Comparative observations of gametes, fertilization, and maturation in sipunculans

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Abstract

A review of characteristic features of sipunculan eggs shows a significant relationship between egg size and development mode, but no significance between egg size and adult size or of developmental mode and size of adult. Specific variations among eggs include size, shape, and structure of the egg envelope. Variations in shape show some relationship to higher taxonomic classification, ovoid eggs being restricted to the families Aspidosiphonidae and Phascolosomatidae. Sipunculan sperm are of the primitive type. Eggs are generally arrested in the first meiotic metaphase at spawning, but two exceptions are reported: *Nephasoma pellucida* and *Sipunculus nudus* spawn eggs with intact germinal vesicles which later break down in sea water. Sperm penetration is demonstrated to occur by the formation of a hole by the sperm in the envelope of the egg.

Keywords: Sipuncula, fertilization, maturation, gametes.

Introduction

Sipunculans are typically dioecious, shedding eggs and sperm into the sea water where fertilization takes place. Reported exceptions include an hermaphroditic species, *Golfingia minuta* (Åkesson 1958) and a parthenogenetic species, *Themiste lageniformis* (Pilger 1987). The gonad, a narrow band of tissue usually located at the base of the ventral retractor muscles, releases gametes at an early stage into the spacious coelomic cavity where the remainder of development occurs. From the coelom the most mature gametes are taken up into the nephridia where they are stored until spawning. Some information on oogenesis is available for a few species (Gerould 1906, Gonse 1956, Sawada, Noda & Ochi 1968, Towle & Giese 1967, Rice 1974). However, little attention has been given to comparisons of mature gametes or to the processes of fertilization and maturation in sipunculans. In studies of developmental patterns of sipunculans over a period of many years (Rice 1966, 1967, 1973, 1975a, 1976, 1988), the author has amassed data on eggs and sperm of numerous species from fortuitous spawnings as opportunities have allowed and has made occasional observations on fertilization and maturation. These observations will be reported in this paper and reviewed in light of previous literature, taxonomic implications, and correlation with developmental patterns.

Materials and methods

Fixation of unfertilized and recently fertilized eggs for light and transmission electron microscopy was carried out in 2.5% phosphate buffered glutaraldehyde followed by postfixation in ice-cold 2% osmium tetroxide, buffered with 0.2 M S-collidine or 0.4 M Millonig's phosphate buffer. In some instances, as noted, fixation was in 2% osmium tetroxide only. For material fixed for scanning electron microscopy, the osmium fixation was generally omitted. Material to be sectioned was dehydrated in alcohol and propylene oxide or acetone and embedded in Epon 812 and serially sectioned at 1 µm. It was stained with Richardson's stain (Richardson, Jarett & Finke 1960) for examination with light microscopy. For transmission electron microscopy, sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 9-S2. Preparation for scanning electron microscopy consisted of dehydrating in an alcohol and acetone series, drying in a critical point dryer with liquid carbon dioxide, coating in a sputtering unit with gold-palladium, and viewing with a NovaScan microscope.

Measurements of eggs were made on living, unfertilized, recently spawned material. The number measured depended on availability of each species and is noted in the text.

Measurements of thickness of ovoid eggs and of thickness of egg envelopes were made on only a few (3 to 10) of the total eggs measured. Precise values for these features are difficult to determine in living eggs and are presented here only as an indication of dimensions for general comparative and descriptive purposes.

Observations

Eggs

A common feature of all sipunculan eggs is the thick egg envelope which is multilayered and perforated by pores. Variations among the eggs of different species are

Family	Species	Size of adult,* length, mm	Size of egg, µm	Type of development**	Shape	Color	Egg envelope thickness, µm	Locality
Sipunculidae	*Phascolopsis gouldi*[5]	165	150-180	L	Spherical	Reddish-brown	3-4	Newport, Rhode Island
	Siphonosoma cumanense[10]	200	122	P	Spherical	Clear	5-6	Puerto Rico
	Sipunculus nudus[6,10]	200	178	P	Spherical	Clear	5	Puerto Rico, Florida (west coast)
Golfingiidae	*Golfingia elongata*[2]	135	125	L	Spherical	Grey-rose	–	Roscoff, France
	G. ohlini[4]	17	177	L	Spherical	White, opaque	–	Argentina (Rio Negra)
	G. vulgaris[5]	155	150-180	L	Spherical	Pale brown	–	Roscoff, France
	Nephasoma minuta[1]	16	260-280 × 214-230	D	Elongate	Grey, opaque	3-4	Kristineberg, Sweden
	Nephasoma pellucida[1]	26	105	P	Spherical	Opalescent Pale grey	6-7	Florida (east coast)
	Thysanocardia pugettensis[7]	150	160	L	Spherical	Pink	5	San Juan Island, Washington
Phascolionidae	*Phascolion cryptus*[8]	30	136	D	Spherical	White, opaque	5	Florida (east coast)
	Phascolion sp.[11]	6	112×124	L	Elongate	Clear	3-4	Florida (east coast)
	P. strombus[1]	40	125	L	Spherical	Grey to reddish-brown	6	Kristineberg, Sweden
Themistidae	*Themiste alutacea*[8]	16	138	L	Spherical	White, opaque	4	Florida (east coast)
	T. lageniformis[12]	26	145	L	Spherical	White, opaque	4	Florida (east coast), Hawaii
	T. petricola[3]	33	156	L	Spherical	White to pale yellow	–	Argentina (Chubut)
	T. pyroides[7]	180	190	D	Spherical	White, opaque	5	Vancouver Island, British Columbia
Aspidosiphonidae	*Aspidosiphon parvulus*[11]	13	107×139	P	Ovoid	Pale yellow	10-12	Florida (east coast)
	A. fischeri[8]	10	94×103	P	Ovoid	Pink	10	Florida Keys, Florida (east coast)
Phascolosomatidae	*Apionsoma misakiana*[9]	14	74×105	P	Ovoid	Clear, opalescent	6-7	Florida (east coast)
	Antillesoma antillarum[8]	48	97×127	P	Ovoid	Yellow	9	Florida Keys
	Phascolosoma agassizii[7]	140	110×140	P	Ovoid	Yellow-orange	10	San Juan Island, Washington
	P. perlucens[8]	35	91×112	P	Ovoid	Pink-red	7	Florida Keys
	P. varians[5]	45	90×104	P	Ovoid	Pale yellow	8	Florida, Belize

References for developmental data: 1. Åkesson 1958; 2. Åkesson 1961; 3. Amor 1975; 4. Amor 1976; 5. Gerould 1906; 6. Hatschek 1883; 7. Rice 1967; 8. Rice 1975a; 9. Rice 1981; 10 Rice 1988; 11. Rice, unpublished; 12. Williams 1972.

* Size of adults is from Stephen & Edmonds 1972 (total length sometimes estimated from length of trunk and relative length of introvert), except for *Golfingia vulgaris* from Théel 1905, *Phascolion cryptus* from Hendrix 1975, and *Antillesoma antillarum*, *Apionsoma misakiana*, *Aspidosiphon fischeri*, *N. pellucida*, *Phascolion* sp., *Phascolosoma perlucens* which were measured by Rice, unpublished.

** P = planktotrophic = development with feeding larvae; L = lecithotrophic = development with non-feeding larvae; D = direct = development with no larval forms.

Table 1.
A summary of characteristic features of sipunculan eggs.

found in size, shape, pigmentation, yolk density, and the number and arrangement of layers in the egg envelope (Figure 1). Descriptions will be given below for recently spawned, unfertilized eggs of 13 species, representing 8 genera and 6 families as recognized in the classification of Gibbs & Cutler (1987). Some of the measurements have been previously reported in review papers as noted in Table 1.

Broader at the animal pole and more tapered at the vegetal pole, the eggs of *Antillesoma antillarum* are ovoid, a typical shape for eggs of many species of sipunculans. Maximum length and width are 127×97 µm (n = 40), the thickness (n = 3) equaling 84 µm. The egg envelope is 9 µm in width with 3 layers, the middle being the most prominent. Inner and middle layers are penetrated by conspicuous pore canals. There is no flattening or depression at the animal pole as occurs in some other sipunculan ovoid eggs. The color is yellow, but in large masses the eggs appear bright orange. The spindle of the first meiotic metaphase is located at the animal pole.

