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Ultrastructure of spermiogenesis in *Vampyroteuthis infernalis* Chun – a relict cephalopod mollusc

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ABSTRACT: Spermiogenesis in the relict deep-sea cephalopod *Vampyroteuthis infernalis* Chun is examined using transmission electron microscopy (TEM), and the results compared with available data on other cephalopods. Early spermatids of *Vampyroteuthis* exhibit an ovoid nucleus (with dense irregular patches), numerous mitochondria and a pair of triplet substructure centrioles (arranged parallel to each other). Subsequently, the following morphological changes take place: (1) nuclear contents condense into a fibrous reticulum, then into thick fibres; (2) the acrosomal vesicle (presumably Golgi-derived) positions itself in a shallow depression at the nuclear apex; (3) the flagellum forms from one of the two centrioles; (4) mitochondria cluster around the flagellum at the base of the nucleus; (5) a dense, fibrous plug forms within the basal invagination of the nucleus. Microtubules surround the acrosome and condensing nucleus of spermatids. The dense plug is of special systematic importance since it also occurs in spermatids and spermatozoa of *Octopus* spp., but not in any investigated species of the Sepiida, Sepiolida or Teuthida. Late spermatids and mature spermatozoa of *Vampyroteuthis* strongly resemble developing spermatids of *Octopus*, suggesting a close phylogenetic relationship between *Vampyroteuthis* (and the Vampyromorpha) and octopods.

INTRODUCTION

The deep sea vampire squid, *Vampyroteuthis infernalis* Chun, occupies a special place in cephalopod classification and phylogeny. This cosmopolitan, but rare, deep-sea species is the sole living representative of the coleoid order Vampyromorpha – a group believed to be closely linked with the origin of the order Octopoda (Donovan, 1977; Bandel & Leich, 1986; Clarke, 1988). Anatomically, *Vampyroteuthis* almost perfectly bridges the gap between the Teuthida and cirrate Octopoda (Pickford, 1939, 1940; Young, 1977). Grimpe (1917) and Robson (1932) considered that the Vampyromorpha belonged within the Octopoda (as suborder [Robson] or subdivision of cirrate Octopoda [Grimpe]). Subsequently, Pickford (1939) gave the group full ordinal status – a decision generally accepted by most workers and in recent classifications (e.g. Voss, 1977; Boss, 1982; Clarke & Trueman, 1988). Recently, however, Fioroni (1981) has established two superorders Octobrachia and Decabrachia (essentially reviving the Decapoda) and placed the Vampyromorpha in the latter superorder together with the orders* Teuthida, Sepiida and new order Sepiolida.

* In accordance with Recommendation 29A of the ICZN Code that the ending – OIDEA be reserved for superfamilies, I adopt Clarke & Trueman's (1988) revised ordinal names Teuthida, Sepiida and Sepiolida and herein modify Coleoidea to Coleoida.

In a previous study (Healy, 1989), I examined the ultrastructure of spermatozoa in *Vampyroteuthis* in an attempt to clarify the relationship of vampyromorphans to other coleoid cephalopods (see also Healy, 1990). Comparison with other cephalopods revealed that sperm morphology supported a close relationship between *Vampyroteuthis* (and the Vampyromorpha) and the Octopoda (Healy, 1989, 1990). Also presented in that account were preliminary observations on spermiogenesis in *Vampyroteuthis* and an un-named species of *Octopus*, which helped to confirm the homology of an interesting sperm component shared by these cephalopods – a large dense plug within the nuclear invagination. This paper examines in detail the events of spermiogenesis in *Vampyroteuthis* which of necessity were only briefly outlined in my earlier study.

