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Culture and development of the polychaete *Perinereis* cf. *nuntia*

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Summary

The polychaete *Perinereis* cf. *nuntia*, a tropical species endemic in Thailand, was cultured in captivity as follows: eggs and sperm from sexually mature (epitokous) *P.* cf. *nuntia*, were artificially fertilized, and settled into a sand bed about 30 cm deep at the nectochaete stage. The sand beds were supplied with seawater at 30-ppt salinity and the nectochaetes reared for five months after which time some adults were becoming epitokes. The culture method yielded 3–4 kg polychaetes at an atokous stage per m^2 of culture area. Because the polychaetes were to be used to feed shrimp broodstock, samples of the worms were screened for the presence of white-spot syndrome and yellow-head viruses using polymerase chain reaction (PCR) methods prior to introduction to the system as founders and at monthly intervals during their culture. Morphological details of *P.* cf. *nuntia* from fertilization to nectochaete stage are described using light microscopy, transmission (TEM) and scanning electron microscopy (SEM). The egg is surrounded by a chorion layer, TEM reveals that the microvillous tip vesicles are putative sites for the binding of the sperm to the egg surface. TEM studies of the cortical reaction show that the cortical alveoli contain a fibrous substance which, after fertilization and membrane fusion, passes into the perivitelline space to form part of the fertilization membrane, as well as being secreted from the egg surface to form a jelly layer surrounding the egg.

Key words: *P.* cf. *nuntia*, polychaete, aquaculture, fertilization, development

Introduction

Polychaetes, segmented marine worms in the phylum Annelida, have recently gained commercial importance as baits for sea fishing (sport angling) and more recently because they are now used as an aquaculture feed either live, in blast frozen form, or as a constituent of formulated feeds; they are especially important as a maturation diet for shrimp broodstock (Olive, 1999). In

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commercial shrimp hatcheries, broodstock fed with live polychaetes are reported to have higher fecundity resulting in higher production of eggs and larvae; the results may be due to the high content of polyunsaturated fatty acids, especially arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid, in the polychaetes (Luis and Passos, 1995; Graeve et al., 1997; Bühring and Christiansen, 2001). The polychaete, *Perinereis* cf. *nuntia,* is probably the predominant species found in Thailand and it has become widely used in commercial shrimp hatcheries, especially for the black tiger shrimp, *Penaeus monodon,* and the white Pacific shrimp, *Litopenaeus vannamei,* broodstock (Withyachumnarnkul et al., 2002; Laoaroon et al., 2005). Most of the polychaetes used in hatcheries are wild-caught, and extensive collection from the wild may seriously deplete the natural sources of this worm, which feeds on detritus on the shore and mangrove areas and which therefore has an important role in maintaining a clean natural environment. In addition, the polychaetes are almost at the bottom of food chain for aquatic animals and they have served this ecologically role for millions of years. Catching wild polychaetes for aquaculture purposes should therefore be avoided. Culturing *P.* cf. *nuntia* for commercial use is obviously a preferable alternative to their harvest from natural sources. Study of the development of the worm is a necessary first step towards development of a commercially acceptable culturing method.

Materials and Methods

Male and female sexually mature adults (epitokes) of *P.* cf. *nuntia* were selected and artificially mated in a 15 cm × 30 cm × 10 cm polystyrene box, in clean seawater (Fig. 1a). The male to female sex ratio was 1:2. The epitokes were cut into pieces to release free sperm and eggs from the coelomic cavity of males and females respectively, which were gently mixed in seawater for 3–5 min. The fertilized oocytes were transferred to a 500 L plastic tank containing clean seawater, with adequate aeration. The larvae reached the stage of nectochaete (three chaetigerous segments) within 48 h, after which they were transferred to 4 m × 4 m × 0.5 m concrete culture tanks at a density of 6,000 individuals m^{-2}. The tanks contained coarse sand (1–3 mm grain size) to a depth of 30 cm from the tank bottom and were filled with filtered (50 m) and ozonized (1 ppm) seawater at a salinity of 30, to a level of approximately 1 cm above the top layer of the sand.