The eggs of *Apionsoma misakiana* are ovoid, the vegetal pole being more tapered and the animal pole flattened. They are 105×74 µm in maximum length and width (n = 200, 50 eggs measured from each of 4 females), clear and opalescent. There is a slight depression in the egg envelope at the animal pole which is characteristic of many ovoid eggs. The metaphase spindle, unlike that in all other known sipunculan eggs, is in a central rather than apical position from the time of spawning until fertilization (Figure 8A). Of moderate thickness, the egg envelope is 6-7 µm. Scan-

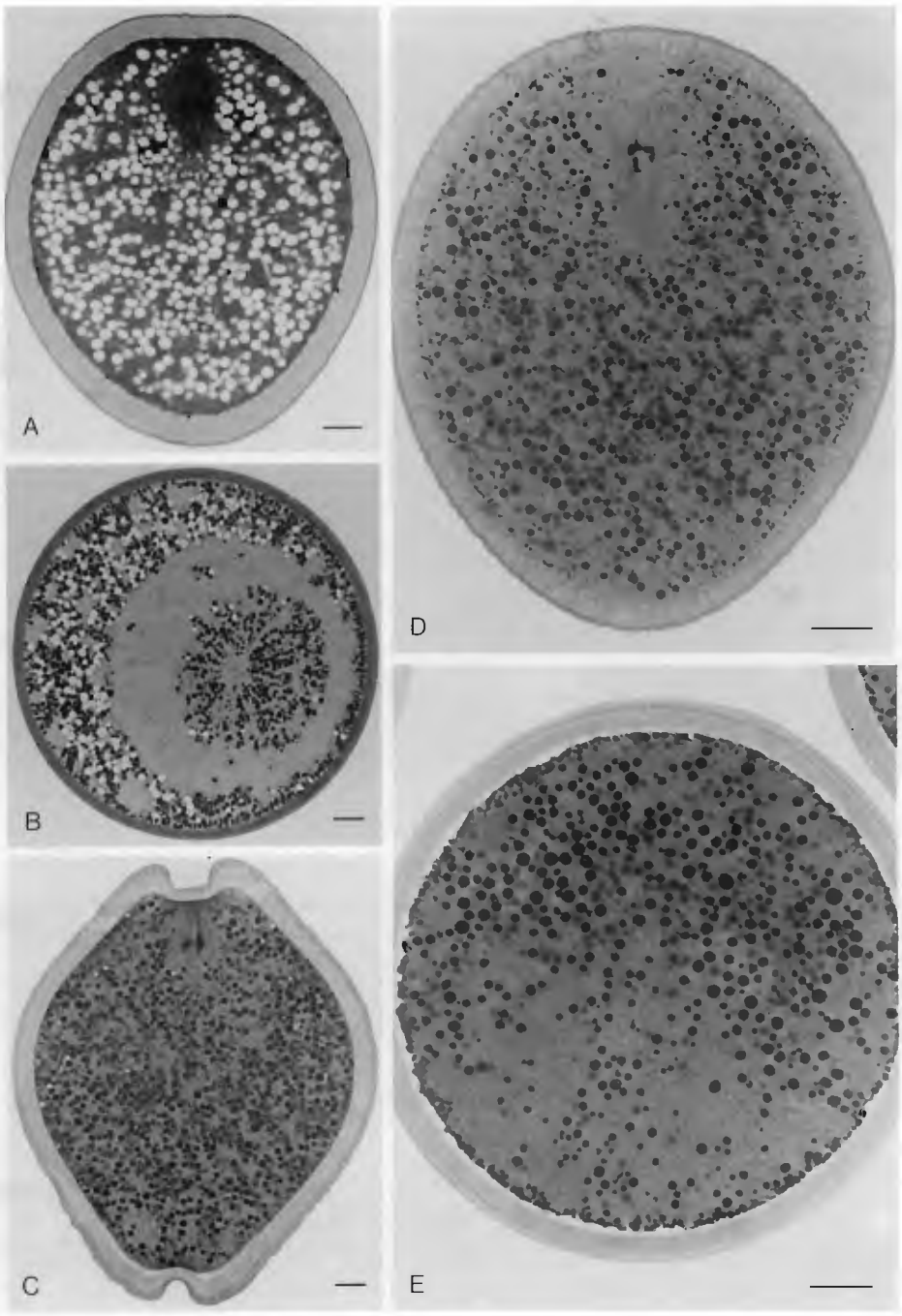

Figure 1.
Sections of recently spawned, unfertilized eggs of 5 species of sipunculans. 1 µm; Epon-embedded; Richardson's stain. Scale bar: 10 µm. A, *Aspidosiphon parvulus*. B, *Phascolion cryptus*. C, *Phascolosoma perlucens*. D, *Aspidosiphon fischeri*. E, *Nephasoma pellucida*.

ning electron micrographs show the surface of the egg to be covered with small depressions, indicating the porous nature of the envelope (Figure 2A-C). Transmission electron micrographs reveal 3 major layers, the middle and widest of which is marked by 4 to 5 electron dense bands interspersed by lighter bands (Figure 3A). Canals, in which microvilli are enclosed, pass through the inner and middle layers, broadening at the outer border of the middle layer where they are covered over by the fibrous material of the outer layer. Where the fibrous covering has been ruptured, scanning electron micrographs reveal the nature of the openings of the pore canals. As the microvilli penetrate the outer layer, they divide into several branches, the electron dense tips of which protrude at the surface of the outer layer (Figure 3A).

Commonly ovoid, the eggs of *Aspidosiphon fischeri* may vary to elongate, without readily distinguishable animal and vegetal poles (Figure 1D). Maximum length and width average 103×94 µm (n=50). The spindle of the first meiotic metaphase is located at the animal pole and the eggs are pale with a pinkish tinge. The egg enve-

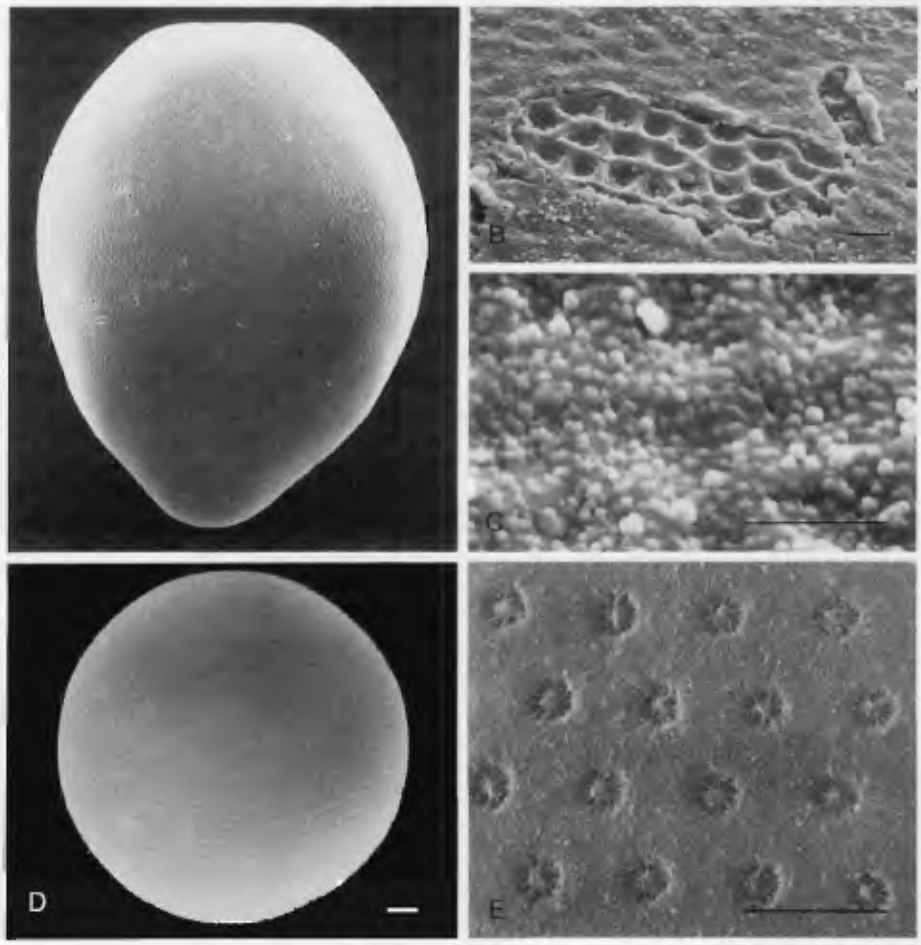

Figure 2.
Scanning electron micrographs of the porous egg envelope of unfertilized eggs of two species of sipunculans. A, egg of *Apionsoma misakiana*. Scale bar: 5 µm. B, Higher magnification of A. Outer layer of egg envelope is ruptured, revealing openings of the pores in the middle layer. Scale bar: 1 µm. C, higher magnification of undamaged surface of B, showing granular nature of the surface of the outer egg envelope. Scale bar: 1 µm. D, egg of *Sipunculus nudus*. Scale bar: 10 µm. E, higher magnification of D, showing characteristic pores of the egg envelope. Scale bar: 5 µm.

lope, approximately 10 µm in breadth, is unusually thick when compared with other sipunculan eggs. With light microscopic examination of living eggs and of 1 µm sections, three layers are obvious, the middle being by far the widest. As seen in electron micrographs, the inner and outer layers are composed of lightly staining granular or fibrous material, whereas the middle layer consists of electron dense clumps of irregular size, shape and arrangement, bordered on its outside by a continuous line of electron dense material, interrupted only by the pores (Figure 3B). Extending through the pores are elongate microvilli which branch in the outer layer.