MATERIALS AND METHODS

Testis tissue was extracted from a seawater-formalin fixed specimen of *Vampyroteuthis infernalis* held in the "wet" collection of the Australian Museum (Sydney) (C. 154223, mid-water trawled, 640 m depth off Newcastle, 33° 07–01′ S, 153° 11–05′ E, by R. V. "Kapala" November 1979). After a thorough rinse in sea water, tissue pieces were placed for 80 min in a 1 % osmium tetroxide solution (prepared in sea water). This was followed by another rinse in sea water, gradual dehydration in ethanol, and embedding in Spurr's epoxy resin. Ultrathin sections were cut with an LKB IV Ultrotome and collected on 200 mesh copper grids. After staining grids with uranyl acetate and lead citrate, they were examined with either a Philips 300 or Hitachi 300 transmission electron microscope operated at 60–80 kV.

RESULTS

Spermatogonia, spermatocytes

Cells here interpreted as being spermatogonia (Fig. 1A) were regularly seen in testicular tissues. They contain a large oblong nucleus (15 µm × 9.5 µm) which features a nucleolus and often a deep invagination – the latter giving gonial nuclei a lobulate appearance (Fig. 1A). At least one triplet substructure centriole has been observed at this stage of development (Fig. 1A inset). In secondary spermatocytes the oval nucleus (maximum diameter 12 µm) shows well developed synaptinemal complexes (Fig. 1B). Numerous, round mitochondria (diameter 0.3–0.4 µm) are observed within the cytoplasm.

Early spermatids

Early spermatids have a round nucleus (containing extensive electron-dense tracts), small mitochondria and two triplet substructure centrioles (Figs 1E, D). The centrioles are