The nectochaetes that were stocked in the tank settled into the sand layer and came up to the surface only during feeding when the water was filled up to 1 cm above the sand level (simulated high-tide). The polychaetes were fed on fishmeal at 4–5 g/tank daily during the first month after stocking the nectochaetes. During the second and third month, the feeding rate was increased to 20 g/tank twice daily; and during the fourth and fifth month, 40 g/tank three times a day. The polychaetes were reared for five months, at which time they had grown to be atokous adults (Fig. 1b); and some had become epitokes (Fig. 1d). Male and female epitokes were selected to provide gametes for artificial fertilizations and the next cycle began.

As the polychaetes were to be used to feed shrimp, there was a requirement that they should be virus-free, especially of viruses that are shrimp pathogens (Flegel, 2001). Although unlikely to possess shrimp viruses, such as white-spot syndrome (WSSV) and yellow-head (YHV) viruses, the worms could still mechanically carry the virus in their gut if they were captured from the wild where they may have fed on virus-bearing crustaceans. Wild caught epitokes, used to establish the cultures, were therefore screened for the presence of WSSV and YHV by polymerase chain reactions (PCR) before being used for breeding. During the culture, samples were obtained monthly and, again, checked for the presence of WSSV and YHV. The detection of WSSV was by PCR and that of YHV was by reverse transcriptase PCR using commercial kits (Intelligenes, Taipei, Taiwan) according to the described manufacturer's protocol.

Development of the fertilized eggs was observed from the point of sperm addition to the nectochaete stage using light (LM), transmission (TEM) and scanning electron microscopy (SEM). The mixture of sperm and eggs was gently mixed for a few minutes. For LM observation, fresh whole mount preparations were used. For TEM, the eggs were collected at 15-s intervals during the first 5 min after fertilization. The samples were fixed for 3 h in 4% glutaraldehyde, dehydrated in a graded ethanol series, embedded in a low-viscosity epoxy resin, and sectioned with diamond knives in an ultramicrotome. Thin sections (60–90 nm) were stained with saturated methanolic uranyl acetate, counterstained with lead citrate, and examined. For SEM, the eggs were fixed as in the TEM process, dehydrated in a series of graded ethanol, and critical-point dried. Samples were coated with gold and examined under SEM.

Results

In the culture tank, the polychaetes grew to the length of 7–15 cm and a body weight of 0.8–1.0 g (Fig. 1b); the survival rate from nectochaete to atokous stage within five months was around 50%, the biomass produced reached 3–4 kg/m^2 of culture area within

