Ultrastructural characteristics of spermatogenesis in three species of deep-sea hydrothermal vent mytilids

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Abstract: To enhance our understanding of the reproductive biology of deep-sea hydrothermal vent mytilids, the histology of the male gonad and the ultrastructure of its gametes were studied in *Bathymodiolus thermophilus*, *B. puteoserpentis*, and *B. elongatus*. Specimens of *B. thermophilus* were collected at the 13°N site on the East Pacific ridge, while *B. puteoserpentis* were sampled from the Snake Pit site of the mid-Atlantic ridge and *B. elongatus* were obtained from the North Fiji Basin. Gonad histology conformed to the typical bivalve profile; the differences in the proportions of acinal and interacinal tissue, as well as differences in acinal fullness in *B. puteoserpentis*, indicate that gametogenesis is discontinuous in these deep-sea mytilids. Evidence of protandric hermaphroditism was observed in *B. elongatus*, which exhibited acini containing both maturing and residual male gametes and immature oocytes. The ultrastructural characteristics of the male gametes conform to those described for littoral bivalve species, and the spermatozoon is of the primitive type. No species-specific differences in spermatozoon ultrastructure were discerned. No evidence of bacterial inclusions was found in either the gametes or the associated gonad cells in any of the species examined. The male gametes are thus probably not vectors for the endosymbiotic bacteria that characterize the nutritional biology of the adults in this genus.

Résumé : L'histologie et l'ultrastructure de la gonade mâle et de ses gamètes ont été étudiées chez. *Bathymodiolus thermophilus, B. puteoserpentis* et *B. elongatus* afin de parfaire nos connaissances sur la biologie de la reproduction chez les Mytilidés des sources hydrothermales profondes. Les spécimens de *B. thermophilus* provenaient du site du 13°N situé sur la dorsale orientale du Pacifique, tandis que les exemplaires de *B. puteoserpentis* ont été recoltés sur le site Snake Pit de la dorsale médio-atlantique, et les individus de *B. elongatus* ont été récoltés dans le bassin nord des îles Fiji. L'histologie de la gonade était semblable au profil typique des bivalves. Les différences dans les proportions des tissus acinal et inter-acinal, ainsi que les différences dans le degré de remplissage des acini chez *B. puteoserpentis* suggèrent l'existence d'un aspect discontinu dans la gamétogenèse de ces Mytilidés des eaux profondes. Les acini de *B. elongatus* contenaient des gamètes mâles matures et d'autres résiduels, ainsi que des ovocytes immatures. Cette observation laisse présager l'existence d'un hermaphroditisme protandrique chez cette espèce. Les caractéristiques ultrastructurales des gamètes mâles sont identiques à celles que l'on observe chez les Bivalves des eaux littorales, et les spermatozoïdes sont du type primitif. Aucune différence spécifique n'a été décelée dans l'ultrastructure des spermatozoïdes des trois espèces. Aucune inclusion bactérienne n'a été retrouvée dans les gamètes ou dans les cellules gonadiques associées chez les trois espèces étudiées. Cela semble exclure les gamètes mâles comme vecteurs potentiels des bactéries endosymbiotiques qui sont une des caractéristiques de la biologie nutritionnelle des adultes de ce genre.

Introduction

The discovery of novel taxa inhabiting deep-sea hydrothermal vents has stimulated research on these organisms, especially the feeding and nutritional biology of species that harbour endosymbiotic bacteria (Cavanaugh 1983, 1985; Le Pennec and Hily 1984; Le Pennec and Prieur 1984; Felbeck et al. 1985; Morton 1986; Fiala-Médioni and Le Pennec 1988; Le Pennec et al. 1988*a*, 1990, 1992; Fiala-Médioni and Felbeck 1990). Conversely, the reproductive biology of these organisms has received very little attention (Le Pennec et al. 1984; Berg 1985; Le Pennec 1988; Tunnicliffe 1991). The relatively ephemeral and unstable conditions of the hydrothermal habitat raise many questions concerning the reproductive strategies of these organisms. Research in this area must begin with detailed investigations into the anatomy, ultrastructure, and gametogenesis of the gonad. The epibenthic bivalve populations inhabiting hydrothermal vent systems are well suited to such study; compared with soft-bodied organisms, they are less subject to injury during sampling by the robot arms of deep-sea submersibles. They are also often numerous, conspicuous, and accessible. In addition, the large body of knowledge concerning reproduction in shallow-water species provides a ready basis for comparison. However, the results obtained to date for hydrothermal vent species are fragmentary and concern principally *Calyptogena magnifica* collected from the same Galápagos ridge (Boss and Turner 1980; Berg 1985), *Bathymodiolus thermophilus* from the same Galápagos site (Kenk and Wil-

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son 1985) and from the 13°N site (East Pacific ridge) (Herry and Le Pennec 1987), as well as *Bathypecten vulcani* from the 13°N site (Herry and Le Pennec 1987).