The eggs of *Aspidosiphon parvulus* are more variable in shape than those of most species of sipunculans. Generally ovoid, they usually show a slight flattening at the animal pole (Figure 1A). Averaging 139×107 µm in length and width (n = 105, 50 eggs from one animal and 55 from the second), they have exceptionally thick egg envelopes of 10 to 12 µm. Examined in 1 µm sections, the envelope consists of one wide, evenly staining layer and a second darkly staining external covering on the outer side of which is a lightly staining fuzz. Living eggs are transparent and have a pale yellow pigmentation. The first meiotic metaphase spindle of the unfertilized egg is in an apical position at the animal pole. In contrast to those of most other sipunculan eggs, the yolk granules stain lightly rather than darkly with Richardson's stain. The cytoplasm, as observed in 1 µm sections with the light microscope, is fine, homogeneous, and granular with scattered darker staining areas of unknown composition. The pores are apparent in the inner layer, but not the outer layer of the egg envelope.

Nephasoma pellucida has spherical eggs, 104 µm in diameter (n = 50). The egg envelope is 6-7 µm thick (Figure 1E). Greyish white in color, the eggs are opalescent in reflected light. The germinal vesicle is present at the time of spawning, an uncommon occurrence in sipunculans. Within 30 minutes in sea water the meiotic spindle has formed (see Maturation and Fertilization).

The spherical eggs of *Phascolion cryptus* are 136 µm in diameter (n = 10) and, compared with most sipunculan eggs of ovoid shape, have a relatively narrow envelope of 5 µm with the typical 3 layers, the outer of which is quite thin. Cover-

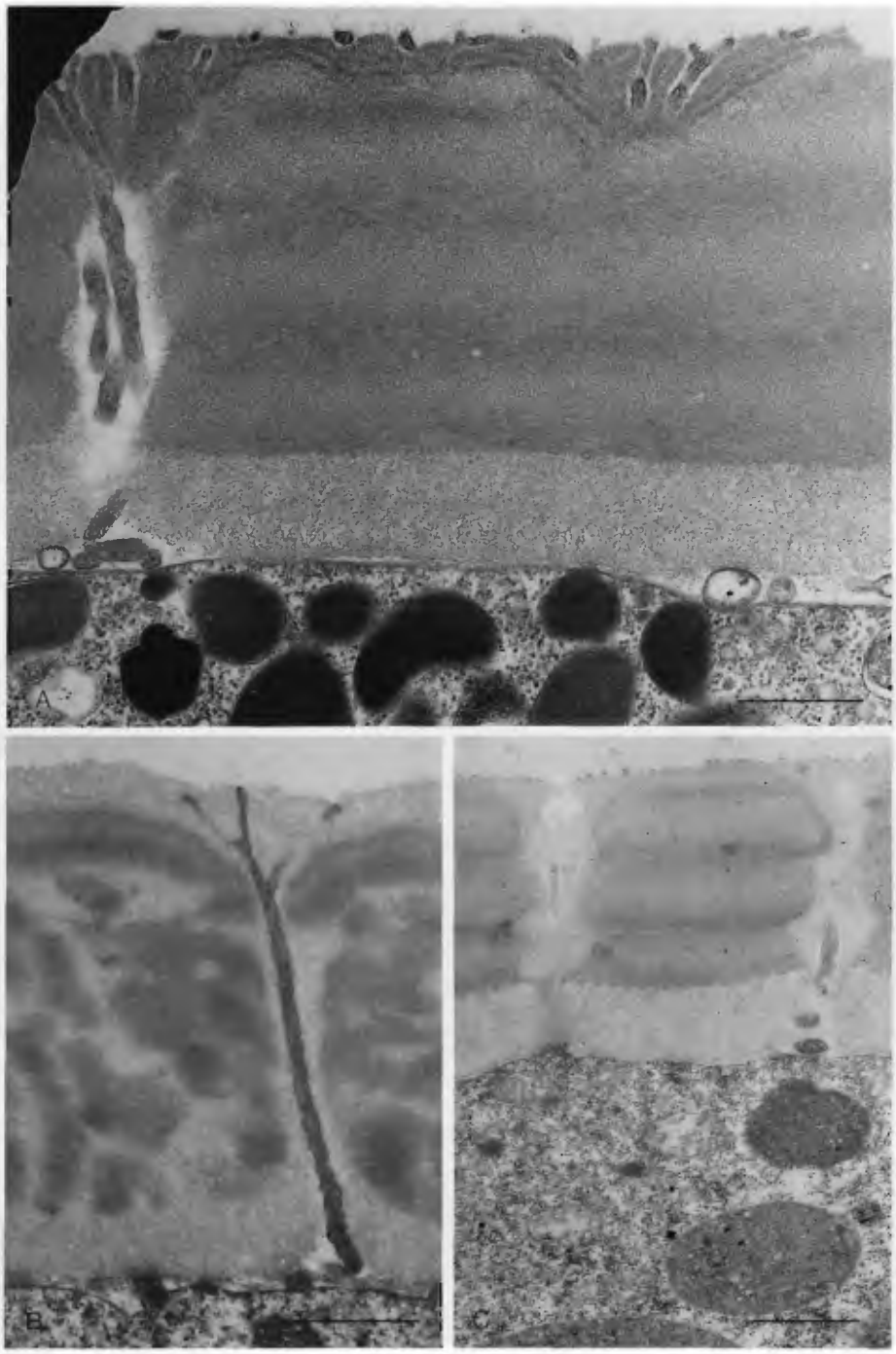

Figure 3.
Transmission electron micrographs of the egg envelopes of unfertilized eggs of three species of sipunculans. Scale bar: 1 µm.A, *Apionsoma misakiana*, microvilli extend through pores in inner and middle layers of the egg envelope, branching in the outer layer and terminating at the surface in electron-dense tips. B, *Aspidosiphon fischeri*. Note the branching microvillus and the patchy organization of the electron-dense fibrous material of the middle layer. C. *Sipunculus nudus*. Outer layer of egg envelope is thin with microvillar tips projecting at the surface. Note pore canals in inner and middle layers enclosing sections of microvilli. Middle layer is characterized by well-defined bands.

ing the surface is a thin jelly coat, about 8 µm wide in living eggs, which lends an adhesive quality. The egg is white and densely concentrated with yolk granules which show varying staining characteristics in 1 µm sections treated with Richardson's stain (Figure 1B).

A small undescribed interstitial species of *Phascolion* , 6 mm in length, has elongate eggs of 124×112 µm . Unlike the ovoid eggs of sipunculans, the animal and vegetal extremities are rounded, showing no tapering. The living eggs are clear and the egg envelope is 3-4 µm thick.

Of the sipunculan eggs that have been described, those of *Phascolosoma perlucens* are unique in having depressions of the egg envelope at both animal and vegetal poles (Figure 1C). Otherwise, the egg is ovoid. Frequently animal and vegetal poles cannot be distinguished, although the depression at the animal pole may be broader and the tapering at the vegetal pole may be greater. The average maximum length and width are 112×91 µm (n = 50) and thickness 86 µm (n = 5). The egg en-