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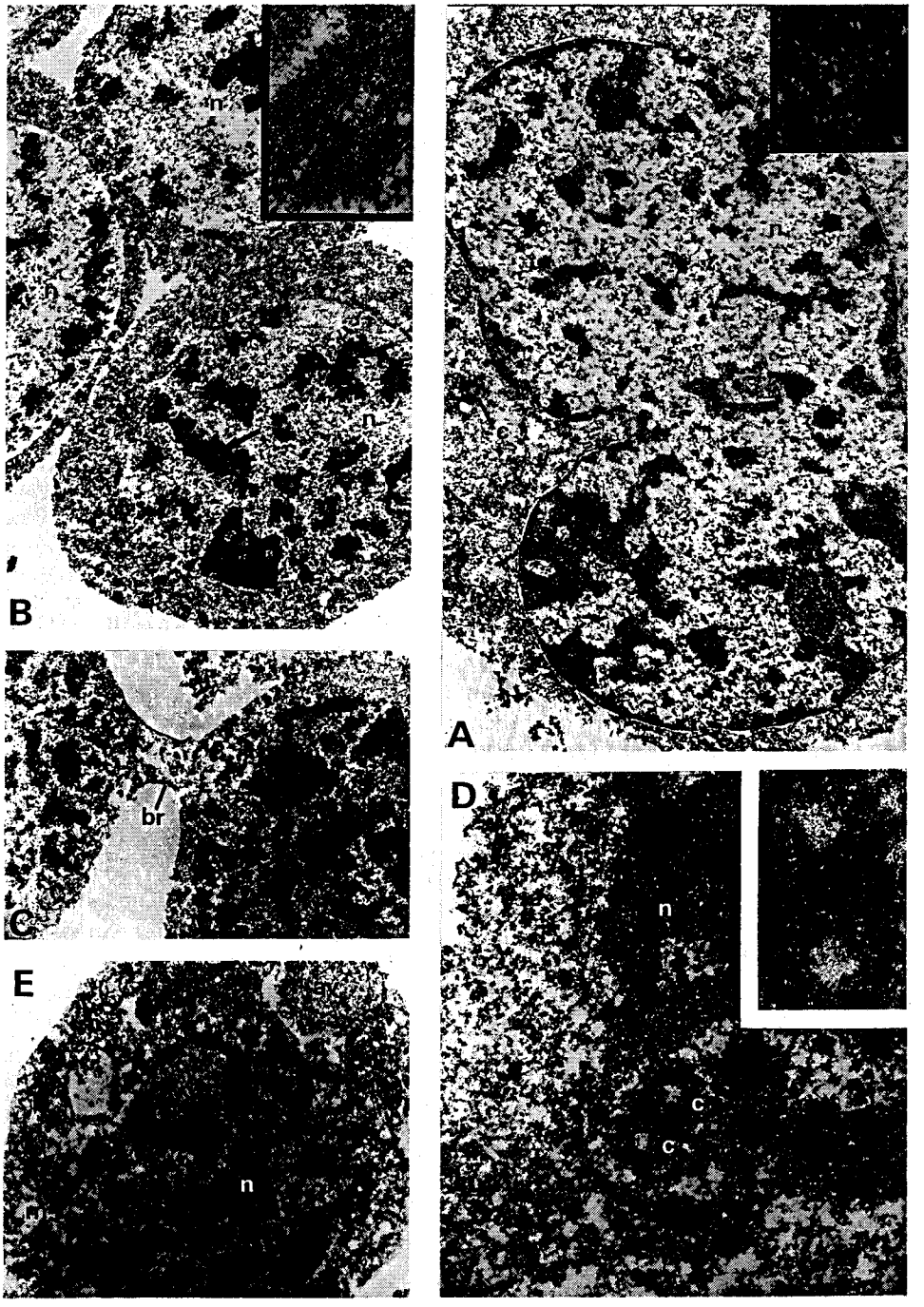
Figs 1A–E. *Vampyroteuthis infernalis*. A: Spermatogonium showing deeply lobulate nucleus and centriole (× 9000). Inset: detail of centriole (× 72 500). B: Spermatocytes. Note (arrows) synaptinemal complexes (× 4000). Inset: Detail of synaptinemal complex (× 18 250). C: Cytoplasmic bridge between early spermatids (× 9000). D: Centriole pair (enveloped by dense material) close to early spermatid nucleus (× 37 300). Inset: detail of centrioles showing triplets (× 85 500). E: Early spermatid showing developing acrosomal vesicle (× 12 700). Abbreviations: av, acrosomal vesicle; br, cytoplasmic bridge(s); c, centriole(s); m, mitochondria; n, nucleus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arranged parallel to each other at the periphery of the nucleus and are partly enveloped by a deposit of fine granular material (Fig. 1D). Although the acrosomal vesicle was observed in early spermatids (Fig. 1E), it has not been possible to identify with certainty an associated Golgi complex. Frequently, two spermatid nuclei were observed within a single cell. While some of these incidents probably represent newly formed spermatids which have as yet to complete cytokinesis (e.g. Fig. 1B), examples of two or, rarely, three mid-spermatids developing within a common cytoplasm have also been found (see Figs 2A, B).