The genus *Bathymodiolus* has until recently been considered monotypic, the type species being *B. thermophilus* Kenk and Wilson, 1985. However, the genus has recently been subdivided to include three additional species: *B. brevior*, *B. elongatus*, and *B. puteoserpentis* (von Cosel et al. 1994). An understanding of the reproductive biology of this genus must therefore include specimens of the novel species.

The present study had two goals: (1) to elucidate the cytological characteristics of spermatogenesis in two new hydrothermal mytilid species, *Bathymodiolus elongatus* van Cosel, Métivier, and Hashimoto sp.nov. and *B. puteoserpentis* van Cosel, Métivier, and Hashimoto sp.nov., as well as in *B. thermophilus*, and (2) to investigate whether the male gametes are transgenerational vectors of the endosymbiotic bacteria that play an important role in the nutritional biology of these species (Le Pennec et al. 1995).

Materials and methods

Sampling

All specimens of *Bathymodiolus* spp. were collected using the submersible *Nautile* (Institut Français de Recherche pour l'Exploitation de la Mer) operated from the N.O. *Nadir*. *Bathymodiolus thermophilus* were obtained in March 1982 and March 1984 during the Biocyatherm and Biocyarise expeditions, respectively, from the 13°N site (12°80′N, 103°56′W) of the East Pacific ridge at −2630 m. *Bathymodiolus puteoserpentis* were acquired between June 12 and July 14, 1988, from the Snake Pit site of the mid-Atlantic ridge during the Hydrosnake expedition, at −3515 m. *Bathymodiolus elongatus* were obtained from the North Fiji Basin (18°49′S, 173°29′W) between June 30 and July 19, 1989, during the Starmer expedition, at −2000 m. Five specimens of each species were examined.

Fixation and dissection

For the 1982 Biocyatherm and 1984 Biocyarise expeditions, all faunal specimens, including *B. thermophilus* sampled at the vent sites, were fixed whole in 10% formalin − seawater within 30 min of on-board recovery. Small (1−2 mm^3) pieces of the gonad were dissected out and postfixed in 3% glutaraldehyde − sodium cacodylate buffer (0.4 M, adjusted to 1300 mosmol with 7% NaCl, pH 7.25) upon reception at the Laboratoire de Biologie Marine. For specimens from the remaining expeditions (*B. puteoserpentis* and *B. elongatus*), pieces of the gonad were dissected out immediately upon arrival of the specimens on board, and fixed directly in glutaraldehyde−cacodylate buffer as above. After several days in the fixation solution, small pieces were removed for processing; these were rinsed twice in cacodylate buffer, postfixed in 1% osmium tetroxide in 0.2 M cacodylate buffer for 2 h, rinsed in buffer, dehydrated in a graded ethanol series, and placed in 50:50 Spurr's resin − ethanol for 6 h. The samples were then immersed in Spurr's resin for 12 h, embedded, and polymerized at 60°C for 48 h. Thin sections were stained with toluidine blue for light microscopy and ultrathin sections were contrasted with uranyl acetate and lead citrate for transmission electron microscope (TEM) observations.

Results

The difference in fixation protocol between *B. thermophilus* and the other two species did not appear to adversely affect the main ultrastructural characteristics of the cells, as shown by the aspect of mitochondria in the spermatids and spermatozoa of all three species. However, some cytoplasmic features did show evidence of poor fixation (e.g., Fig. 5); the following account therefore relies only on well-defined features.

In the *Bathymodiolus* species examined, the male gonad consisted of acini surrounded by inter-acinal connective tissue. Each acinus comprised the germinal epithelium, which included the spermatogenetic cell line, resting on a basal lamina (Fig. 1). These acini emptied into spermatic ducts that were lined with a simple ciliated cuboidal epithelium. Microvilli were often present between the cilia. Numerous spermatozoa were observed in the lumen of these ducts (Figs. 1, 3).

In *B. elongatus* the male gonad was dominated by inter-acinal connective tissue comprising vacuolated adipo-granular cells (Fig. 2). In addition to male gametes, the *B. elongatus* acini presented large (up to 47 μm) globular cells whose cytological profile was identical with that of immature oocytes described for the same species from the same site (Herry and Le Pennec 1987). These cells had a well-developed nucleus, a large nucleolus, and numerous inclusions in the cytoplasm (Figs. 4, 16, 19). Oogonia were also observed in spent acini of this species (Fig. 17).