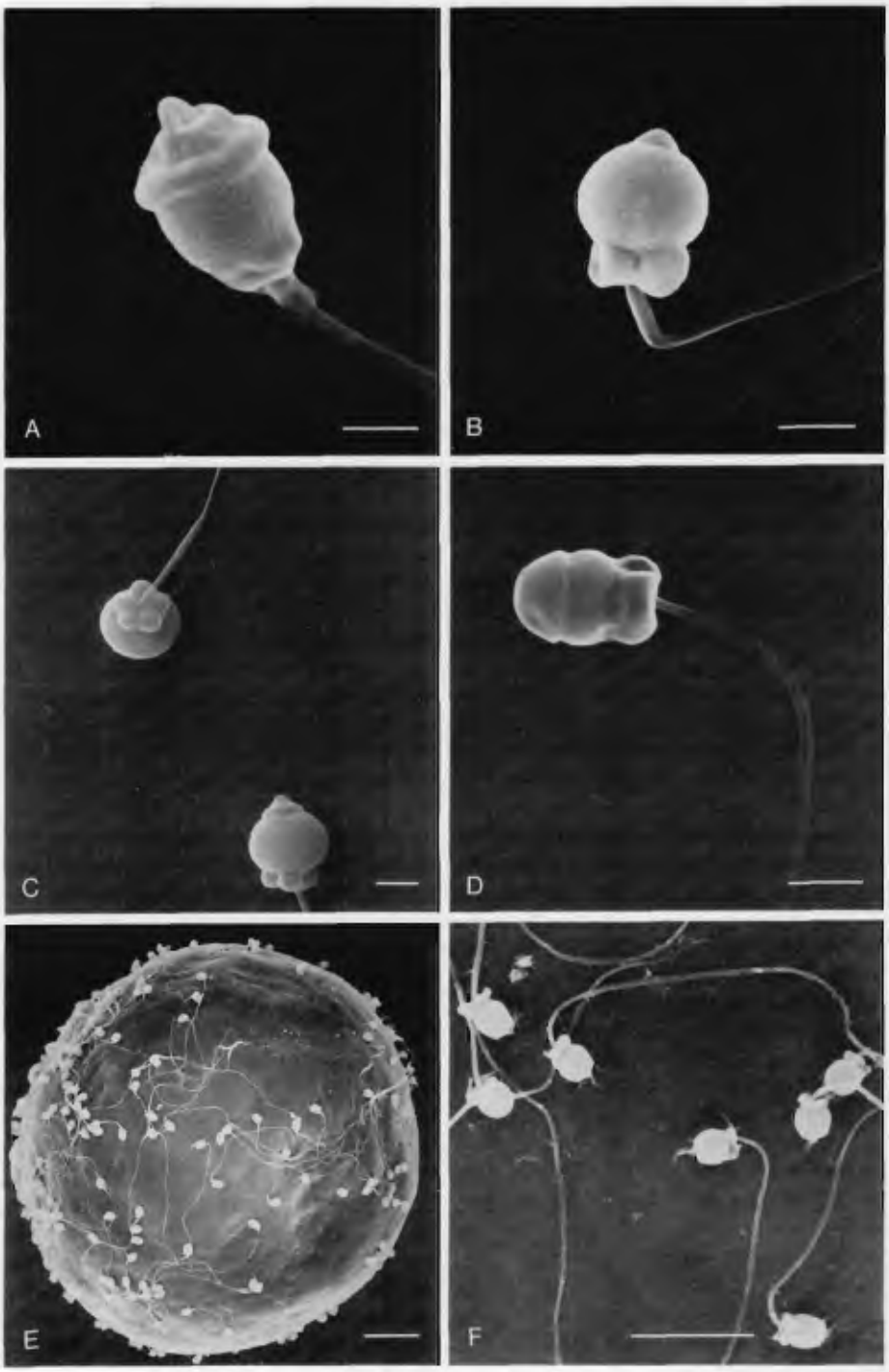

Figure 4.
A-D, scanning electron micrographs of sperm of four species of sipunculans. Scale bar: 1 µm. A, *Themiste pyroides*. B, *Apionsoma misakiana*. C, *Aspidosiphon fischeri*. D, *Siphonosoma cumanense*. E, egg of *Nephasoma pellucida* with attached sperm. Scale bar: 10 µm. F, higher magnification of E. Note extended acrosomal filaments of sperm. Scale bar: 5 µm.

velope, 7 µm in thickness, consists of 3 layers, the outer and inner being much thinner than the middle; the outer layer appears only as a fine dark line in live eggs. The color of the eggs is pink to red. At the time of spawning the spindle of the first meiotic metaphase is at the animal pole.

Eggs of *Phascolosoma varians* are ovoid and broader at the animal pole than vegetal. A slight depression in the egg envelope at the animal pole is not as deep as in *P. perlucens*. Appearing somewhat rounder than the eggs of other species of *Phascolosoma* they are 104×90 µm in length and width (n = 10). The egg envelope is 8 µm thick, the inner and middle layers being approximately equal in width and the outer more narrow. Eggs are pale yellow. The spindle of the first meiotic metaphase is at the animal pole.

The eggs of *Sipunculus nudus* are greyish and rather clear. They are spherical, 178

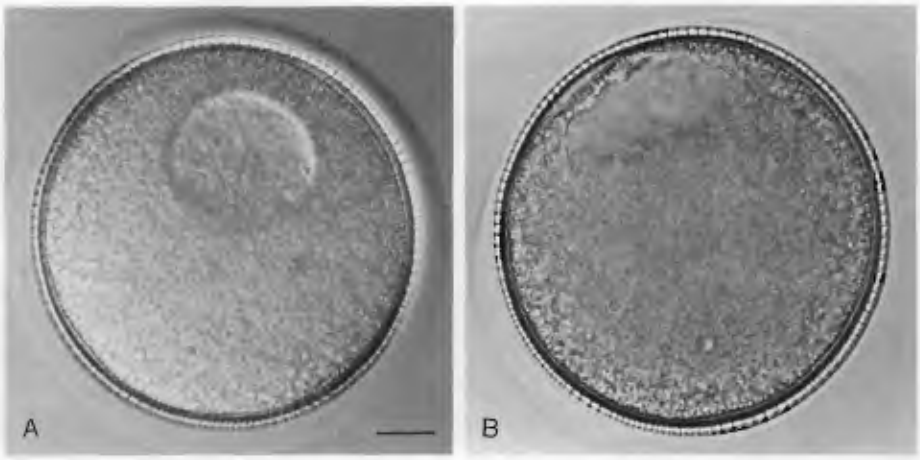

Figure 5. Unfertilized eggs of *Sipunculus nudus*. Light micrographs taken with Nomarski optics. Scale bar: 25 µm. A, recently spawned egg with germinal vesicle and outer jelly coat. B, egg after 2 hours in sea water. Germinal vesicle is in the process of dissolution.

µm in diameter (n = 200, 50 eggs measured from each of 4 females), and exhibit a sparkling opalescence under reflected light (Rice 1988). As in *Nephasoma pellucida*, the germinal vesicle is present at spawning. The germinal vesicle, usually central but sometimes eccentric, commonly has one nucleolus (Figure 5A). The egg envelope is 5 µm thick and is surrounded by an outer jelly coat 12 to 13 µm in width. Prominent pore canals in the egg envelope are readily apparent in the living egg and in scanning electron micrographs (Figures 5A; 2D,E). Transmission electron micrographs show a well developed, lightly staining inner layer which is about one-fourth of the width of the thicker, darker middle layer (Figure 3C). Three to four bands, composed of apparently fibrous material, make up the middle layer. The outer layer is extremely thin and inconspicuous, except at the openings of the pores. Microvilli extend through the pores and, after branching, terminate at the surface of the outer envelope.

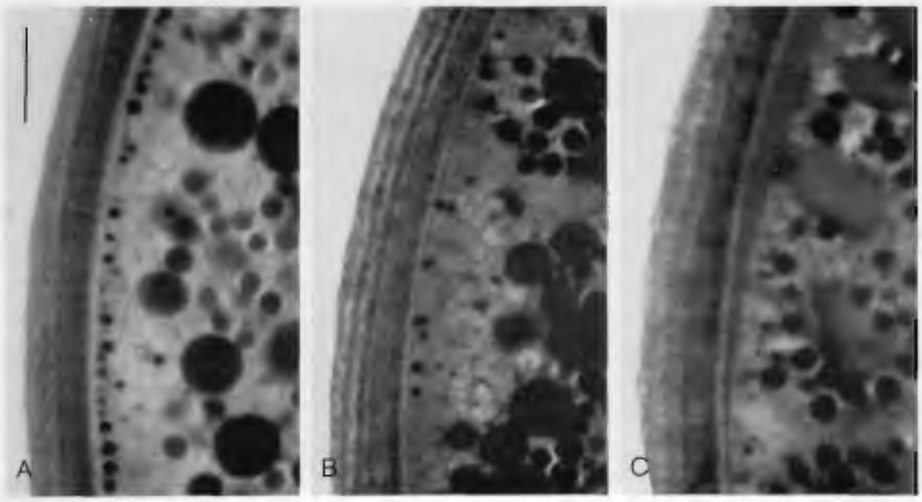

Figure 6.
A-C, sections of the egg envelope and cortex of fully developed coelomic oocytes of *Thysanocardia pugettensis* that have been removed from the coelom, rinsed and placed in sea water, sectioned at 1 µm; embedded in Epon; stained with Richardson's stain. Scale bar: 5 µm. A, immediately after placement in sea water. Cortical granules are apparent in the cytoplasmic cortex beneath the egg envelope.
B, after approximately 6 hours in sea water (10 °C). Note decrease in abundance of cortical granules. C, after approximately 10 hours in sea water (10 °C). Cortical granules have disappeared and a newly-formed space is apparent between the egg envelope and cytoplasm.

The eggs of *Themiste alutacea* are spherical, 138 µm in diameter (n = 108, 50 eggs from each of 2 animals, 8 from a third). Densely concentrated with yolk, they are white and opaque. The egg envelope of 4 µm is relatively narrow. The spawned egg adheres to the substratum or any available surface on contact, although no jelly coat has been detected in light or electron microscopic examination.

Similar to the eggs of *Themiste alutacea*, those of *Themiste lageniformis* are spherical, white, and adhesive. They are 139 µm in diameter (n = 50) with an egg envelope of 4 µm that is surrounded by a jelly coat about 10 µm thick.

