Nephasoma pellucidum: A Model Species for Sipunculan Development?

Anja Schulze and Mary E. Rice

ABSTRACT. Recent developments in metazoan phylogeny, especially with regard to the position of the Sipuncula in the annelid clade, have sparked a renewed interest in sipunculan development. If Sipuncula are annelids, they must have secondarily lost segmentation. By comparison with segmented annelids, they could provide important clues for the evolution of segmentation. A sipunculan model species is needed to examine fundamental developmental processes. Here we describe the development of *Nephasoma pellucidum* and explore its potential as a model species for sipunculan development. Like other sipunculans, *N. pellucidum* produces eggs with a thick, porous, multilayered egg envelope. Cleavage in *N. pellucidum* is spiral, holoblastic, and unequal. The species shows the most common, and likely ancestral, developmental mode in the group. Its life cycle includes a lecithotrophic trochophore and a planktotrophic pelagosphera larva. The trochophore is enclosed in the egg envelope, with cilia growing through the envelope's pores. The trochophore larva metamorphoses into the pelagosphera larva at approximately 60 h. Pelagosphera larvae reached metamorphic competence at about five weeks. Metamorphosis to the juvenile was induced by supplying sediment that had been inhabited previously by conspecific adults. Juveniles were observed for several weeks. We conclude that *N. pellucidum* is a good model species for sipunculan development, although rearing conditions in the laboratory still need to be optimized.

INTRODUCTION

During the past two decades, our understanding of metazoan relationships has changed radically, starting with the first use of ribosomal RNA sequences for phylogenetic analysis (Field et al., 1988). Many taxa for which evolutionary origins have long been mysterious or controversial can now be placed with more certainty into the metazoan tree of life (Dunn et al., 2008; Halanych, 2004). Among those, two groups that were long regarded as distinct phyla have been absorbed into the Annelida: the Echiura, or spoon worms, and the Siboglinidae, previously called Pogonophora and Vestimentifera (McHugh, 1997; Rouse and Fauchald, 1997).

The Sipuncula, commonly known as peanut worms or star worms, have had a complex taxonomic history but now appear to be following the same route as the echiurans and siboglinids. Nearly 50 years after Hyman (1959) affirmed phylum status for the group, recent authors place them into the annelid clade, based on

Anja Schulze, Texas A&M University at Galveston, 5007 Avenue U, Galveston, Texas 77551, USA. Mary E. Rice, Smithsonian Marine Station at Fort Pierce, 701 Seaway Drive, Fort Pierce, Florida 34949, USA. Corresponding author: A. Schulze (schulzea@tamug.edu). *Manuscript received 9 June 2008; accepted 20 April 2009.*

phylogenetic analyses of mitochondrial gene order (Bleidorn et al., 2005; Boore and Staton, 2002), sequence data from several genes (Struck et al., 2007), and expressed sequence tags (Dunn et al., 2008). Although there is a growing consensus on the annelid affinities of sipunculans, it remains to be determined which of the incredibly diverse annelids is the sister group to the Sipuncula. With a simple body, consisting of a trunk and a retractable introvert with an array of tentacles at the anterior end, they show little similarity to any other polychaete group. In the molecular analyses, support for a sister group relationship with any other polychaete taxon is low. The monophyly of the Sipuncula is uncontested, and solid hypotheses of within-group relationships have been published (Maxmen et al., 2003; Schulze et al., 2005, 2007; Staton, 2003).

Sipuncula are an interesting case in the field of "EvoDevo," or the interface of evolution and development. Embryonic and larval characters have often been cited as support for phylogenetic hypotheses. Rice (1985) listed several similarities between sipunculan and annelid development, such as the larval prototroch and metatroch and the retention of the egg envelope to form the larval cuticle. She also noted that in some sipunculan larvae the ventral nervous system develops in paired cords, similar to most polychaetes. On the other hand, Scheltema (1993), comparing embryos and larvae of annelids, mollusks, and sipunculans, argued that sipunculan development shows more similarity with that of mollusks. The development of all three taxa includes spirally cleaving embryos and a trochophore larva. A long-held view is that annelid and mollusk embryos can be distinguished at the 64-cell stage by the arrangement of the micromeres around the animal pole: they form either an "annelid cross" or a "molluskan cross." Reproducing Gerould's (1906) drawing of the embryo of *Golfingia vulgaris* with a molluskan cross, Scheltema concluded that sipunculans and mollusks were sister groups. However, Maslakova et al. (2004) showed that the annelid and molluskan crosses are far from universal within the respective taxa and probably hold no phylogenetic significance.