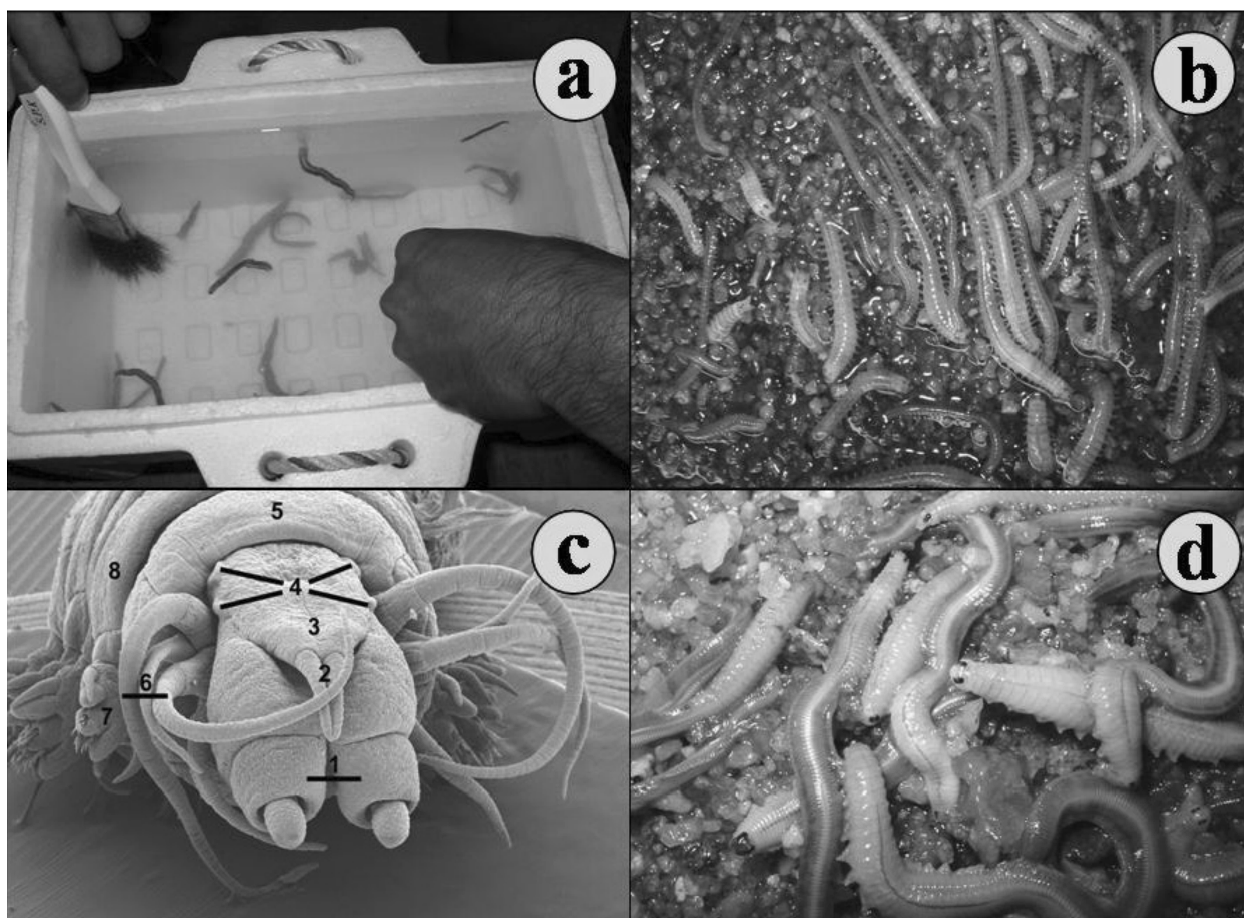

Fig. 1. Artificial fertilization of the polychaete *Perinereis* cf. *nuntia* and their gross structures. (a) Artificial fertilization of the sperm and eggs in a polystyrene box. (b) The adult (atokous) polychaetes at five months, ready for harvest. (c) SEM showing the proximal region of the atokous polychaete: 1, palp; 2, antenna; 3, prostomium; 4, eyes; 5, peristomium; 6, tentacular cirri; 7, parapodium; 8, first chaetiger. (d) Sexually mature (epitokous) polychaetes are differentiated by the color of the proximal body region, males being creamy colored, while females are green.

5 months at which point the worms were harvested. Fig. 1c shows the head portion of an atokous specimen with palps, antennae, prostomium, eyes, peristomium, tentacular cirri, parapodium and the first chaetiger as is typical for the family (Pettibone, 1963). Two pairs of eyes located on either side of the dorsal surface of the prostomium could be distinguished clearly by SEM (Fig. 1c), but barely by examination of gross specimens (Fig. 1d). The body length of epitokes was rather uniform at 5–8 cm, which is slightly shorter than the atokous form. The most striking development when becoming epitokous was a change in body colour, from pink to creamy in the male and to light greenish colour in the female (Fig. 1d). The colour changes were more pronounced in the anterior portion of the body. With the technique of artificial fertilization described, (Fig. 1a) the fertilization rate was c. 90%.

