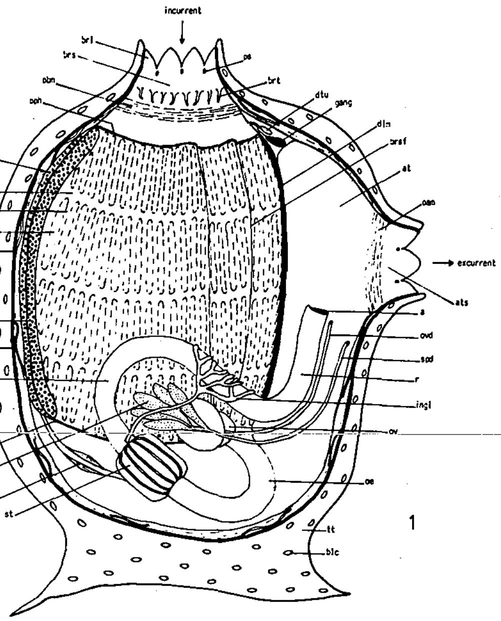
1. *Amaroucium californicum adult*.
2. *A. californicum* larva.
3. *A. solidum* adult.
4. *A. solidum* larva.
5. *A. propinquum* adult.
6. *A. propinquum* larva.
7. *A. arenatum* adult.
8. *A. arenatum* larva.

PLATE IV

1. Synoicium par-fustis adult.
2. S. par-fustis larva.
3. Sigillinaria pulchra adult.
4. S. pulchra larva.
5. Didemnum carnulentum adult.
6. D. carnulentum larva.
7. Cystodytes lobatus adult.
8. C. lobatus larva.

PLATE V

1. Distaplia occidentalis adult.
2. D. occidentalis larva.
3. Undescribed species adult.
4. Undescribed species thorax showing left side and front types of atrial openings.
5. Undescribed species larva.
6. Ascidia ceratodes adult.
7. A. ceratodes larva.



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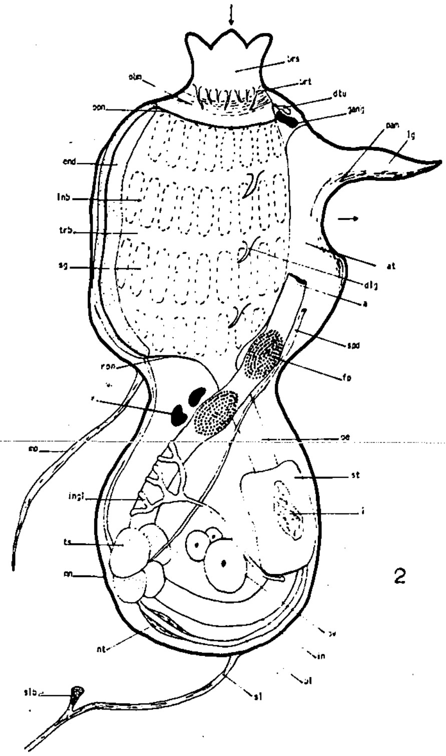

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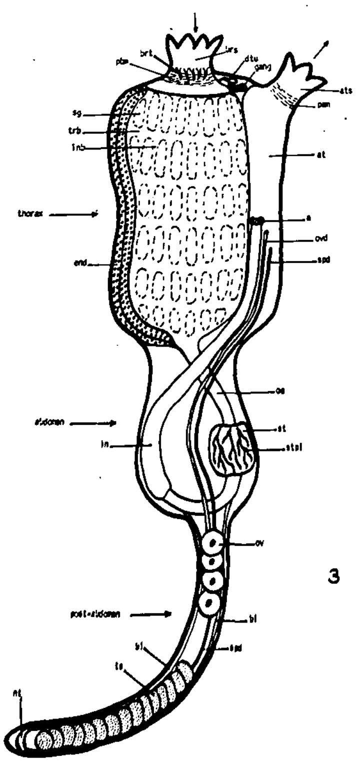