The primary reason why few past researchers have recognized sipunculans and echiurans as annelids is that adults of both taxa show no sign of segmentation, either externally or internally. It took advanced techniques in immunohistochemistry and confocal laser scanning microscopy to demonstrate segmentation in the nervous system of echiuran larvae (Hessling and Westheide, 2002). Similar techniques initially failed to show segmentation in sipunculan larvae (Wanninger et al., 2005) but a recent study showed a segmental mode of neural patterning in the early pelagosphera stage (Kristof et al., 2008).

If the Sipuncula fall into the annelids, they must have secondarily lost segmentation in the later larval stages and the adult. If no morphological segmentation is evident, what happened with the molecular pathways responsible for segment formation in other annelids? By comparison with other species, sipunculans are valuable for the identification of the genetic and cellular basis of segment formation in annelids.

The recent developments in metazoan phylogeny have thus sparked a renewed interest in sipunculan development. A model species is needed to study fundamental developmental processes. A good model species has to be readily available, be easy and inexpensive to maintain in the laboratory, lend itself to a variety of examination techniques, and be representative for its taxonomic group. Here we describe the development of *Nephasoma pellucidum* and explore its potential as a model species. *N. pellucidum* is a relatively common species that inhabits cracks and crevices in hard substrates in shallow warm waters. The species exhibits the most common developmental mode within the Sipuncula, which includes a lecithotrophic trochophore stage and a planktotrophic pelagosphera larva (Rice 1967, 1975a, 1975b, 1976, 1989). We have accumulated these data between 1972 and 1984 and, more recently, between 2003 and 2006.

MATERIALS AND METHODS

Specimens of *Nephasoma pellucidum* were collected from numerous localities offshore from Fort Pierce, Florida, extending from Capron Shoal and Pierce Shoal 4 and 6 miles, respectively, southeast of the Fort Pierce Inlet to the Sebastian Pinnacles approximately 32 miles north of the Inlet. At the Pinnacles, worms inhabited rubble of oculinid coral at depths of 70 to 100 m, whereas on the more southern shoals they occurred in depths of 9 to 15 m in rubble composed of mollusk shells, sand dollar tests, and rocks. Occasionally specimens were found in the Fort Pierce Inlet in intertidal clumps of oyster shells. The worms were carefully removed from the rubble with hammer and chisel. Multiple adults from each collection were kept in glass dishes in approximately 200 mL seawater at room temperature. Spawning occurred in the lab, generally after changing the water. Whenever eggs were observed in the culture dishes, they were pipetted into a clean dish and observed for development. Larval cultures were kept until the larvae either died or metamorphosed. Water was changed at least every two days. The larvae were periodically fed with unicellular algae or diatoms (*Isochrysis*,

Dunaliella, or *Nanochloropsis*). To induce metamorphosis, larvae were pipetted into dishes with muddy sediment previously inhabited by conspecific adults.

For scanning electron microscopy (SEM) and transmission electron microscopy (TEM), specimens were fixed in 2.5% glutaraldehyde in Millonig's phosphate buffer (Millonig, 1964) for at least 1 h and up to several days at 4°C. Fixation was followed by three washes in a 1:1 mixture of Millonig's phosphate buffer and 0.6 M sodium chloride and postfixation in 1% osmium tetroxide (1:1:2 mix of 4% OsO_4 : Millonig's buffer : 0.75 M NaCl). Samples were then dehydrated in an ethanol series up to 100%. For SEM, they were critical point dried and mounted on SEM stubs using double-sticky tape and viewed in either a Nova Scan or a JEOL 6400 Visions scanning electron microscope. Images were either scanned from negatives or stored digitally. For TEM, the dehydrated specimens were transferred to propylene oxide and subsequently embedded in Epon resin and sectioned. Thin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL 100CX transmission electron microscope.