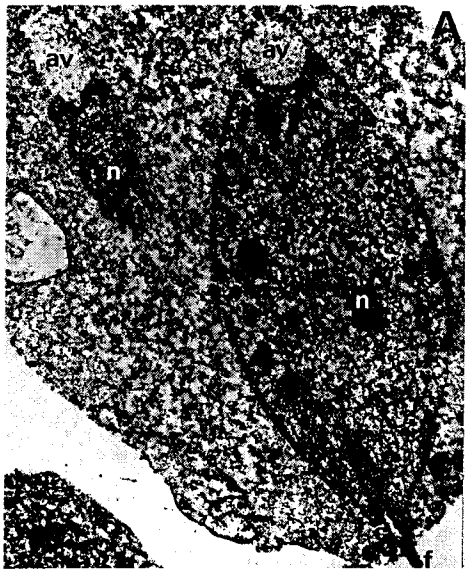
Mid-spermatids

The most significant morphological events of spermiogenesis occur in mid-spermatids. The round, acrosomal vesicle becomes partly embedded in the apex of the condensing nucleus (Fig. 2D). Condensation of the mid-spermatid nucleus involves conversion of a granular/patchy nuclear fabric (Figs 2B [bottom], 3A–C) to a fibroreticulate one (Figs 2A, B [top], D, E, 3D, E). As this takes place, the nucleus becomes fusiform, depressed apically (to accommodate the acrosomal vesicle – Figs 2A, D) and deeply invaginated basally (Figs 2E, 3A–D). Both centrioles remain outside the basal invagination of the nucleus, but appear to be involved in the production of a coarsely fibrous plug which fills the invagination (Figs 2E, 3E). Longitudinal sections through the developing nuclear invagination indicate that microtubules which penetrate the fibrous plug originate from the centrioles (Figs 3B, D, E) and are present even before the fibrous material of the plug begins to form (Figs 3B–D). Similarly, the sheath of microtubules, which envelopes the condensing nucleus (Fig. 2E) and the acrosomal vesicle (Fig. 2D), has almost certainly been derived through centriolar activity (e.g. see Figs 3B, D, E). The distal centriole also gives rise to the flagellum at the plasma membrane (Figs 2A, E).

Late spermatids

In the final stage of spermiogenesis, mitochondria which have been positioned near the base of the nucleus (see Figs 2E, 3E, 4F) finally become organized into the pericentriolar midpiece (Fig. 4G). Since the number of mitochondria of mid-spermatids greatly exceeds the number observed in mature sperm (3–4), midpiece formation undoubtedly involves some degree of mitochondrial fusion, though without loss of cristate substructure (Fig. 4G). In late spermatids, the nucleus becomes coarsely fibrous in texture, as does the dense plug within the basal invagination (Figs 4C, D, F). Microtubules continue to form a sheath around the nucleus and acrosomal vesicle (Figs 4A, B, E), and those penetrating the dense plug are clearly visible (Fig. 4F).

Figs 2A–E. *Vampyroteuthis infernalis*. A: Two advanced spermatids (one cut obliquely) sharing a common cytoplasm (× 7250). B: Transverse sections of double spermatids at differing stages of nuclear condensation (× 9000). C: Cytoplasmic bridge linking advanced spermatids (× 10 000). D: Acrosomal vesicle and condensing nucleus (fibrous stage) (× 21 400). E: Longitudinal section through centrioles, flagellum, developing nuclear invagination (containing fibrous plug and microtubules). Note also perinuclear microtubules (× 21 400). Abbreviations: av, acrosomal vesicle; br, cytoplasmic bridge(s); c, centriole(s); dp, dense (fibrous) plug; f, flagellum; m, mitochondria; mt, microtubules; n, nucleus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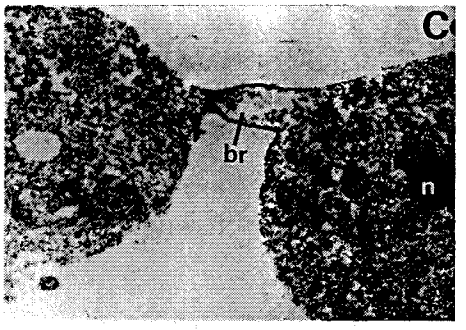
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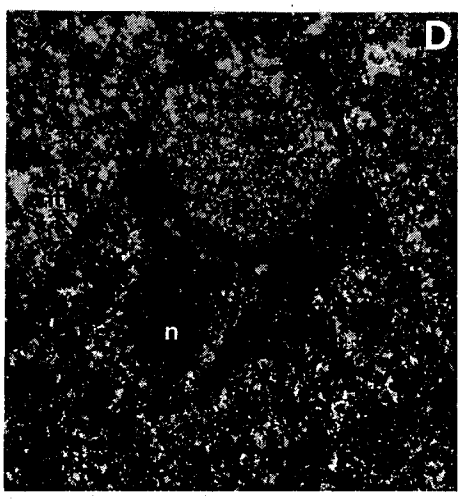
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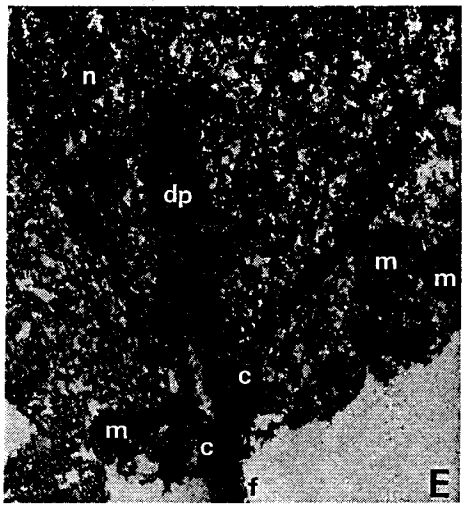
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Mature spermatozoa