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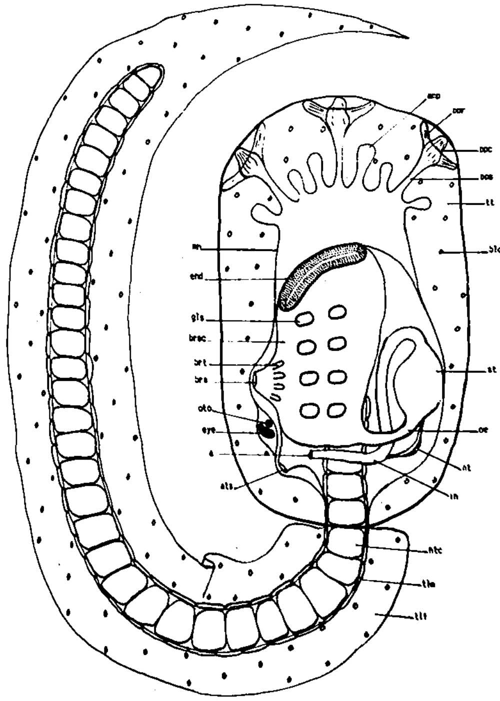

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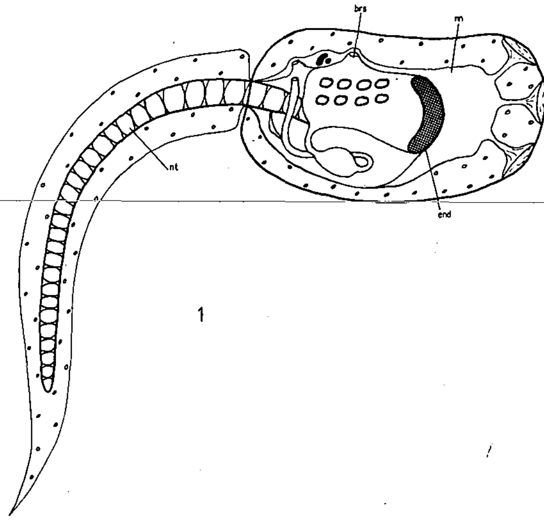
PLATE 1.



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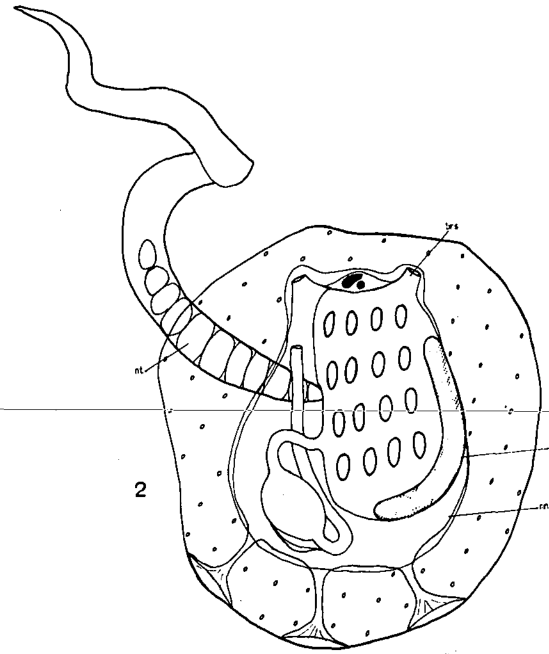

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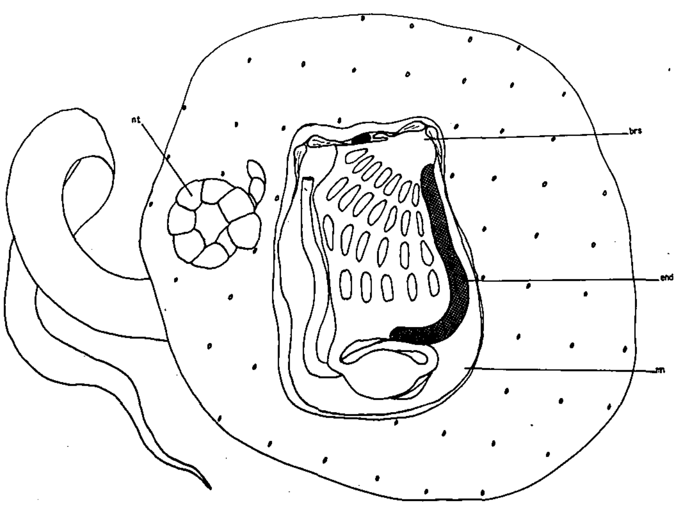