RESULTS

Gametes

The spermatozoan of *Nephasoma pellucidum* is of the primitive type according to Franzén's classification (Franzén, 1958). The nuclear region is rounded and capped by a doughnut-shaped acrosome with a central nipple-like protuberance. The head, including nucleus and acrosome, measures 1.5 × 1.7 μm. Posterior to the nucleus, four mitochondrial spheres are arranged in a circle, from the center of which extends the flagellum (Figure 1A).

The egg at the time of spawning is spherical, measuring 105 μm in diameter (Figures 1B, 2A). In direct light the surface appears opalescent, and the color is pale gray. The egg envelope, up to 6 μm in thickness, is multilayered and perforated by numerous pores (Figure 3).

Spawning

As in most sipunculans, sexes are separate; eggs and sperm are spawned freely via the nephridipores into the surrounding water where fertilization occurs. From data accumulated on spawning in the laboratory, two spawning peaks are evident: one in the spring (April–May) and the other in the fall (September–November). Observations of spawning were carried out on animals in the laboratory, usually for a period of one month after collection from the field: 139 spawnings were recorded over a period of 8 years (1972–1980), and spawning occurred every month of the year except January. Although a few animals were observed to spawn after maintenance in the laboratory for as long as 18 months, 88% of the spawnings were recorded within 30 days of collection.

Cleavage

The eggs at spawning may be arrested in the first meiotic metaphase, or they may possess an intact germinal vesicle. In the latter case, the germinal vesicle breaks down soon (within at least 30 min) after spawning, regardless of whether the egg is fertilized. Within 40 min after fertilization (23°C), the first polar body is formed (Figure 2B), and at 55 min the second polar body makes its appearance. The first cleavage, occurring at 90 min, is unequal, the CD cell exceeding the AB cell in size (Figure 2C). The next three cleavages occur at approximately half-hour intervals, and the 16 cell stage is attained within 3 h after fertilization. The third cleavage, from 4 to 8 cells, is spiral and unequal. The A, B, and C cells, all approximately the same size, divide simultaneously, preceding the initiation of division of the larger D cell by about 1 min and completing their divisions 5 min before that of the D cell. In the 8 cell stage, the micromeres and macromeres of the A, B, and C quadrants are approximately the same size, the C sometimes being slightly larger, but all are smaller than the d cell which, in turn, is smaller than the D cell.

After the first few cleavages, the divisions are more frequent, and by 7 h after fertilization the egg has developed into an early blastula; cilia from the prototrochal cells protrude through the pores of the egg envelope and the embryo begins to rotate slowly on the bottom of the container. By 16 h the embryos are swimming throughout the dish, no longer confined to the bottom. At this time the stomodaeal invagination is evident, and the embryos show the first signs of positive phototropism (Figure 2D).

Trochophore: Morphology and Metamorphosis to the Pelagosphera

By 48 h the embryo has reached the stage of trochophore. The shape has changed from spherical to oval, as a result of a slight posttrochal elongation (see Figure 1C). A pair of dorsolateral red eyespots is present in the pretrochal hemisphere. Prototrochal cavities are evident to the inner side of the prototroch cells, and the gut is differentiated into three regions: esophagus, stomach, and intestine.