The ultrastructure of mature spermatozoa of *Vampyroteuthis* has been detailed in a previous study (Healy, 1989). However, for the sake of completion, the following description and Figure 5I are presented.

The acrosomal vesicle is spherical, 1 μm in diameter and set in a curved depression of the nuclear apex. Contents of the vesicle are granular with a dense peripheral layer (80 nm thick) lining the posterior, internal surface of the vesicle. Sperm nuclei are short (8.5 μm) and fusiform with a deep basal invagination (2.2–2.7 μm) filled by a plug of dense material. Visible within the plug are numerous round-oblong cavities and microtubules – the latter originating from both centrioles (positioned outside the nuclear invagination). Oblong cavities are also present within the nucleus. The centrioles are arranged parallel to each other and show typical triplet substructure. Three or four mitochondria surround the centrioles to form the midpiece region. The sperm flagellum is 130–135 μm long and continuous with the "distal" centriole.

Figures 5A–I summarize in semi-diagrammatic form the events of late spermatogenesis and spermiogenesis and the morphology of mature spermatozoa in *Vampyroteuthis*.

DISCUSSION

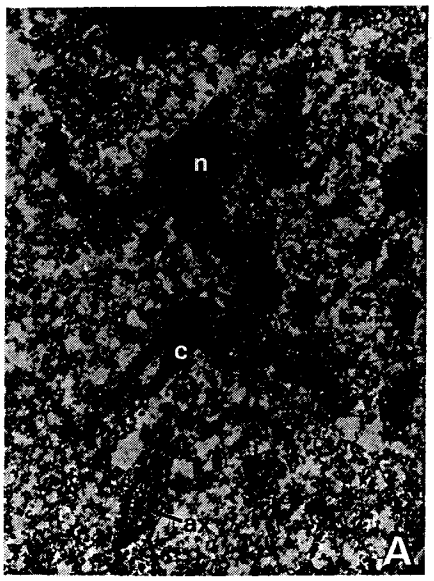
Spermiogenesis – comparison with other cephalopods

The ultrastructure of spermiogenesis has been studied in only a few cephalopod taxa (*Nautilus* – Arnold & Williams-Arnold, 1978; Tsukahara, 1985; *Alloteuthis, Loligo, Eusepia* – Maxwell, 1975; *Spirula* – Healy 1989, 1990; *Eledone* – Maxwell, 1974; *Octopus* – Galangau & Tuzet, 1968a, b; Galangau, 1969; Healy, 1989). However, these data, in conjunction with light microscopic accounts by Franzén (1955, 1967), provide a reasonably firm basis for comparison with *Vampyroteuthis*.

The developing and mature acrosomal vesicle of *Vampyroteuthis* (spherical with a dense layer lining the posterior inner surface of the vesicle) strongly resembles those of mid-spermatids in the Sepiida, Teuthida and *Spirula* (for example compare Figs 2D, 4B, C, 5 with Figs 2a, b of Maxwell, 1975). A spherical vesicle also occurs initially in octopod spermatids (Galangau & Tuzet, 1968a; Healy, 1989) but like the Sepiida, Teuthida and *Spirula* (Maxwell, 1975; Healy, 1990), the vesicle subsequently undergoes varying degrees of elongation and internal restructuring.