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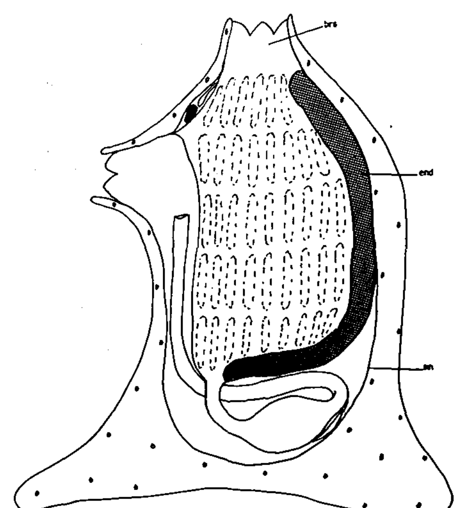
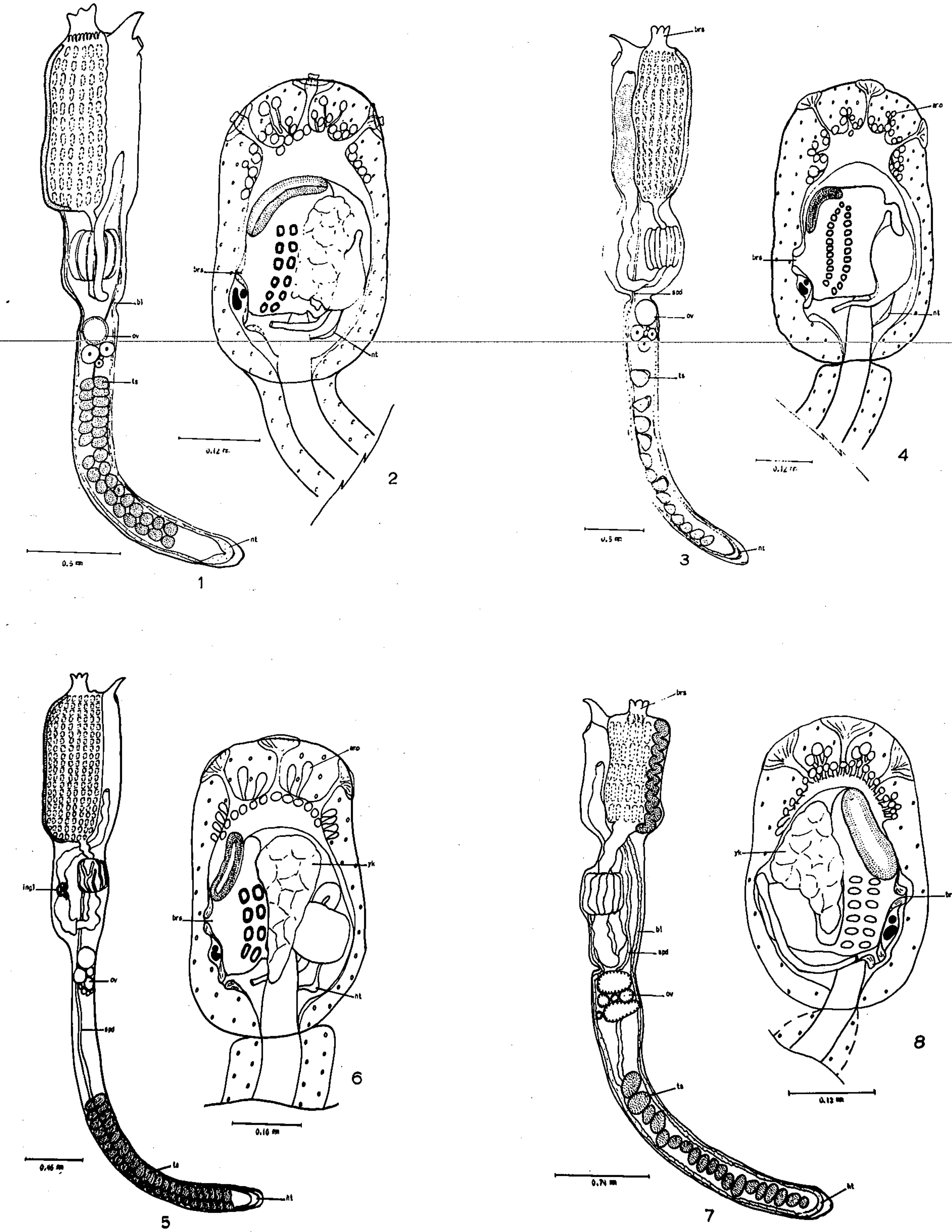


4

PLATE II.