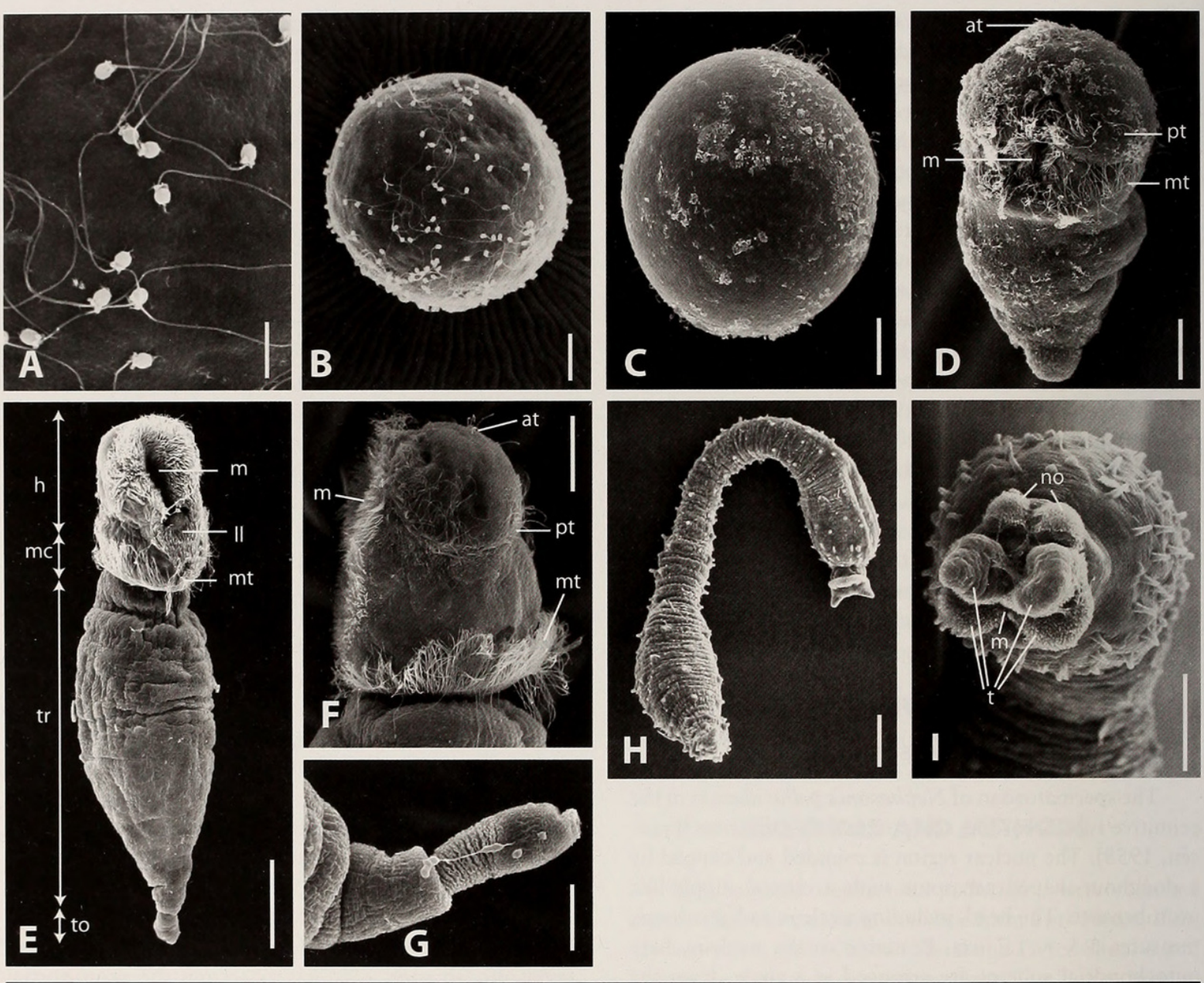