Like other cephalopods, nuclear condensation in *Vampyroteuthis* involves formation of a fibrous reticulum which later becomes modified into longitudinally orientated thick fibres (Galangau, 1969; Maxwell, 1975; Arnold & Williams-Arnold, 1978; Healy, 1990).

Figs 3A–E. *Vampyroteuthis infernalis*. A: Early stage in development of basal invagination of nucleus. One of the two centrioles and a portion of the axoneme visible (× 21 300). B: Microtubules from both centrioles are inserted into the developing nuclear invagination (× 25 700). C: Microtubules visible in developing nuclear invagination (× 24 400). D: Microtubules originating from the centrioles not only penetrate the nuclear invagination (1) but also form a sheath (2) around the condensing nucleus (× 25 800). E: Oblique longitudinal section showing later stage spermatid than Fig. 3D. Note microtubules penetrating the developing fibrous plug (1) and also surrounding the nucleus (2) (× 21 400). Abbreviations: ax, axoneme; dp, dense (fibrous) plug; c, centriole(s); G, Golgi complex; m, mitochondria; mt, microtubules; n, nucleus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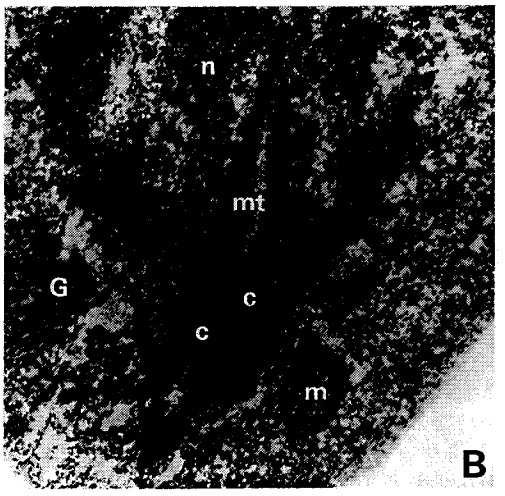
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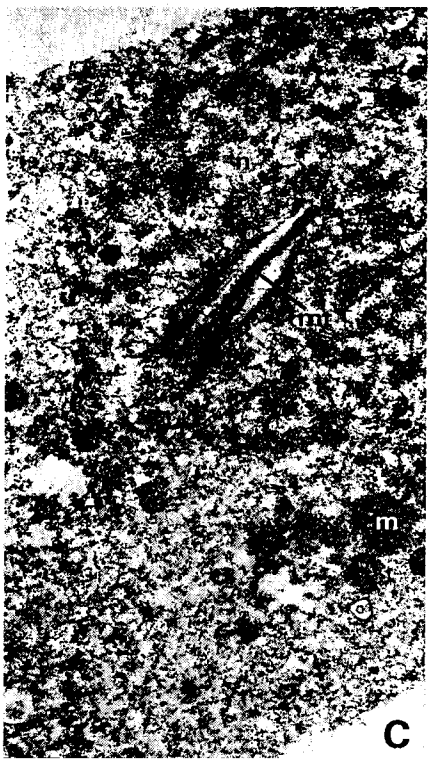
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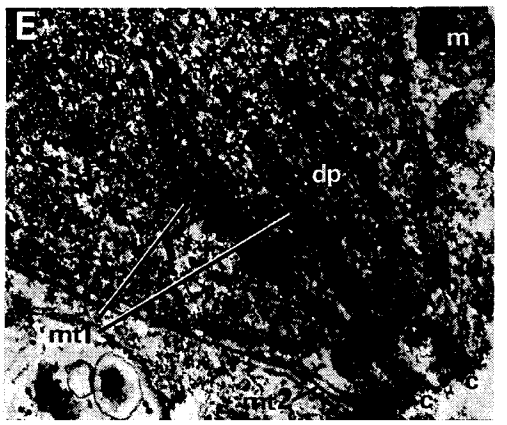
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Nuclear lamellae such as those occurring during late spermiogenesis in many internally fertilizing gastropods (Walker & MacGregor, 1968; Eckelbarger & Eyster, 1981; Healy, 1982a, b, 1988; Kohnert & Storch, 1984; Koike, 1985) have not been observed in *Vampyroteuthis* or any other cephalopod, though Maxwell (1975) refers to the closely-packed fibrous substructure of sepoid and teuthoid sperm nuclei as "pseudolamellar". The presence of a microtubular sheath ("manchette") around the condensing nucleus of mid- and late spermatids of *Vampyroteuthis* appears to be a regular feature of spermiogenesis in cephalopods (see Galangau, 1969; Maxwell, 1975), as indeed it is in many gastropods and other phyla (Franzén, 1988). In *Nautilus* more than one layer of microtubules is present (Arnold & Williams-Arnold, 1978; Tsukahara, 1985). Whether or not perinuclear microtubules perform any role in shaping the sperm nucleus in cephalopods is still debatable. Current opinion seems to favour the view (Fawcett et al., 1971) that the shape of sperm nuclei is controlled genetically rather than by extrinsic forces such as those presumably exerted by cytoplasmic microtubules. Nevertheless, the work of Bergstrom & Arnold (1974) showing spatial alignment of microtubules with chromatin fibres in spermatids of *Loligo pealii* suggests that shaping of the sperm head in some cephalopods may involve a combination of intrinsic (genetic) and extrinsic (in this case, microtubular) factors.