Under TEM, the unfertilized egg size was about 150 μm in diameter and contained a nucleus (picture not shown) and cytoplasm. On the periphery of the cytoplasm, several interconnected cortical alveoli containing fibrous material were observed (Fig. 2a). Interspersed among the cortical alveoli are electron-dense fine granules. The ooplasm was surrounded by a thin oolemma, which lies beneath an extra-ovarian chorion or vitelline membrane which has a variable width of 0.7–1.0 μm and which is composed of three layers (Fig. 2b). The outermost layer is a row of small spheres (about 10 nm in diameter), the second layer is a thin electron-lucent layer about 10 nm wide and the third innermost layer, about 200 nm wide, was composed of fine granules similar to those inside the ooplasm, interspersed by rows of fibrous material similar to that inside the cortical alveoli. Beneath this complex vitelline membrane there is a perivitelline space containing a fibrous material. Microvilli, with the diameter of 0.1 μm wide and 0.5 μm long, traverse the whole depth of the perivitelline space and extend through the extra-

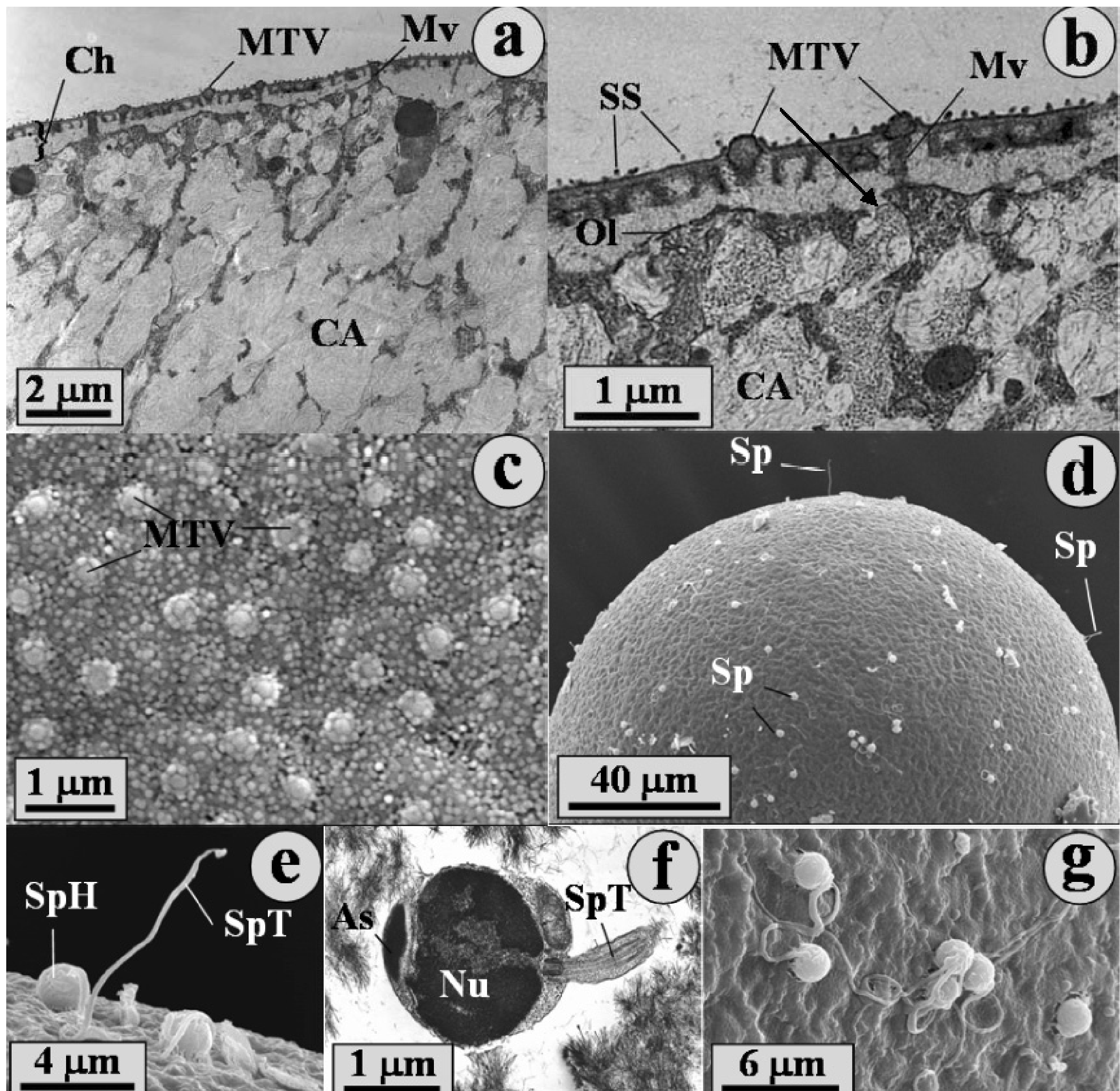

Fig. 2. Ultrastructure of the unfertilized egg and sperm. (a) TEM of the unfertilized egg showing chorion, microvilli, microvillus tip vesicles and cortical alveoli. (b) Enlarged picture of the chorion showing the microvillus tip vesicles and small spheres. (c) SEM