Considerable individual variation was observed in the ripeness of the *B. puteoserpentis* gonads.

No species-specific differences were detected in the ultrastructure of the gametes observed in the male gonad. The following description thus applies to all three species examined; the figures refer to particular species but are representative of all specimens studied.

The spermatogonia were the largest of the cell line, the size range being 6−8.4 × 5−8.1 μm. Their nucleus was large (5 μm) and had particularly well-developed euchromatin. Two large nucleoli were visible in some sections (Fig. 5).

The primary spermatocytes were grouped together in the acini, the size range being 3.5−5.2 × 4.4−8.7 μm. Several stages of prophasic spermatocytes were observed, based on the morphology of the nuclear chromatin (Figs. 5, 6). Secondary spermatocytes were not observed (the second meiotic interphase is extremely brief in bivalves; Sastry 1979).

The spermatids, produced through division of the secondary spermatocytes, were round or oval and were grouped together in the acini (Figs. 7, 8). They were easily recognizable because of their small size (2.3−3 × 1.5−2.5 μm) and their nucleus (approximately 2.2 μm), which contained areas with highly condensed chromatin. These spermatids presented ultrastructural characteristics that differed according to the five developmental processes associated with spermiogenesis: (1) formation of the acrosomal vesicle, (2) development of the flagellum, and cellular modifications of (3) the mitochondria, (4) the cytoplasm, and (5) the nucleus.

Formation of the acrosomal vesicle began with the elaboration of acrosomal granules (Fig. 7), which condensed to form the membrane-bound acrosomal vesicle containing electron-dense material (Figs. 7, 8). This vesicle migrated toward the anterior pole of the spermatid, extended to the nuclear surface, and then invaginated to form the subacrosomal region (Fig. 7). This subacrosomal region contained material that was less electron dense than that observed in the acrosomal vesicle (Fig. 9). After a period of elongation and

Fig. 1. *Bathymodiolus thermophilus* (13°N), semithin section showing an acinus (AC♂) with the different spermatogenetic cell lines. SPi, spermatogonia, SPC, spermatocytes; SPT, spermatids; SPZ, spermatozoa. Inter-acinal connective tissue (ICT) surrounds the acinus. **Fig. 2.** *Bathymodiolus elongatus* (North Fiji), semithin section of a spermatic duct (SD) surrounded by storage tissue consisting of adipo-granular cells (AGC). CE, cuboidal epithelium; SPZ, spermatozoa. **Fig. 3.** *Bathymodiolus puteoserpentis* (Snake Pit), TEM detail of the ciliated cuboidal epithelium (CE) of the spermatic duct. BL, basal lamina; Ci, Cilia; MV, microvilli; SPZ, spermatozoon in duct lumen. **Fig. 4.** *Bathymodiolus elongatus* (North Fiji), semithin section. Immature oocytes (IO) are present at the periphery of a male acinus (AC♂). AGC, adipo-granular cells; BL, basal lamina; N, nucleus; Nu, nucleolus. Toluidine blue. **Fig. 5.** *Bathymodiolus thermophilus* (13°N), transmission electron micrograph of primary spermatogenetic cell line. Spermatogonia (SPi) possess a voluminous nucleus (N) and two nucleoli (Nu). SPC, primary spermatocytes in first meiotic prophase (single arrow) and anaphase (double arrow). BL, basal lamina. **Fig. 6.** *Bathymodiolus puteoserpentis* (Snake Pit), transmission electron micrograph of spermatocytes (SPC) in pachytene stage of prophase (triple arrows). SPT, spermatids undergoing spermiogenesis.

Figs. 7–13. Transmission electron micrographs of male gonad cells in the three *Bathymodiolus* species examined. Fig. 7. *Bathymodiolus puteoserpentis* (Snake Pit). Spermatids (SPT) undergoing spermiogenesis. The nucleus (N) elongates, the cytoplasm (CY) is lysed and exocytosed and the acrosomal granules (AG) are elaborated. IAV, invagination of the acrosomal vesicle. Fig. 8. *Bathymodiolus puteoserpentis* (Snake Pit), spermatids with their acrosomal vesicle (AV). Some of the spermatids have differentiated into spermatozoa (SPZ). F, caudal flagellum. Fig. 9. *Bathymodiolus thermophilus* (13°N) mitochondria (Mi), grouped under the nucleus (N), at the posterior pole of the spermatid (SPT). The cytoplasm (CY) is exocytosed. SPZ, spermatozoa, showing the acrosomal vesicle (AV) and the subacrosomal region (SAR). Figs. 10 and 11. *Bathymodiolus elongatus* (North Fiji) and *Bathymodiolus puteoserpentis* (Snake Pit) spermatozoa (SPZ), respectively, showing their ovoid shape. A, acrosome; SPT, spermatids. Figs. 12 and 13. *Bathymodiolus puteoserpentis* (Snake Pit) and *Bathymodiolus thermophilus* (13°N), respectively, showing details of the anterior and posterior poles of spermatozoa. A, acrosome; AV, acrosomal vesicle; DC, distal centriole; F, caudal flagellum; Mi, mitochondria; MP, midpiece; N, nucleus; SAR, subacrosomal region.