The occurrence in *Vampyroteuthis* of two spermatids developing within a common cytoplasm (and enveloped by a common plasma membrane) has not previously been reported for the Cephalopoda. Grieb & Beeman (1978) noted binucleate secondary spermatocytes in the squid *Loligo opalescens*, but these were cells in a pre-cytokinetic phase and did not produce double spermatids. Within the Mollusca, development of two or more spermatids in a common cytoplasm has been observed in the prosobranch *Turritella communis* (see Franzén, 1955) and in various heterobranch gastropods (Healy, 1982b, unpublished data). Although this phenomenon is probably connected with sperm conjugation in some cases (for example, *Turritella* – Afzelius & Dallai, 1983), conjugated sperm were not observed in *Vampyroteuthis*.

In spermatids and mature sperm of *Vampyroteuthis* and *Nautilus*, two centrioles are retained – each arranged parallel to the longitudinal axis (centrioles of *Nautilus* composed of doublets instead of triplets; see Arnold & Williams-Arnold, 1978; Tsukahara, 1985). In other cephalopods only a single triplet centriole is usually observed (Maxwell, 1974, 1975), though Fields & Thompson (1976) identify two centrioles in spermatozoa of the sepiolid *Rossia pacifica*, and two centrioles initially occur in spermatids of *Octopus* spp. (Galangau, 1969; Healy, 1989). Galangau (1969) suggests that one of the two