of the surface of the unfertilized egg showing microvillous tip vesicles and small spheres. (d) SEM of egg showing two sperm with tails (flagella) perpendicular to the egg surface and those that lie flatly on the surface. (e) Enlarged SEM picture of the sperm with perpendicular flagellum. (f) TEM of the sperm showing various components. (g) SEM of sperm with "laying-down" flagella (sperm tails). As, acrosome; Ch, chorion; CA, cortical alveoli; Mv, microvilli; MTV, microvillus tip vesicles; Nu, nucleus; Ol, oolemma; Sp, sperm; SpH, sperm head; SpT, sperm tail; SS, small sphere.

vitelline membrane. The tips of the microvilli are found interspersed with the small granules to form the uppermost surface of the vitelline membrane. The tips of the microvilli form spherical electron-lucent structures, the microvillus tip vesicles (MTV); each is about 0.3 μm in diameter (Fig. 2b). Under SEM, several MTVs were seen to be surrounded by the more numerous small spheres (Fig. 2c).

On the surface of the eggs that were mixed with the sperm, several sperm were observed (Fig. 2d). Many sperm lay flat on the surface of the egg (Fig. 2d, 2g) but a few sperm had their flagella perpendicular to the surface (Fig. 2d, 2e). The sperm with such an orientation were thought to be those that had penetrated the egg (see Epel, 1978). Under TEM, the sperm, about 2 μm in size, was seen to be composed of a flagellum containing a