Spermatozoa

The mature spermatozoa of the 4 species, *Apionsoma misakiana*, *Aspidosiphon fischeri*, *Siphonosoma cumanense*, and *Themiste pyroides*, examined in this report are essentially similar in form, consisting of a rounded head, a short midpiece with mitochondrial spheres, and a long filamentous tail (Figure 4A-D). The chief difference among these species is in the degree of development of the acrosome. In *Siphonosoma cumanense* it is rounded and large, approaching the size of the head

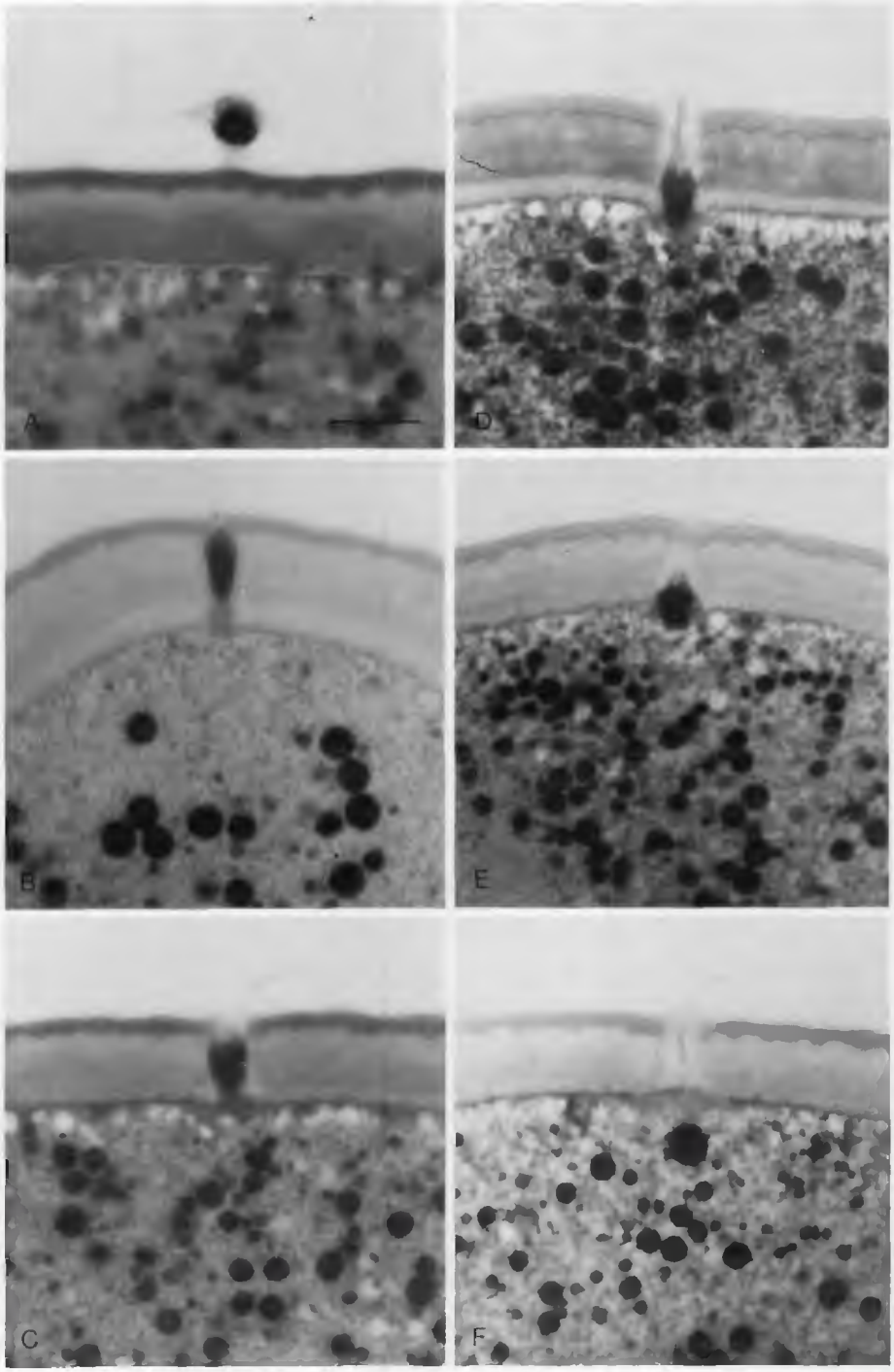

Figure 7.
Sections of six eggs of *Phascolosoma agassizii* fixed in 2% osmium tetroxide within 15 minutes after combination with sperm. 1 µm thick; Epon-embedded; Richardson's stain. Note sperm entry holes in egg envelopes, D-F. Scale bar: 5 µm. A, sperm is attached to outer egg envelope by short acrosomal filament. B-E, sperm is in various stages of penetration of egg envelope. F, sperm head is totally incorporated into egg cytoplasm.

itself. The caps of *Apionsoma misakiana* and *Aspidosiphon fischeri* are both pointed, but that of the latter is offset from the head by a basal rim. The most elaborate development of the acrosomal cap is found in *Themiste pyroides* in which the prominent cap possesses two components: a basal circular ridge and a pointed apical portion. Three of the species have 4 mitochondrial spheres, but the fourth, *Aspidosiphon fischeri*, has 5 distinctive spheres in the midpiece.

When the sperm makes contact with the surface of the egg, it forms an acrosomal filament (Figure 4E, F). In the case of *Themiste pyroides*, the egg of which possesses a thick jelly coat 50 µm in width (Rice 1967), the acrosomal filament is extended to a length of 50 µm through the jelly coat to the egg envelope (Rice, in press). Many sperm attach to a single egg in this manner with heads at the surface of the jelly coat and tails bent at an angle of 90° from the head. The penetrating sperm leaves a trail through the jelly.