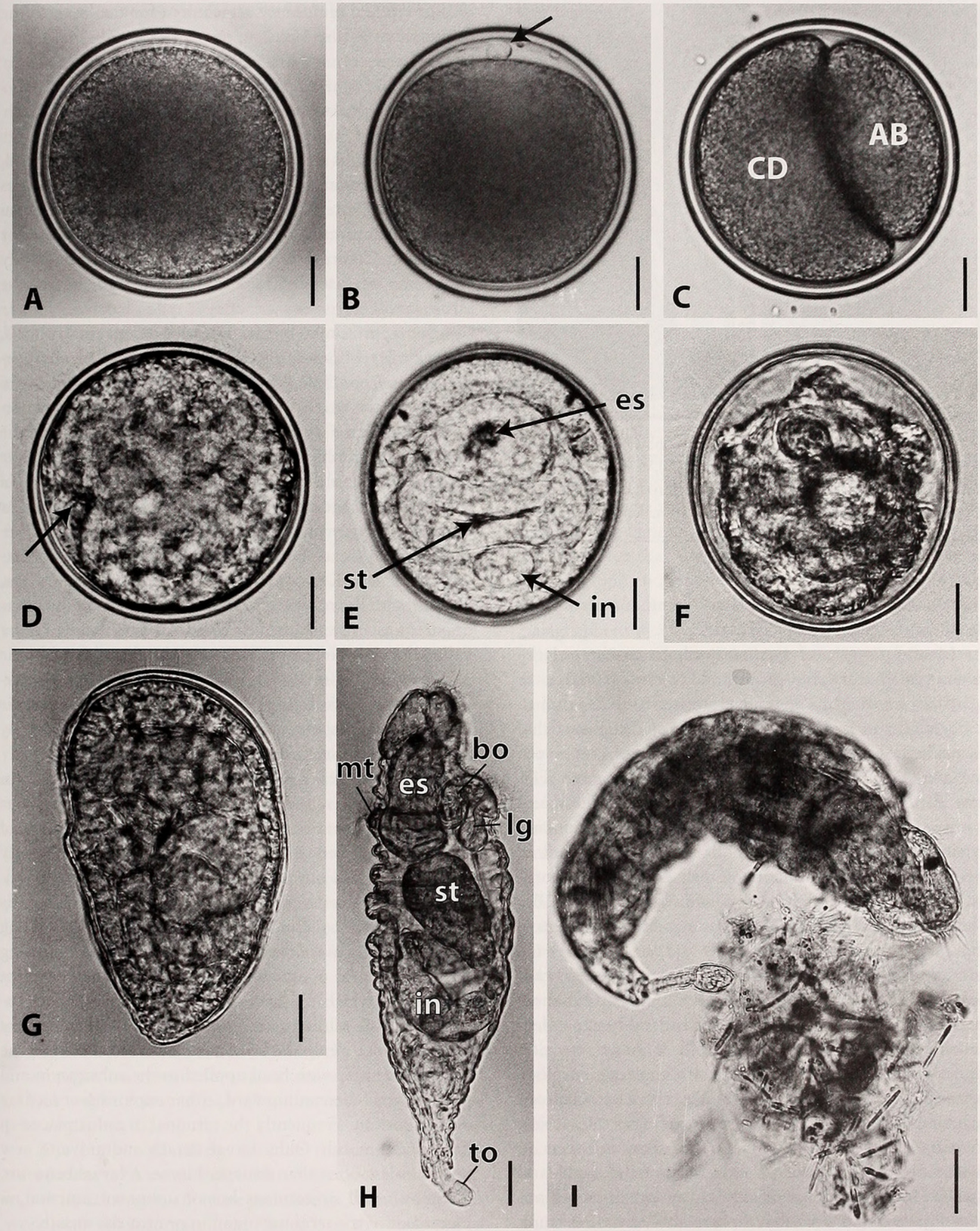