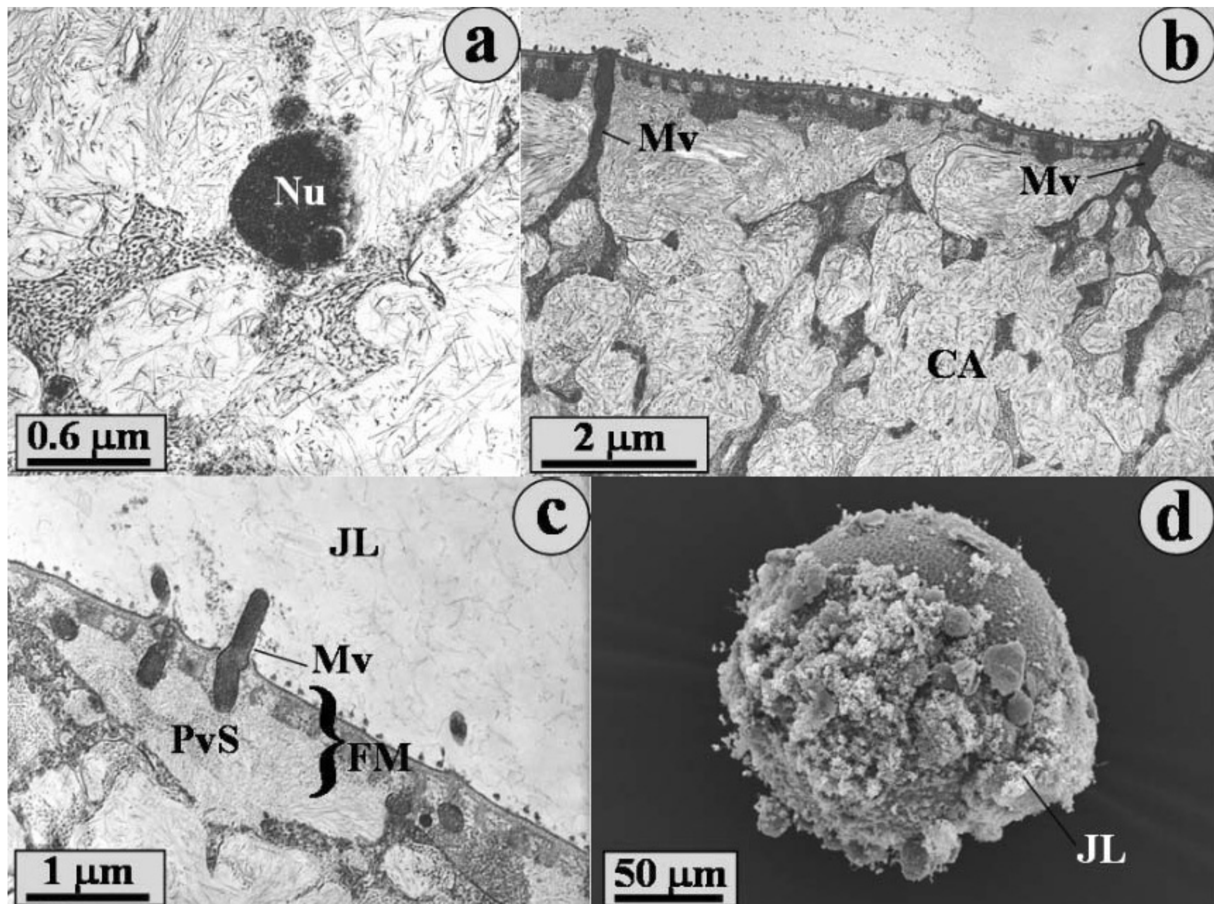

Fig. 3. (a) TEM showing Sperm DNA mass inside the ooplasm. (b) TEM of the fertilized egg showing the absence of mucrovillous tip vesicles. The microvilli were slightly elongated and the cortical alveoli (arrow) are shown after membrane fusion releasing material into the space between the oolemma and the fertilization membrane. (c) The surface of the fertilized egg showing protrusion of the microvilli, accumulation of fibrous materials in the perivitelline space, forming fertilization membrane, and eventually extruding through the vitelline membrane leading to the formation of the jelly layer. (d) SEM of the fertilized egg partially covered by the jelly material. CA, cortical alveoli; FM, fertilization membrane; JL, jelly layer; Mv, microvilli; Nu, nucleus; PvS, perivitelline space.

series of microtubules, and the head, being composed of nuclear material, acrosome and mitochondria (Fig. 2f). The nuclear material was composed of mostly electron-dense material interspersed by some less dense areas.

In samples fixed around 15 s after fertilization, the nuclear mass of the sperm could be observed inside the ooplasm (Fig. 3a) interspersed with the material forming the cortical alveoli. Fig. 3b shows the process of raising the vitelline membrane and the separation of the oolemma from the chorion, the arrow in Fig. 3b points to a region where fusion of the cortical alveoli membrane and the oolemma appears to have occurred allowing the release of the contents into the perivitelline space. The MTV began to disappear and, within 1 min, had completely disappeared from the egg surface (Fig. 3b). This was due to an expansion of the MTV to form an extended microvillus in which the tip vesicles had everted to form a slightly elongated form which protruded out of the chorion (Fig. 3c). The cortical alveoli open at the surface of the oolemma and release their content into the space between the oolemma and fibrous material of the chorion, to greatly increase the perivitelline space. The material formerly contained in the alveoli appears to pass through the vitelline membrane around the extended microvilli to form the jelly layer (Fig. 3c). SEM observations of the egg surface 45 s after fertilization shows the material derived from the alveoli on the surface of the egg, it is as if the cortical alveoli have secreted their fibrous material contents which have passed through the vitelline membrane to cover certain areas of the egg surface and form a loose jelly layer surrounding the egg (Fig. 3d) which would of course have been dehydrated during fixation.