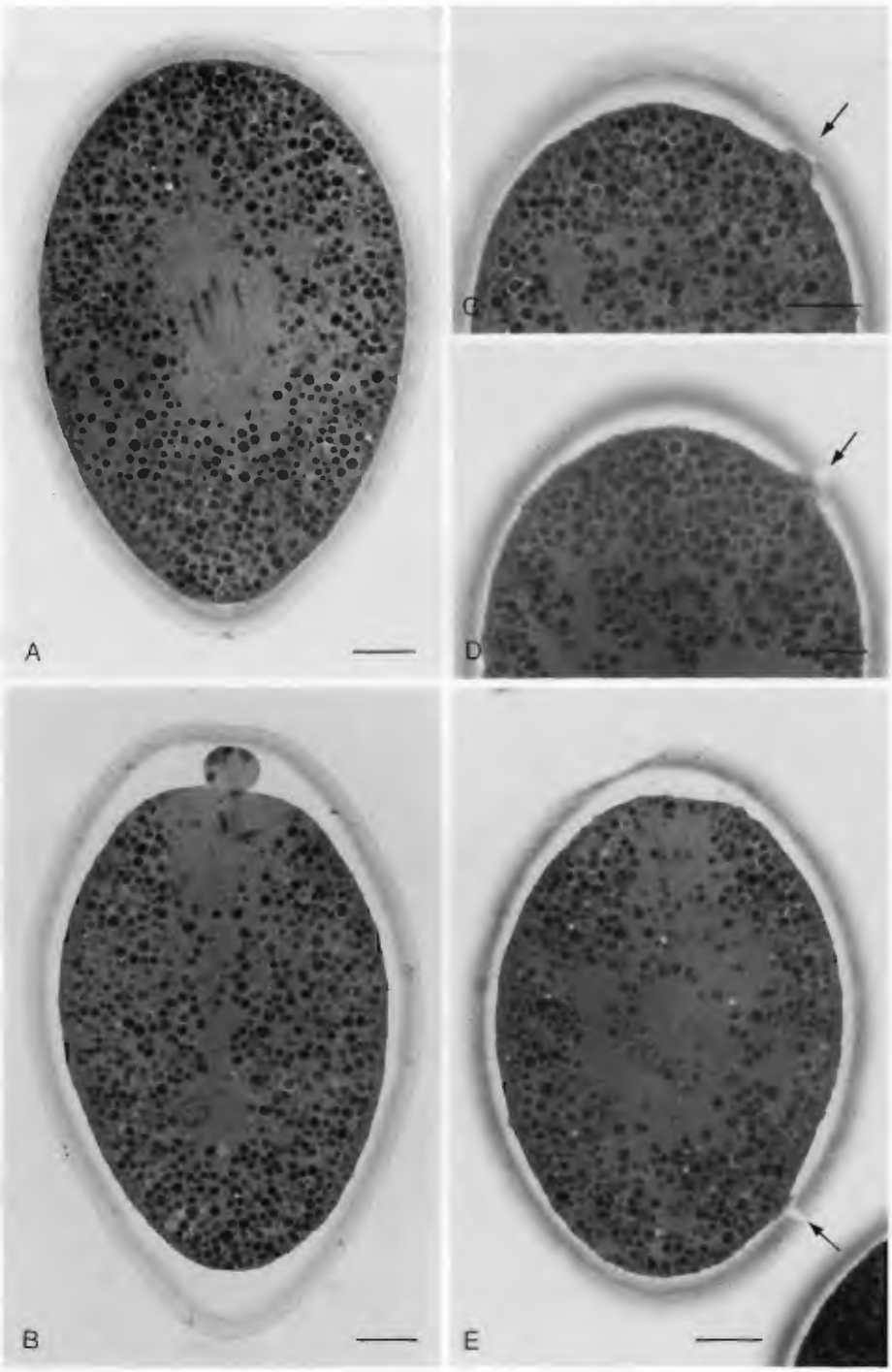

Figure 8.
Sections of eggs of *Apionsoma misakiana*. 1µm; Epon-embedded; Richardson's stain. Scale bar: 10 µm.
A, unfertilized egg showing first meiotic metaphase in central position. B, fertilized egg in the first polar-body stage. Male pronucleus is visible in lower third of egg. C-E, fertilized eggs showing sperm entry holes (arrows) in the egg envelopes. Cytoplasmic fertilization cones can be seen in C and D beneath the egg envelope at the point of sperm entry.

Fertilization and maturation

Maturation in sipunculan eggs commonly begins in the coelom with the breakdown of the germinal vesicle and is completed after sperm penetration with the extrusion of the first and second polar bodies. Previous reports have suggested that the breakdown of the germinal vesicle occurs prior to the uptake of coelomic oocytes into the nephridia where the eggs are stored until spawning (cf. Rice, in press). At spawning eggs are generally arrested in the first meiotic metaphase.

The process of fertilization is concurrent with and dependent on the completion of maturation. Following sperm penetration, the egg undergoes two meiotic divisions, giving off two polar bodies after which the female pronucleus is formed. Fertilization is concluded when female and male pronuclei unite to form the zygote

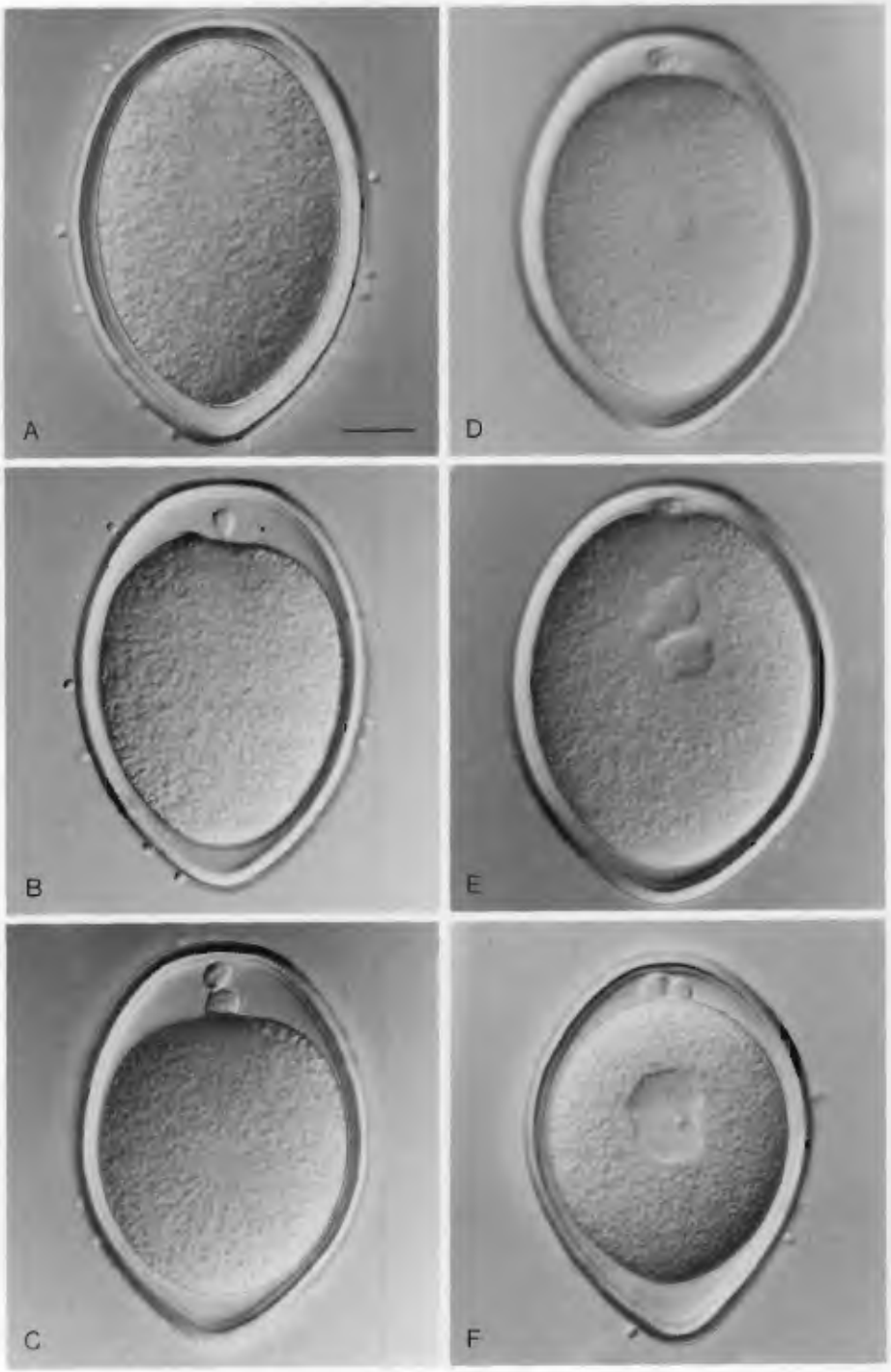

Figure 9.
Completion of maturation and fertilization in the egg of *Apionsoma misakiana*. Light micrographs taken with Nomarski optics. Scale bar: 20 µm. Time is in minutes after combination of eggs and sperm. A-E are same egg. A, 9 minutes. Cytoplasm has withdrawn from egg envelope at vegetal pole and first meiotic metaphase spindle has moved toward animal pole. B, 29 minutes. First polar body stage. C, 52 minutes. Second polar body stage. Clear central area is site of formation of male pronucleus. D, 60 minutes. Female pronucleus has formed at animal pole and male pronucleus in the central egg. E, 75 minutes. The vesicular female pronucleus and the male pronucleus have made contact. F, approximately 85 minutes. Female and male pronuclei have united to form zygote nucleus.

nucleus. Observations on various aspects of these two processes are reported below for 5 species of sipunculans. The information, gathered fortuitously over a period of years from spontaneous spawnings in the laboratory, differs in comprehensiveness of coverage for the 5 species.

Sipunculus nudus. Spawning was observed in this species on two occasions. At the time of spawning the eggs possessed an intact germinal vesicle which, within two hours in the absence of sperm, broke down in approximately 25 % of the eggs, resulting in the appearance of the first meiotic metaphase (Figure 5A, B). Within 4 hours about 75 % of the eggs were in the first meiotic metaphase. As no sperm were spawned on these occasions, the fertilizability of these eggs could not be tested.