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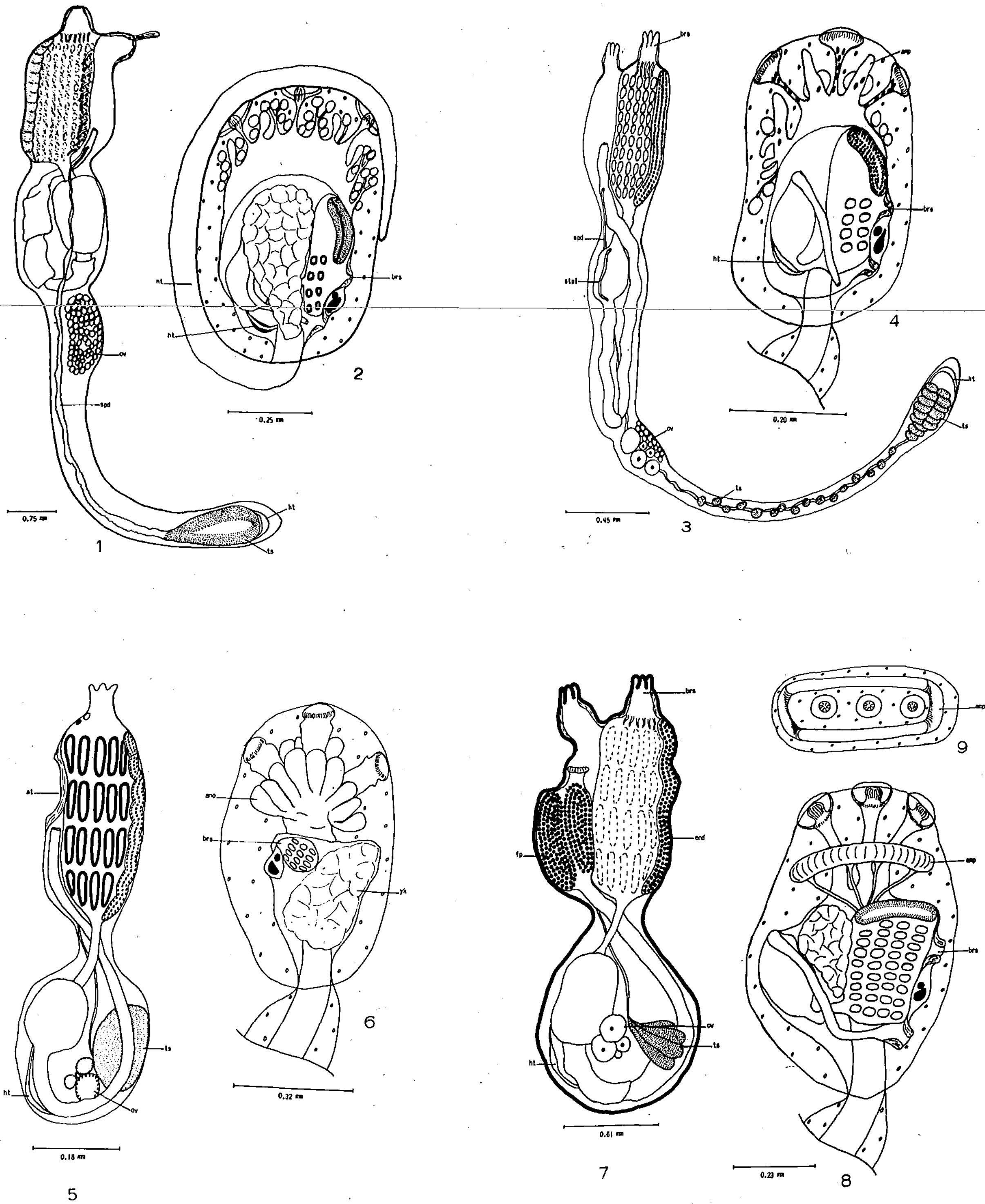
1
2
3
4
5
6
7
8
9
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0.25 mm
0.45 mm
0.20 mm
0.18 mm
0.32 mm
0.61 mm
0.23 mm
ov
spd
ht
ts
brs
stpl
at
ano
yk
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end

PLATE IV.