Under LM, the fertilization membrane is clearly visible above a clear zone surrounding the egg (Fig. 4a). The thick jelly layer is not easily visible with light

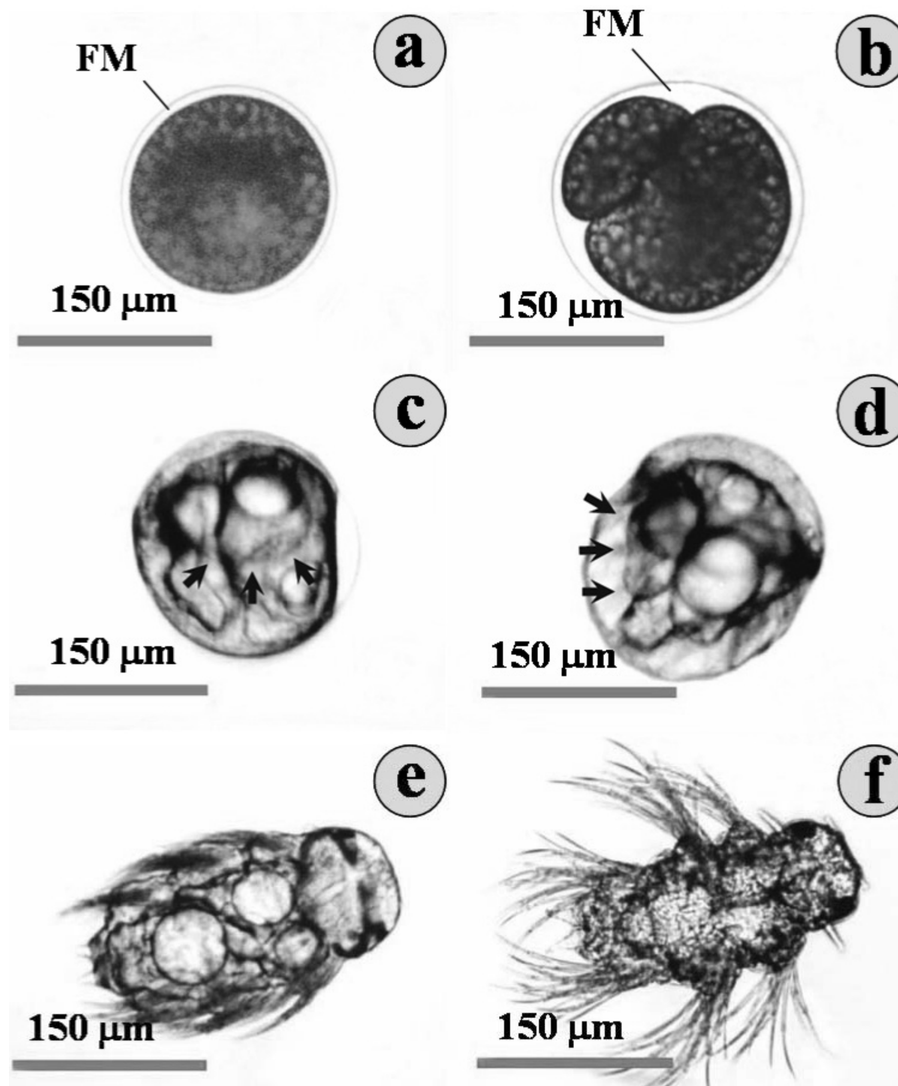

Fig. 4. Development from egg to nectochate stage. (a) LM of the fertilized egg at 1 h, covering with fertilization membrane. (b) The two-cell stage, at 2 h. (c) Monotrochophore showing an equatorial ring of cilia (arrows) at 24 h. (d) Trochophore showing three pairs of chaetigerous sacs (arrows). (e) Metatrochophore. (f) Nectochaete. Bar = 150 µm. FM, fertilization membrane.