FIGURE 1. Scanning electron micrographs showing development of *Nephasoma pellucidum* (scale bar lengths here in parentheses). A. Sperm on the surface of the egg (5 μm). B. Egg with sperm on surface (20 μm). C. Trochophore larva; note cilia extending through egg envelope (20 μm). D. Early pelagosphera larva (20 μm). E. Fully formed pelagosphera larva, ventral view (50 μm). F. Head of a pelagosphera larva, lateral view (20 μm). G. Terminal organ of the pelagosphera larva (10 μm). H. Metamorphosed juvenile (100 μm). I. Tip of juvenile introvert with tentacle buds and lobes of nuchal organ (50 μm). Abbreviations: at = apical tuft; h = head; ll = lower lip; m = mouth; mc = metatrochal collar; mt = metatroch; no = nuchal organ; pt = prototroch; t = tentacles; to = terminal organ; tr = trunk. (Images A, B from Rice, 1989: fig. 4E,F; used with permission)

FIGURE 2. (*facing page*) Light micrographs showing development of *Nephasoma pellucidum* (scale bar lengths here in parentheses). A. Unfertilized egg (20 μm). B. Egg with polar body (arrow) (20 μm). C. Two-cell stage; note size difference between CD and AB blastomeres (20 μm). D. Blastula stage; beginning invagination of stomodaeum (arrow) (20 μm). E. Early trochophore; note eyespots at anterior end (top) (20 μm). F. Trochophore shortly before metamorphosis to pelagosphera (20 μm). G. Early pelagosphera in the process of elongation (20 μm). H. Fully metamorphosed pelagosphera larva (20 μm). I. Feeding pelagosphera, 10 d old (50 μm). Abbreviations: bo = buccal organ; es = esophagus; in = intestine; lg = lip gland; mt = metatroch; st = stomach; to = terminal organ.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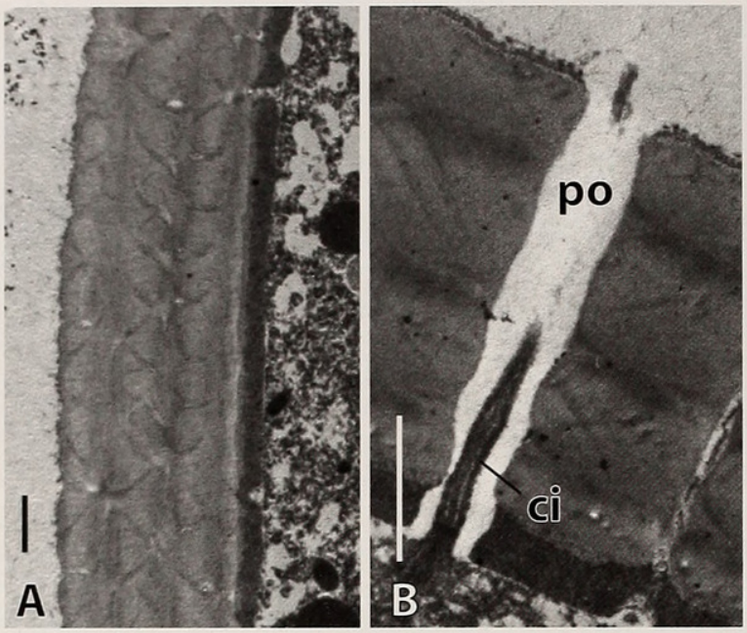

FIGURE 3. Transmission electron micrographs showing egg envelopes of *Nephasoma pellucidum* (scale bars = 1 μm). A. Section through multilayered egg envelope. B. Cilium growing through pore in egg envelope of trochophore larva. Abbreviations: ci = cilium; po = pore.

The trochophore is lecithotrophic, being completely enclosed by the egg envelope (Figure 2E,F).

Metamorphosis of the trochophore to the pelagosphera larva occurs at 60 to 65 h (23°C) and extends over a period of 6 to 8 h. The body elongates from 140 μm to 250 μm, by an increase in the length of the posttrochal hemisphere. The lumen of the gut is completed, and mouth and anus break through the overlying egg envelope (Figures 1D, 2G). The ventral ciliated surfaces of the head and the lower lip are formed apparently by an evagination and expansion of the anterior stomodaeum. Larval organs of the lower lip become functional: the buccal organ is protrusible, and the pore of the lip glands opens (Figure 2H). The metatrochal cilia project from a prominent metatrochal collar posterior to the mouth and lower lip. As the retractor muscles become functional, the entire pretrochal body is retractable into the posterior or posttrochal region of the larva. The coelom is considerably expanded, and the posttrochal body is capable of great extension and contraction. Whereas the posttrochal egg envelope is transformed into the larval cuticle, the pretrochal egg envelope is gradually sloughed off, leaving a thin cuticle covering the head. The terminal organ appears first as an evagination of the posterior extremity of the trunk and within a few hours differentiates into a more discrete elongate structure (40 μm) that is retractable into the trunk and provides a temporary attachment for the larva to the substratum (Figure 1G).

Pelagosphera: Morphology, Behavior, and Metamorphosis to the Juvenile

Four regions of the body can be distinguished: head, metatrochal collar, trunk, and terminal organ (see Figures 1E,F, 2H). The terminal organ is well developed with an unusually long neck, terminated by a bulbous posterior expansion (Figure 1G). The terminal organ of a 10-day larva may be extended to a length one-third that of the entire larva.

For approximately two weeks after metamorphosis, the majority of larvae are attached by their terminal organs; some continue to swim, or else attach and swim intermittently. At two weeks there is a high rate of mortality and, in the absence of substratum, most larvae die within two months; the maximum survival time of larvae reared in culture dishes is 103 days. Surviving larvae of one month of age attain a maximum size of 1.2 mm. At this age the body proportions have changed, the head being relatively smaller than in the younger stages. The external body wall is smooth, glistening in reflected light, and through the relatively transparent body wall the gut is apparent as an elongate dark yellow stomach and a lighter yellow recurved intestine, ending at the dorsal anus in the anterior trunk. Usually larvae are still attached by the terminal organ at these later stages, although some may lie on the bottom, relatively quiescent. Swimming occurs only rarely, although metatrochal cilia are still present.