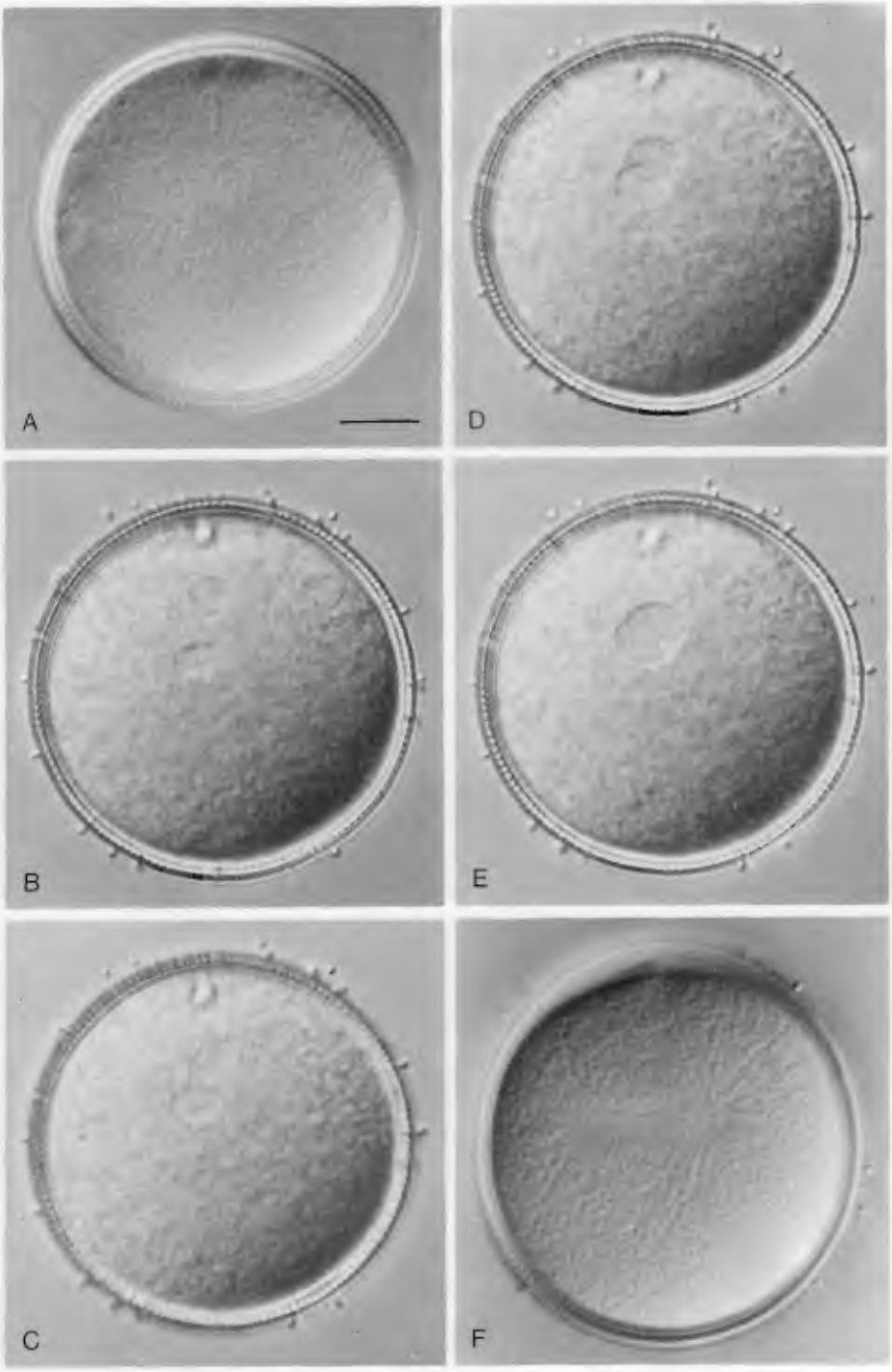

Figure 10.
Completion of maturation and fertilization in the egg of *Siphonosoma cumanense*. Light micrographs taken with Nomarski optics. Scale bar: 25μm. Time is in minutes after combination of eggs and sperm. A, unfertilized egg with spindle of first meiotic metaphase at animal pole. B, 33 minutes. Female pronucleus is upper and male pronucleus lower. C, 35 minutes. Female and male pronuclei in contact. D, 37 minutes. E, 39 minutes. Zygote nucleus. F, anaphase spindle prior to first cleavage.

Nephasoma pellucida. In 8 observations of recently spawned eggs germinal vesicles were intact. In 5 of the 8 spawnings sperm were also present and the eggs were surrounded by attached sperm (Figure 4E,F). Dissolution of the germinal vesicle occurred within 30 minutes in both the presence and absence of sperm. In the presence of sperm, polar body formation occurred within one hour after spawning.

Thysanocardia pugettensis. Spawning was not observed in this species, however, coelomic oocytes were found to undergo initial maturation with the breakdown of the germinal vesicle and thus to become fertilizable after 6 to 10 hours in sea water (Rice 1967). Eggs were fertilized with coelomic sperm which became active after 2 to 3 hours in sea water. Sperm attached to the eggs after the breakdown of the germinal vesicle and the subsequent formation of the first meiotic metaphase. In living eggs the formation of the meiotic metaphase was indicated by the appearance of a

clear polar cap and accompanied by the emergence of a narrow space between the egg envelope and the enclosed cytoplasm. After sperm penetration a ring of pink pigment appeared around the polar cap.

Coelomic oocytes, before and during exposure to sea water, were examined by light microscopy after fixation in 2% osmium tetroxide, embedment in epoxy resin, and sectioning at 1 µm. It was found that the cortical granules of the coelomic oocyte, described by Rice in 1973, underwent a dissolution along with the breakdown of the germinal vesicle. Subsequent to the disappearance of the cortical granules and presumably associated with this event, the space between egg envelope and cytoplasm made its appearance (Figure 6A-C).

Phascolosoma agassizii. As previously reported for this species and as is usual for most sipunculans, the egg is arrested in the first meiotic metaphase at the time of spawning (Rice 1967). The first visible changes in living eggs after the addition of sperm to the surrounding sea water are the rounding of the egg cytoplasm within the egg envelope followed by the extrusion of the first and second polar bodies. The cytoplasm separates from the egg envelope at the animal and vegetal poles. The interval between the addition of sperm to the culture and the extrusion of the second polar body may vary from 1½ to 4 hours at 12 °C in a single batch of eggs (Rice 1975a).

During a study of development of this species (Rice 1967), in a single specimen, dissected during the breeding season, eggs were discovered in the nephridium. Nephridial eggs were in the first meiotic metaphase, the condition of spawned eggs, whereas coelomic oocytes either showed an intact germinal vesicle or were in various stages of vesicle dissolution.

In a study of fertilization, spawned eggs from 2 females, 20 and 30 minutes after the combination with sperm, were fixed in 2% osmium tetroxide, embedded in epoxy resin and sectioned at 1 µm for light microscopic examination. Of 50 sectioned eggs, 15 were found in which sperm were in the process of penetrating the egg envelope. Others, in which sperm penetration had been completed, exhibited persistent sperm entry holes. In no case was more than one sperm entry hole observed in a single egg. Even when eggs were surrounded by dense concentrations of sperm, no evidence of polyspermy was found. Sperm entry holes or penetrating sperm were observed in different positions over the entire egg with the exception of the apical depression at the animal pole. In non-penetrating sperm adhering to the surface of the egg, a short acrosomal filament was detected in contact with the outer layer of the egg envelope, spreading out at its point of attachment (Figure 7A).

In the sectioned material, sperm were found in various phases of penetration, ranging from initial penetration in the outer half of the egg envelope to total incorporation within the cortical cytoplasm of the egg (Figure 7B-F). One section of a sperm within the outer egg envelope revealed that the portion of the inner envelope in the path of the sperm was markedly changed in stainability, appearing darker and more homogeneous (Figure 7B). In other sections cortical cytoplasm of the egg in contact with the tip of the penetrating sperm was also marked by an increased homogeneity. Sperm tails, presumably not incorporated into the egg, were observed often in the sperm entry hole. Sections of 2 to 3 day embryos, still surrounded by the unchanged egg envelope, showed persistent sperm entry holes. At this stage, the outer layer of the envelope had covered the hole, but the gap remained in the middle and inner layers.

In later stages of fertilization studied in sectioned eggs, the sperm head, of similar size and staining properties as yolk granules, was not identified after penetration into the cytoplasm. After its enlargement to form the male pronucleus it was distinguished in the center of sectioned eggs as a well formed nucleus having a prominent chromatin network. The female pronucleus, formed after the extrusion of the second polar body at the animal pole, was observed to consist of numerous individual vesicles. From the available material it was not possible to determine the number of vesicles or their relationship to one another. Prior to the union of male and female pronuclei, the latter appeared as a single large, rounded nucleus, apparently formed by a coalescence of the vesicles.

Apionsoma misakiana. Observations were made on both living and fixed material from the stage of germinal vesicle breakdown in coelomic oocytes through the union of male and female pronuclei to form the zygote. The opportunity to examine normal breakdown of the germinal vesicle was afforded during a study on reproductive biology in which coelomic oocytes of 160 females were measured over a period of 16 months. In all except one of the 160 females germinal vesicles of coelomic oocytes were intact. In a single specimen the oocytes were in various stages of

germinal vesicle dissolution. Dissection of this individual revealed the presence within the nephridia of oocytes, all of which were in the first meiotic metaphase. This observation was made in November during the known breeding season of the species. Dissection of another specimen, the coelomic oocytes of which all possessed unaltered germinal vesicles, showed no eggs in the nephridia. This finding is consistent with previous reports that germinal vesicle breakdown in sipunculans occurs in the coelom prior to nephridial uptake (Gerould 1906, Gonse 1956, Åkesson 1958, Gibbs 1975, Rice 1975a). Attempts to induce germinal vesicle breakdown by placing coelomic oocytes in sea water (16 h, 25 °C) or in 10^{-1} to 10^{-3}M dithiothreitol in sea water (4 h, 25 °C) were unsuccessful.