Figs. 14–19. Transmission electron micrographs of the cell types found in the male gonad of the three *Bathymodiolus* species examined. Fig. 14. *Bathymodiolus puteoserpentis* (Snake Pit), adipo-granular cells (AGC). Their nucleus (N) is acentric and their cytoplasm is filled with lipid-like droplets (Li) and protein-like granules (PG). ICT, inter-acinal connective tissue. Fig. 15. *Bathymodiolus puteoserpentis* (Snake Pit), lysis of adipo-granular cells (AGC). Arrows indicate lysis of the plasma membrane (PM). LM, lysed material; N, nucleus; SPT, spermatids. Fig. 16. *Bathymodiolus elongatus* (North Fiji), oocyte (O) adjacent to the basal lamina of an acinus (BL). AGC, adipo-granular cells; Ci, cytoplasmic inclusions; MV, microvilli; N, nucleus; SPZ, spermatozoa. Fig. 17. *Bathymodiolus elongatus* (North Fiji), inter-acinal connective tissue (ICT) containing adipo-granular cells (ACG) and lysed material (LM). Li, lipid-like inclusions. Some oogonia (Oi) are observed. Figs. 18 and 19. *Bathymodiolus elongatus* (North Fiji), atretic material containing spermatozoa (SPZ), lipid-like substances (Li), and immature oocytes (IO). A phagocyte (PH) with pseudopodium-like extensions is visible. N, nucleus.

in the final stages of spermiogenesis, the acrosome formed the anterior extremity of the spermatozoon (Figs. 9, 11).

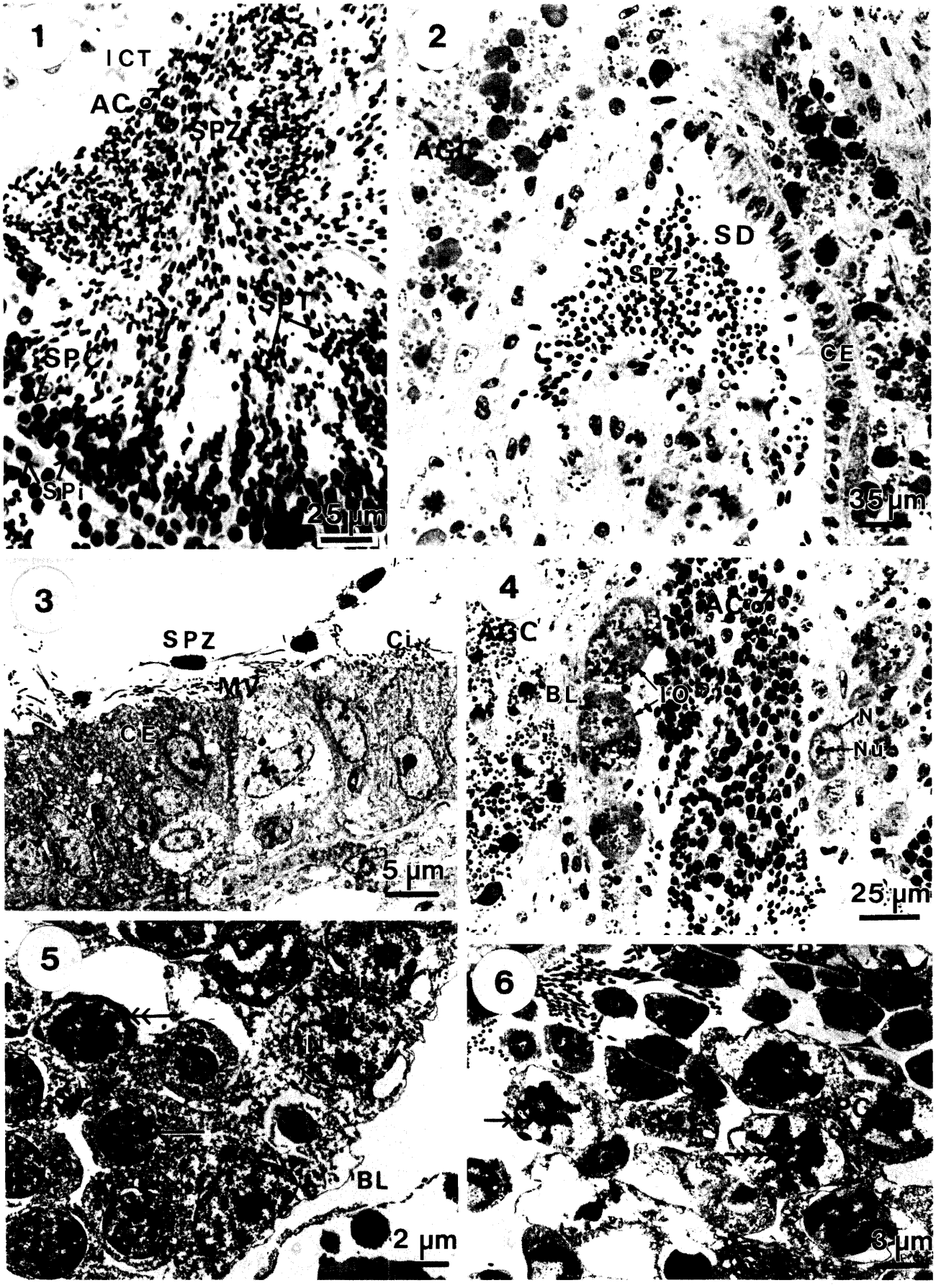
The process of flagellum development was contemporaneous with acrosome formation. This was accomplished by the migration of two centrioles to the posterior pole of the spermatid. The proximal (closest to the nucleus) centriole was perpendicular to the distal centriole, from which the flagellum later developed (Figs. 12, 13). The centriolar apparatus occupied the centre of the midpiece of the future spermatozoon.