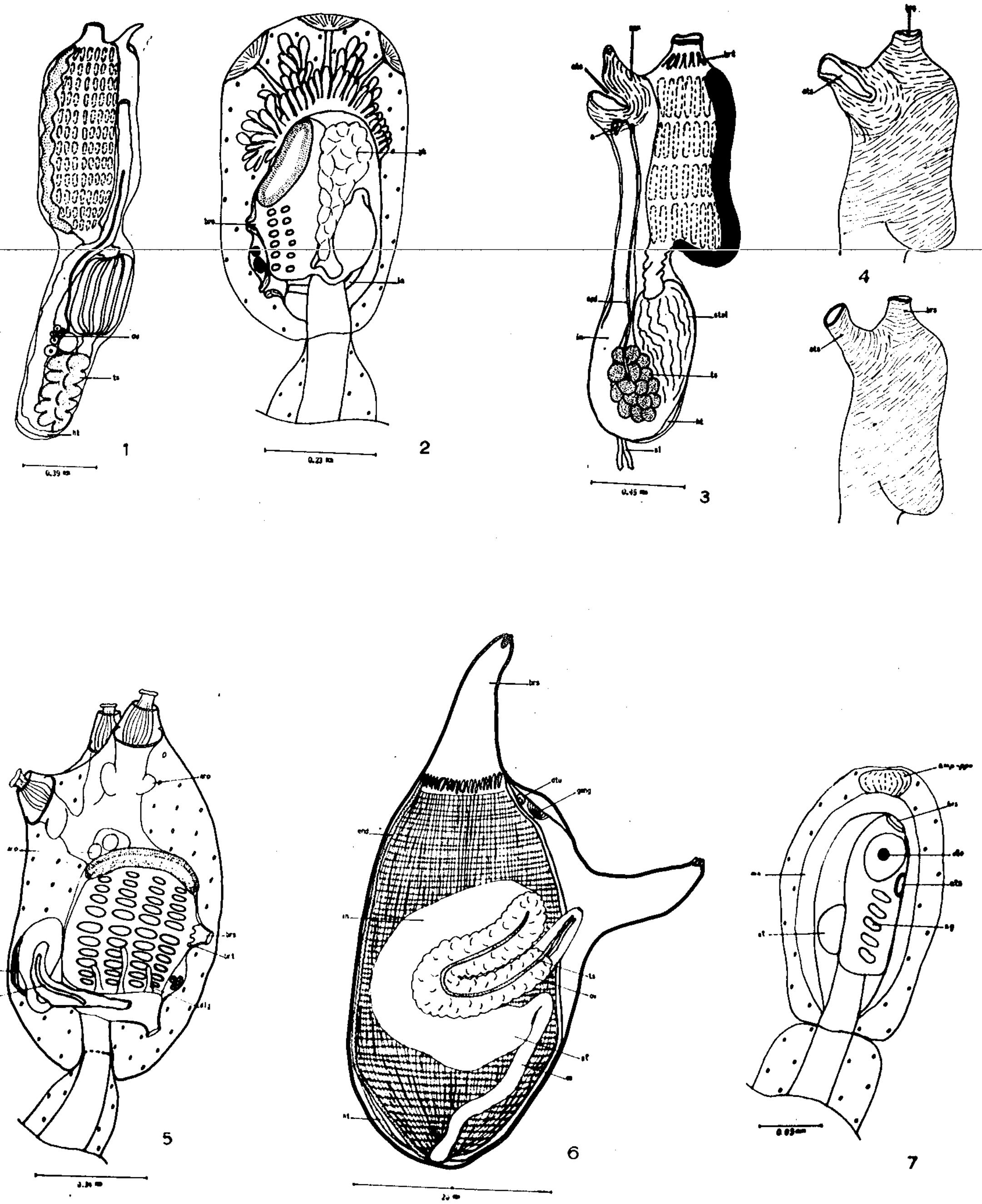

PLATE V