As part of other studies on development of this species, 66 spawnings of unfertilized or recently fertilized eggs in polar body stages were observed and recorded in the laboratory. At the time of spawning the eggs were arrested in the first meiotic metaphase. The location of the metaphase spindle, in a central position in the egg, differed from the usual apical position of other sipunculans. After 4 1/2 hours in sea water, unfertilized eggs showed a migration of the spindle to the apical position. Within 20 minutes after eggs were exposed to sperm, and presumably after sperm penetration, the spindle migrated apically where a meiotic division resulted in the first polar body.

The completion of maturation, initiated by sperm penetration, was followed in both living eggs and those sectioned at 1 µm (Figures 8A-E; 9A-F) concurrent with or slightly prior to the formation of the first polar body, the cytoplasm rounded up, withdrawing from the egg envelope at the vegetal pole. By 23 minutes after the addition of sperm, the first polar body was released and shortly thereafter the spindle of the second meiotic metaphase was formed. The male pronucleus was first distinguishable at 35 minutes in the center of the egg. The second polar body was formed at 52 minutes and by 55 minutes the lobulated female pronucleus was obvious at the animal pole. The two pronuclei then migrated toward one another, first making contact at 75 minutes and undergoing complete fusion to form the zygote nucleus at 90 minutes. As in *Phascolosoma agassizii*, sperm entry holes were observed in sectioned as well as in living eggs of *Apionsoma misakiana*.

Cinematographic studies have shown that the relationship of the cytoplasm to the egg envelope is a dynamic one. The first indication that fertilization had been initiated was the withdrawal of the egg cytoplasm from the posterior egg envelope. The cytoplasm was found to round up as polar bodies were formed, then to stretch out, although not filling the posterior space until later stages of cleavage.

Siphonosoma cumanense. Eggs were collected and microscopic observations begun immediately following the observed spawnings of female and male animals (Rice 1988). Differing from other sipunculans, the fertilized egg showed little, if any, withdrawal of cytoplasm away from the egg envelope. Polar bodies were relatively small and inconspicuous. The process of maturation proceeded more rapidly than for other species described above. Within 40 minutes following male and female spawnings, male and female pronuclei in the egg had fused and at 50 to 55 minutes the first cleavage began (25 °C). The second polar body had appeared at 30 minutes and at 35 minutes the male and female pronuclei were distinguished as well-formed nuclei (Figure 10B-E). At 37 minutes they made their first contact and within 3 more minutes they had fused to form the zygote nucleus (Figure 10F).

Discussion

Egg characteristics

Compiled from the literature and from the data presented here, characteristic features of sipunculan eggs, along with developmental features and approximate sizes of adults, are summarized in Table I. Relations were tested statistically between egg size and adult size, egg size and developmental mode, and body size and developmental mode. A test for correlation of adult body size and egg size shows no significance ($r = 0.237$, $P > 0.05$). Similarly, the data show no significance in the relationship of adult body size and developmental mode: i.e., planktotrophic development with feeding larvae, lecithotrophic development with non-feeding larvae, and direct development without larval forms (ANOVA, $F = 0.00$, $P = 0.99$). However, the relationship between egg size and developmental mode is significant ($F = 7.20$, $P = 0.004$). An analysis using pairwise comparisons indicated that there were differences in mean egg size only between species with planktotrophic and direct development (GT-2 method of *a posteriori* pairwise comparisons, $\alpha = 0.05$).

Certain relations between egg shape and taxonomic classification are apparent

in Table 1. Ovoid eggs and thick egg envelopes comprised of 3 layers are restricted to two families, Aspidosiphonidae and Phascolosomatidae, and the genera *Antillesoma*, *Apionsoma*, *Aspidosiphon*, and *Phascolosoma*. Gibbs & Cutler (1987) consider the species formerly known as *Golfingia misakiana* to be *Apionsoma misakiana* and they place it in the family Phascolosomatidae. The shape of the eggs of this species is consistent with this taxonomic arrangement.

Nephridial uptake of coelomic oocytes

Prior to spawning, the more advanced coelomic oocytes, through a little understood process of nephridial selectivity, are taken into the nephridium from the coelom by way of the ciliated nephrostomal funnels. After a short period of storage, gametes are expelled to the exterior sea water by way of the nephridiopores. It has been suggested that germinal vesicle breakdown may be a prerequisite for nephridial uptake and that the regulatory mechanism for nephridial selectivity might be associated with this process (cf. Rice 1975a; in press). Observations reported here for *Phascolosoma agassizii* and *Apionsoma misakiana* in which oocytes in the stage of the first meiotic metaphase were found in both the nephridia and coelom of the same individuals lend support to this presumption. However, contrary observations for *Sipunculus nudus* and *Nephasoma pellucida* in which eggs were found to be spawned with intact germinal vesicles present conflicting evidence. Thus, the previous generalization regarding the presumed association of germinal vesicle dissolution and nephridial uptake is no longer valid and other mechanisms must be considered for the regulation of selection of oocytes from the coelom for storage in the nephridia and subsequent spawning. Dissolution of the germinal vesicle of oocytes in the coelom in advance of nephridial uptake has been reported by Gerould (1906) for *Golfingia vulgaris* and *Phascolopsis gouldi* and by Åkesson (1958) for *Phascolion strombi* and Gibbs (1975) for *Nephasoma minuta*.

Dissolution of the germinal vesicle

Breakdown of the germinal vesicle of coelomic oocytes in sea water has been reported in *Golfingia elongata* (Selenka 1875), *Golfingia vulgaris* (Gerould 1906), and *Thysanocardia pugettensis* (Rice 1967). Attempts to induce germinal vesicle breakdown in sea water have been unsuccessful in *Phascolopsis gouldi* (Andrews 1889), *Phascolion strombi* (Åkesson 1958), *Nephasoma minuta* (Gibbs 1975), *Golfingia elongata* and *G. rimicola* (Gibbs 1976), *Phascolosoma agassizii* (Rice 1967) and *Apionsoma misakiana* reported here. The eggs of both *Sipunculus nudus* and *Nephasoma pellucida* are spawned with intact germinal vesicles, and, at least in the case of *N. pellucida* these eggs are fertilizable. It is not known whether oocytes taken directly from the coelom without passage through the nephridium are fertilizable in these two species.

Associated with the dissolution of the germinal vesicle in *Thysanocardia pugettensis* is the breakdown of the cortical granules. Gonse (1956) also reported the disappearance of cortical granules with the initiation of maturation in *Golfingia vulgaris*. Thus the cortical granules in sipunculan eggs must serve a different, although still not understood, function than the contribution to the formation of the fertilization membrane widely studied in echinoderms and some other invertebrates (Schuel 1985).

Sperm penetration

Both Gerould (1906) and Åkesson (1958) supposed that sperm penetration of sipunculan eggs was accomplished by sperm entry through a pore of the egg envelope. However, in *Phascolosoma agassizii* the width of the sperm head exceeds that of the pore by as much as 8 times, so that this explanation alone is not tenable. The present study has demonstrated that sperm penetration in the eggs of *Phascolosoma agassizii* and *Apionsoma misakiana* is effected by the formation of a hole in the egg envelope. Although actual penetration of sperm has not been documented in other sipunculans, the persistent sperm entry holes have been observed in fertilized eggs or early embryos of many species, including *Phascolosoma perlucens*, *Phascolosoma varians*, *Thysanocardia pugettensis*, *Nephasoma pellucida*, and *Themiste pyroides* (Rice 1975b). Thus sipunculans can be added to the list of numerous invertebrates in which sperm penetration has been shown to occur by hole formation in the egg coverings (Austin 1968).

Completion of maturation

Although he did not observe sperm penetration, Gerould (1906) described the formation of male and female pronuclei and subsequent fusion in great detail in two species of sipunculans, *Golfingia vulgaris* and *Phascolopsis gouldi*. He noted the 180°

rotation of the sperm after entrance into the egg and the appearance of an associated astrosphere with centrosome at the base of the sperm. The astrosphere was observed to migrate first to the center of the egg and to be followed by the sperm. Once in the center, both sperm and astrosphere increased in size. The egg nucleus, after two maturation divisions in which two polar bodies were formed, was separated into 10 chromatic vesicles which later united to form the female pronucleus. The male and female pronuclei, each with a single aster, migrated toward each other and underwent fusion in a position between the animal pole and the center of the egg. Observations are reported in the present paper on formation of polar bodies, male and female pronuclei and the zygote nucleus for three species, *Apionsoma misakiana*, *Phascolosoma agassizii*, and *Siphonosoma cumanense*. In the two former species, both of which have ovoid eggs, there was a marked cytoplasmic withdrawal from the egg envelope at the animal and vegetal poles associated with polar body formation. In the present study it was not possible to follow the path of the sperm through the cytoplasm to the center of the egg. However, the fusion of male and female pronuclei was observed. The female pronucleus when first formed at the animal pole consisted of several vesicles, the number and relationship of which was not determined. The vesicles coalesced to form a single pronucleus which migrated toward the center of the egg to join the male pronucleus.

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