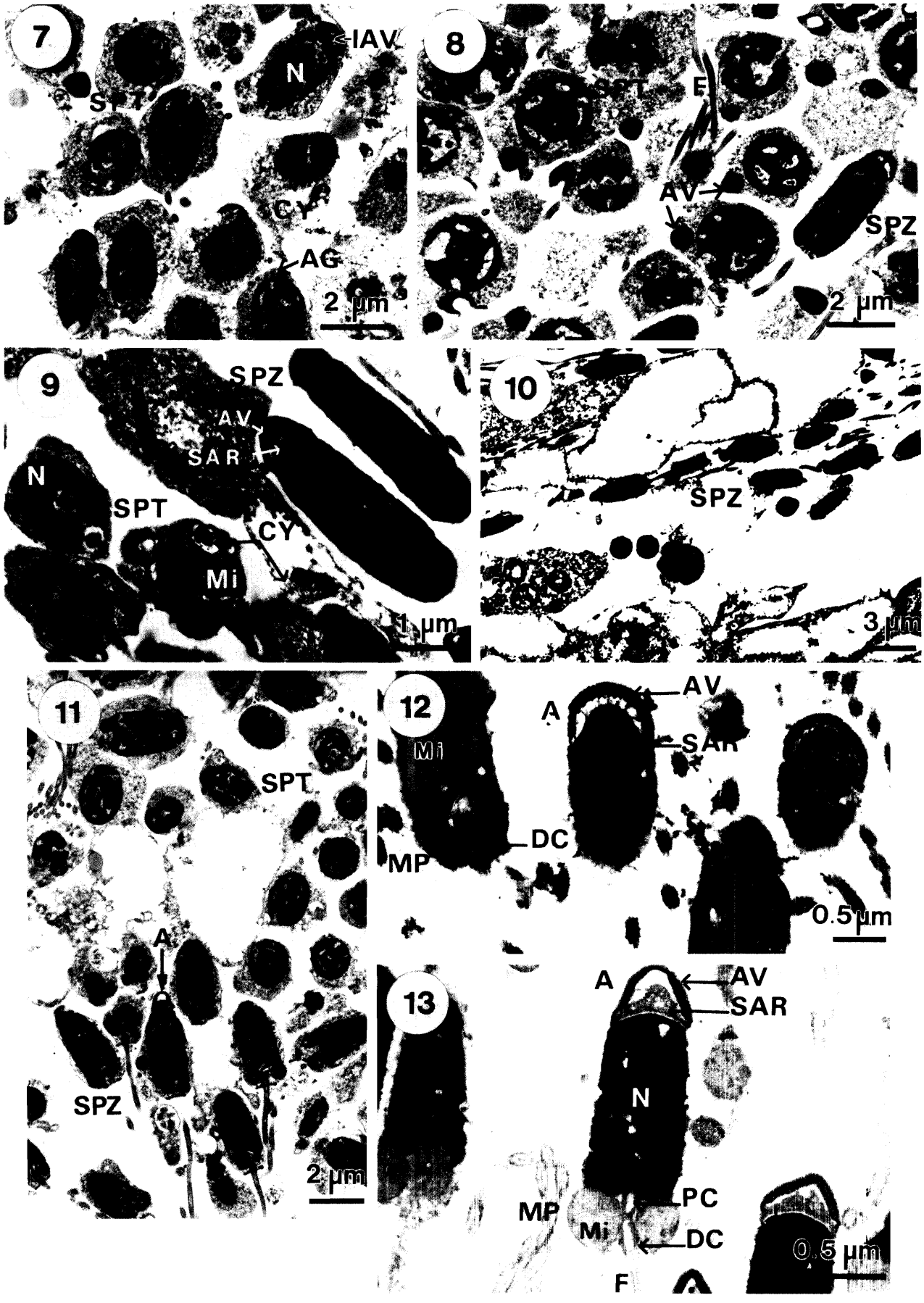
During modification, the mitochondria began by forming a group beneath the nucleus, at the posterior pole of the spermatid. They then fused to produce four large mitochondrial spheres that formed a ring at the base of the nucleus. Elongation of the nucleus and condensation of the chromatin began later during spermiogenesis, giving the spermatozoon its characteristic head shape. At the end of spermiogenesis the nucleus occupied the greater part of the sperm head and a small invagination was observed at its posterior pole; this invagination was occupied by the proximal centriole (Figs. 10–13).