Attached larvae are observed to feed on the substratum surrounding their points of attachment (Figure 2I). The body may be bent downward so that the ventral surface of the head is applied to the bottom of the dish, or the body may be stretched out from the point of attachment parallel to the substratum. In culture dishes in which there is an algal growth covering the bottom, the area surrounding the attached larva is often bare, indicative of larval grazing activity. The area of attachment is often marked by clumps of feces on which the larva may graze and ingest. Occasionally larvae release themselves from the attachment and swim or move along the bottom to a new site. Free larvae sometimes move with head applied to the substratum and posterior end directed upward, either exploring or feeding on the bottom. Frequently the terminal organ is placed in or near the mouth. Older larvae detach and move to new locations less often than younger larvae. A larval behavior, common to all sipunculans but of unknown function, is placement of the terminal organ in or near the mouth.

Metamorphosis of larvae reared in culture dishes could be induced at the age of 5 to 6 weeks by exposure to a fine, muddy sediment that had been occupied previously by adults. Attempts to induce metamorphosis before this age were not successful. Before metamorphosis, larvae buried themselves in the sediment and in 3 d underwent metamorphic changes to the juvenile stage.

The process of metamorphosis is initiated by the loss of the metatrochal cilia, reduction in the size of the lower lip, narrowing of the head, and elongation of the pretrochal body. At the end of 3 d, both posttrochal and pretrochal regions of the body are narrowed and elongated, the metatrochal collar is reduced, the terminal organ and lip are partly regressed, the mouth moves to a terminal position, and dorsal to the mouth a pair of developing tentacular lobes is apparent (Figure 1H,I). These morphological modifications, along with the behavioral changes of initiation of burrowing and cessation of swimming, mark the beginning of the juvenile stage. Regions of the body of the juvenile are reduced from the four found in the larva to two: (1) the broader and longer posterior trunk, formed from the posttrochal larva, and (2) the more narrow anterior introvert, which is terminated by mouth and developing tentacles and formed from the pretrochal larva. Similar to the pretrochal larval body, the introvert of the juvenile is retractable into the trunk.

The most immediate modifications are found in the head and metatrochal regions. As the mouth becomes terminal, the dorsal surface of the head is foreshortened. The ventral lip is lost, but ciliation of the ventral surface of the head persists to surround the mouth and the ventral surface of the developing dorsal lobes. On the dorsal head two heavily ciliated patches that will give rise to the paired nuchal organ have moved further anteriorly as the head foreshortens. In the 7- to 9-day-old juveniles the buccal organ is no longer apparent. One to four rings of simple hooks appear in the region of the former metatrochal band. Papillae, already apparent in older larvae, are more prominent and numerous. Scattered among the hooks, the papillae are dome shaped and, as seen in scanning electron micrographs, have central pores from which several cilia protrude. Papillae of similar structure, but somewhat larger, cover the entire trunk (Figure 1H). A vestigial terminal organ may persist for one or two weeks. Within two to four weeks a second pair of rudimentary tentacles appear ventral to the mouth.

The body wall of the juvenile thickens, losing its transparency. Externally circular constrictions, also seen in late larval stages, are more prominent. Juveniles of one week also show longitudinal "folds" of the body wall, resulting in a checkered appearance of the integument in some regions.

DISCUSSION

Nephasoma pellucidum is one of the few sipunculan species in which the life cycle has been observed from spawning to juvenile stage. Other species are *Siphonosoma cumanense* (Rice, 1988), *Thysanocardia nigra* (Rice, 1967), *Themiste pyroides* (Rice, 1967), *Themiste lageniformis* (Pilger, 1987), *Themiste alutacea* (Rice, 1975c), *Phascolion strombus* (Åkesson, 1958; Wanninger et al., 2005), and *Phascolion cryptum* (Rice, 1975c). Most of these species show abbreviated development, either omitting both the trochophore and pelagosphera stage, or omitting the pelagosphera stage, or having a lecithotrophic pelagosphera (Rice, 1976). The oceanic, planktotrophic pelagosphera larvae of many aspidosiphonid and phascolosomatid larvae have been recovered in plankton tows; however, their complete life cycles are unknown (Rice, 1981; Hall and Scheltema, 1975).