microscopy. Embryonic development and cleavage takes place inside the vitelline membrane; the first and the second cleavage were observed at 1–2 h and 3–4 h respectively (Fig. 4b). The embryo developed an equatorial ring of cilia at 24 h, and became a monotrochophore moving inside the egg jelly (Fig. 4c). At 30 h, the monotrochophore became the trochophore, which grew by elongation of the trunk, and subsequently three pairs of chaetigerous sacs appeared laterally (Fig. 4d). At 36 h, the trochophore hatched out to become the meta-trochophore and swam freely in the water (Fig. 4e) and developed to the nectochaete stage, with three chaetigerous segments, by 48 h (Fig. 4f). The survival rate from fertilized eggs to nectochaete stage was about 50%. The nectochaetes stopped swimming and settled down into the sand bed and grew up to juvenile and atokous adults within five months.

Discussion

The culture procedure of *P.* cf *nuntia* described in this article may be one of the culture methods that could be adopted for large scale production of this species; other methods may be developed that differ in detail. Although the method described herein is simple and practical, it could be improved by some minor adjustments. For instance, improvement of survival rate from fertilized eggs to nectochaete stage from the current

percentage (50%) would obviously help the production. Sexual maturation of the male and female polychaetes at the same time is a requirement for mating success; quite often, the two sexes did not become mature at the same time, or the number of matured males and females was not in the right proportion. Since each sex survived only for 24 h after reaching sexual maturation, pairing may not occur or the number of fertilized eggs may be lower if the number of the sperm is low in proportion to the number of the eggs. Further research on preservation of the sperm or the eggs, either by cryopreservation (Olive and Wang, 1997; Wang and Olive 2000) or even for a few days in the refrigerator (Gwo, 2000; Hamaratoğlu et al., 2005) may help significantly improve the production.

The methods described, as well as the disease screening program, are important for the control of diseases that might spread to the shrimp from the use of wild collected polychaetes. Outdoor and uncontrolled feeding of wild collected polychaetes is likely to pose a risk of acquiring viruses from the environment since when collected from regions where there is extensive shrimp farming, this type of polychaete that naturally feeds on detritus and dead marine species, may feed on dead crustaceans that contain viruses, especially the most dangerous WSSV. Therefore indoor culture, or an outdoor one with biosecurity, is the method of choice to produce polychaete for shrimp broodstocks, or for additives for shrimp nutrition.

The ultrastructure and development of *P*. cf. *nuntia* described in this study are similar to those of other nereid species (Sato and Osanai, 1986; Porchet and Spik, 1978). One of the striking resemblances was the detail of chorion layers and the presence of MTV, which served as a sperm receptor in the polychaete, *Neanthes japonica* (Sato and Osanai, 1986). It is likely that the structure in *P*. cf. *nuntia* serves the same function. In sea urchins, the process by which sperm penetrate the egg begins with the sperm tail (flagellum) being lifted perpendicular to the egg surface (Epel, 1978); it is thus likely that this feature indicated an early stage of sperm penetration, and probably the movement of the sperm axoneme and translocation of the sperm nucleus through the egg cytoplasm (Schatten, 1981; Misamore et al., 1996; Misamore and Lynn, 2000). Obviously, further studies are needed to answer the mechanisms and purpose of this peculiar phenomenon. As more than one sperm showed this phenomenon, it is possible that polyspermy may occur in some eggs; whether the chromosome of the extra sperm is destroyed, or whether survival is possible with more than two sets of chromosomes, is not known.

Although the fibrous material in the cortical alveoli was not determined in this case, in the polychaete, *Perinereis cultrifera,* Porchet and Spik (1978) indicated that it is composed of glycoconjugates such as glucuronic acid and neutral monosaccharides, especially xylose, fucose and rhamnose. In sea urchins, the fibrous and hyaline material was found to consist of proteases, mucopolysaccharides and peroxidase (Vacquier et al., 1973; Foerder and Shapiro, 1977; Glabe and Vacquier, 1978; Hylander and Summers, 1982; Mozingo and Chandler, 1991). These substances form part of the fertilization membrane and jelly layer surrounding the fertilized egg. It is possible that their function is to protect the developing embryo from harmful external environment impacts such as a difference in ionic strength, osmolarity and pH, and may even provide protection against pathogens.

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