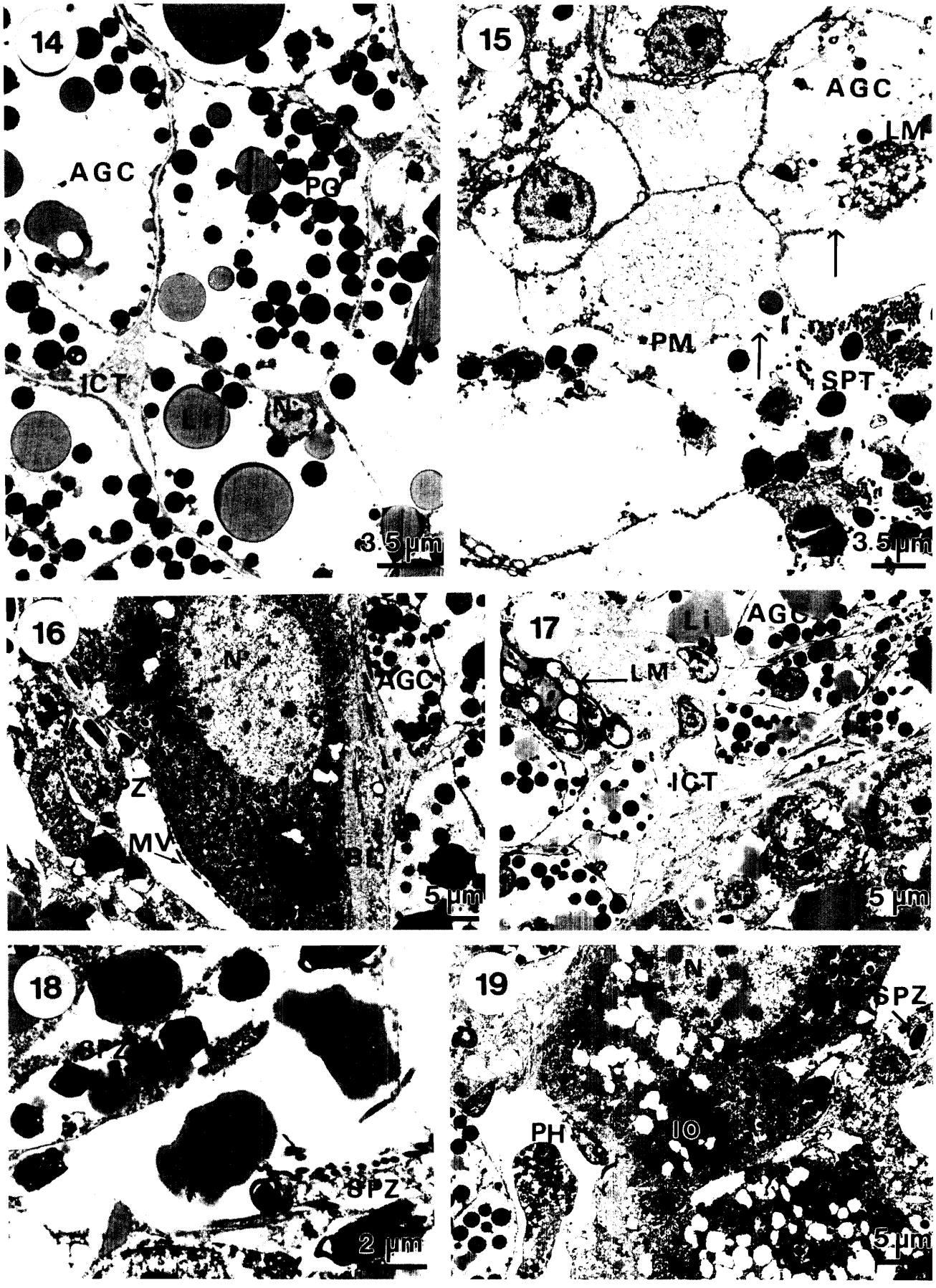
The spermatozoa were ovoid and possessed a caudal flagellum. The head, 3.6–3.7 μm long, was capped by a short (0.6 μm) acrosome. The nucleus contained dense, homogeneous chromatin. The height and diameter of this nucleus were 2.4–2.5 and 0.9–1.2 μm, respectively. The midpiece was composed of four mitochondria (observed in transverse section), each measuring 0.6 μm in diameter. The flagellum possessed the classical 9 + 2 microtubular structure. Its length could not be determined from the sections (Figs. 8, 10, 11, 13).