We argue that a model species should show the ancestral developmental mode for the taxon. Cutler (1994) concluded that indirect development with a planktotrophic pelagosphera was ancestral in Sipuncula. The most recent phylogenetic analyses (Schulze et al., 2007; Schulze and Rice, 2009) seem to confirm this view. The genera *Sipunculus* and *Siphonosoma*, which to our present knowledge only contain species with planktotrophic pelagosphera larvae, represent the two basal clades in both analyses. The remaining three major clades have species of *Phascolosoma* and *Apionsoma* as their basal branches, two additional genera in which abbreviated development is unknown.

Of the species listed above, only the life cycle of *Siphonosoma cumanense* includes a planktotrophic pelagosphera as *N. pellucidum* does. *Siphonosoma cumanense* is a large, sand-burrowing species. Like other sipunculans, it survives well under laboratory conditions, when supplied with sediment and adequate aeration. However, its potential for use as a model species is limited by two factors. First, even though the species has a wide geographic distribution, it is rarely found in significant numbers, and the establishment of a viable population would require major efforts. Second, larvae do not seem to be competent to metamorphose until about 8 weeks old (Rice, 1988).

Nephasoma pellucidum is geographically widespread, mostly in shallow warm waters, although it does not seem to be as abundant in most places as at our collecting station

near Fort Pierce, Florida. Collection of a significant number of individuals can be time consuming because they have to be carefully removed from the cracks and crevices of rubble; the removal process can damage the animals, often causing their death. After successful retrieval, however, adults are easy to maintain in laboratory conditions. Removed from their shelter, they survive in simple glass bowls without aeration or food supplement for at least a year, feeding only on the biofilm at the bottom of the dish. We assume that, left in their shelter or in sediment, with proper aeration and occasional food supply, they would survive for years. This assumption is based on the longevity of other sipunculans: individuals of *Apionsoma misakianum* have been kept in holding tanks at the Smithsonian Marine Station for nearly 30 years.

Nephasoma pellucidum spawns frequently during the warmer months of the year. Spawning can be induced by changing the seawater in the dish, although this procedure does not reliably yield the desired results, leaving some uncertainty as to when the spawning occurs. Embryos and larvae are easy to observe with different microscopic techniques. Mortality before the first metamorphosis, from trochophore to pelagosphera, is minor. The pelagosphera larvae are more transparent than other sipunculan larvae, facilitating observation by light and confocal laser scanning microscopy. In contrast to the pelagosphera larvae of some other sipunculan species, they relax relatively well when temporarily cooled to 4°C and treated with menthol, magnesium chloride, or 10% ethanol, leaving their head and terminal organ exposed.

The increase in mortality during the prolonged pelagosphera phase presents some difficulties. By the time metamorphic competence is reached, the percentage of surviving larvae is low. A further reduction in numbers occurs at metamorphosis, because not all larvae respond to the settlement cue, that is, adult-conditioned sediment. Therefore, postmetamorphic juveniles are only rarely observed. Common metamorphosis-inducing agents such as potassium chloride, cesium chloride, gamma-aminobutyric acid, 3,4-dihydroxy-L-phenylalanine (L-dopa), and isobutylmethylxanthine (Bryan et al., 1997; Morse et al., 1979; Yool et al., 1986) seem to have no effect on metamorphosis in *N. pellucidum*.

As a conclusion, among the sipunculans for which development has been studied, *N. pellucidum* is a good candidate for a model species. Rearing larvae through metamorphosis still presents some difficulties, and future work should focus on optimizing the conditions. Recently the cold-water species *Phascolosoma agassizii* from the Sea of Japan, which also has a planktotrophic pelagosphera, has been reared through metamorphosis (A. S. Maiorova and A. V. Adrianov, Institute of Marine Biology, Far East Branch of the Russian Academy of Sciences, Russia, personal communication) and might be another appropriate candidate for a model species, even though metamorphosis could never be observed in individuals of the same species collected in the Pacific Northwest and reared at the University of Washington Friday Harbor Laboratories.

Acknowledgments

We thank the staff at the Smithsonian Marine Station at Fort Pierce for their research support (SMSFP Contribution No. 750). We are particularly grateful to Julie Piraino, Hugh Reichardt, and Woody Lee for their support with specimen collection, microscopy, and imaging. Valuable assistance was provided in the earlier phases of this study by Douglas Putnam and Cindy Hunter. This work was partially supported by a Smithsonian Marine Station postdoctoral fellowship granted to AS. We thank the organizers of the Smithsonian Marine Science Symposium for the opportunity to present our research.

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