The adipo-granular cells of the inter-acinal connective tissue contained osmiophilic granules as well as electron-translucent vacuoles that probably contained lipid (Fig. 14). The nucleus, when visible, was generally not centred within the cell (Figs. 14, 15). The shape of these adipo-granular cells varied and depended on the abundance of the cytoplasmic inclusions. Their dimensions were approximately 18 × 8 μm. Rupture of the plasma membrane and release of the cytoplasmic inclusions into the connective tissue or into the lumen of the gonadal acini was observed, although this could have been a consequence of shipboard fixation in 10% formalin – seawater (Figs. 15, 17).

Male gametes undergoing lysis were observed within both the lumen and the ducts of spent acini and within the inter-acinal connective tissue, presumably having traversed the ruptured acinus wall. The degeneration of the spermatozoa was marked by densification of the mitochondrial spheres and deformation of the acrosome. The lysed gametes were observed in close proximity to large pools of a lipid-like substance (Fig. 18). Phagocytes (approximately 15 × 6 μm) were also observed nearby (Fig. 19).







Discussion

Spermatogenesis in the three *Bathymodiolus* species examined was similar to that previously described for various coastal mytilids such as *Mytilus edulis* (Longo and Dornfeld 1967), *Musculus discors* (Franzén 1983), and *Perna perna* (Bernard and Hodgson 1985).

As in most bivalves, the male gametes of *Bathymodiolus* spp. correspond to the primitive spermatozoon type as defined by Tuzet (1950) and Franzén (1956). These spermatozoa, "primitive" because of their simple morphology, are characteristic of animal groups that rely on broadcast of male gametes, as is the case for all bivalve species (Franzén 1956, 1977, 1983).

Comparative studies on bivalve spermatozoa reveal that their morphology and ultrastructure may be useful taxonomic characters, as least at the family level and above (for a review see Healy 1996). Franzén (1983) suggested that probably no two bivalve species have identical spermatozoa. In some cases, spermatozoon ultrastructure has been useful in classification at the species level. For example, in littoral mytilids, such as *M. edulis* (Longo and Dornfeld 1967), *Mytilus perna* (Bourcart et al. 1965), *Mytilus galloprovincialis* (Hodgson and Bernard 1986), and *P. perna* (Bernard and Hodgson 1985), the male gametes are similar in general structure, although in each species they possess a unique set of characteristics. The same is true for the teredinids *Bankia australis* and *Bankia carinata* (Popham et al. 1974). Although no differences were discernible in the ultrastructure of the three *Bathymodiolus* species examined here, the results of preliminary electrophoretic study of the enzyme system of *B. thermophilus* and *B. elongatus* suggested considerable genetic divergence between the two species (Moraga et al. 1994). It is thus possible that speciation in these three taxa is still underway. Future genetic studies may shed more light on the taxonomic relationships of the different species of *Bathymodiolus* in various areas of the oceanic hydrothermal ecosystem.

TEM observations of the male gametes of bivalves reveal that the most conservative spermatozoan structures are the midpiece and the flagellum. The nucleus varies in both shape and size, and the acrosome structure varies considerably among taxa (Fawcett 1970). Different nucleus shapes have been described: cylindrical in *M. perna* (Bourcart et al. 1965) and *Pecten maximus* (Dorange and Le Pennec 1989); barrel-shaped in *Spisula solidissima* (Longo and Anderson 1969); long, tapering, and curved in *Tapes decussatus* (Pochon-Masson and Gharagozlou 1970); round in *Crassostrea virginica* (Galtsoff and Philpott 1960); and elongated and conical in the *Bathymodiolus* species of the present study.

The differences in relative proportions of the acini and the inter-acinal connective tissue, as well as in the degree of fullness of the acini in the three *Bathymodiolus* species examined, suggest that there is a discontinuous component to the reproductive process in these deep-sea organisms, but obviously for financial reasons it may not be possible to ascertain whether this represents a cyclic phenomenon. The reciprocal relationship of gametogenesis to the inter-acinal tissue has been documented in the reproductive cycle of littoral bivalves (Coe 1943; Lubet et al. 1976; Beninger 1987). The role of the various cell types comprising the inter-acinal tissue in the transfer of metabolites to the developing gametes has been demonstrated for *P. maximus* (Le Pennec et al. 1991). In particular, the adipo-granular cells in the inter-acinal tissue of mytilids supply metabolites to the developing gametes (Lubet 1959; Herlin-Houtteville 1974; Mathieu 1979). It should be noted that, in contrast to littoral bivalve species (Lubet 1959; Eble 1969; Gabbott 1975; Lowe et al. 1982; Pipe 1987), no vesicular connective tissue cells were observed in any of the *Bathymodiolus* species studied; these cells accumulate glycogen in littoral species.

The presence of female gametes in spent acini of *B. elongatus* suggests that this species may be a protandric hermaphrodite. A similar finding was made for a hydrothermal mytilid from the Galápagos vents (Berg 1985). Further studies are necessary to evaluate the extent of hermaphroditism in hydrothermal mytilids.

The presence of degenerating spermatozoa and phagocytes within the lumen and ducts of the acini and the surrounding inter-acinal connective tissue suggests that residual material may be resorbed following spawning, as has already been observed in the littoral scallop *P. maximus* (Dorange and Le Pennec 1989; Le Pennec et al. 1991).

The acquisition of bacterial gill endosymbionts by bivalves has received relatively little attention. Endow and Ohta (1990) demonstrated the presence of inclusions similar to gill endosymbionts in the primary oocytes of *Calyptogena soyoae*, and Gustafson and Reid (1988) observed inclusions similar to the gill endosymbionts of *Solemya reidi* in larvae of this species; they hypothesized that the bacteria are cryptically packaged in the gametes. Although the existence of variously modified procaryotic cells cannot be totally ascertained by means of visual observation, Cary and Giovannoni (1993) demonstrated the presence of endosymbiont 16S rRNA in the oocytes of *Calyptogena magnifica, C. phaseoliformis*, and *C. pacifica*. Transovarial inheritance of endosymbionts thus appears fairly well established in these species. This transovarial mechanism of symbiont transmission would be similar to that observed for certain sponges (Gallissian and Vacelet 1976; Levi and Levi 1976). It is as yet not known whether transovarial transmission occurs in the genus *Bathymodiolus*; however, the data obtained in the present study do not reveal any obvious procaryotes in the spermatozoa of the three species studied.

Conversely, considerable evidence exists to support the hypothesis that gill endosymbionts are captured by the gill epithelium and "cultured" by the host bivalve. Environments inhabited by host species contain very high concentrations of bacteria (10^5-10^9 cells/mL) compared with oceanic seawater (10^2-10^3 cells/mL; Francheteau and Laubier 1982). Le Pennec et al. (1988*b*) clearly demonstrated bacterial endocytosis by the gill epithelium in *B. thermophilus* as well as in the littoral species *Thyasira flexuosa* and *Lucinella divaricata*. These results, which tend to support the hypothesis that chemoautotrophic gill bacteria are captured from the external environment, are similar to the results of Cary et al. (1989, 1993), who concluded that the tube worm *Riftia pachyptila* obtained its symbionts directly from free-living bacterial populations. Indeed, recent observations show that early juveniles of *R. pachyptila* lack bacteria (Jones and Gardiner 1988). *Bathymodiolus* spp. are less

dependent on gill endosymbionts than the genus *Calyptogena* (Le Pennec et al. 1995), and this may have a bearing on endosymbiont transmission mechanisms. Further studies are required to ascertain how *Bathymodiolus* spp. acquire and maintain their endosymbiont populations.

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