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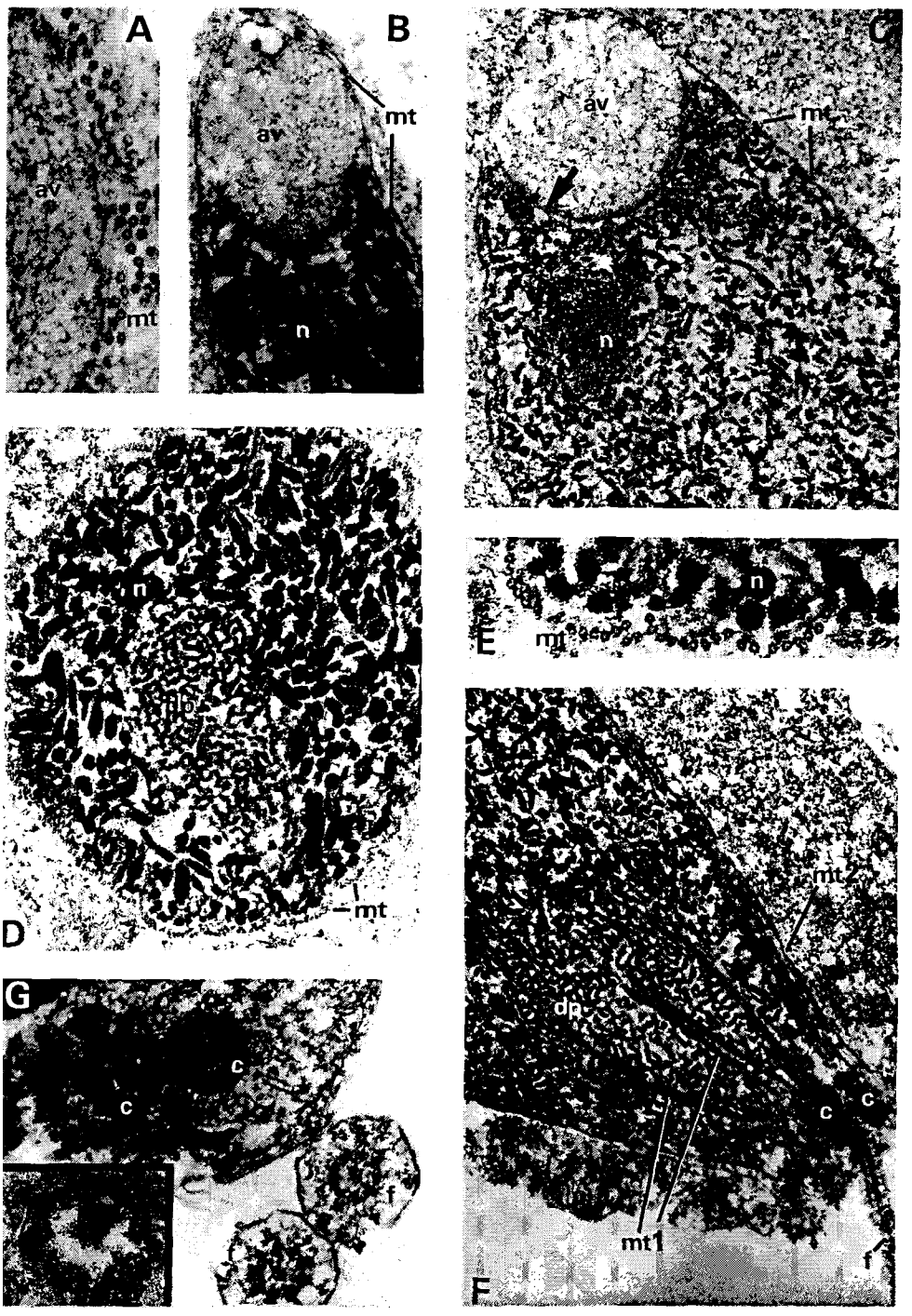
Figs 4A–G. *Vampyroteuthis infernalis*. A: Detail of microtubules (× 67 500). B: Longitudinal section through nuclear apex and acrosomal vesicle of late spermatid. Note surrounding microtubules (× 21 400). C: Acrosome and nuclear apex of late spermatid. Arrow indicates dense basal layer of acrosomal vesicle (× 21 200). D: Transverse section through basal invagination of nucleus showing dense (fibrous) plug, perinuclear sheath (× 27 000). E: Detail of perinuclear microtubule sheath of Fig. 4D (× 67 500). F: Longitudinal section through nuclear invagination, dense plug, centrioles and flagellum of late spermatid. Microtubules originating in centrioles penetrate plug (1) and surround nucleus (2) (× 21 400). G: Midpiece of mature spermatozoon with parallel centrioles visible (× 43 700). Inset: detail of centriole showing triplet substructure (× 70 700). Abbreviations: av, acrosomal vesicle; c, centriole(s); dp, dense plug; f, flagellum; m, mitochondria; mt, microtubules; n, nucleus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spermatid centrioles of *Octopus vulgaris* is transformed into the extensive fibrous plug within the nuclear invagination. As I have recently demonstrated (Healy, 1989), *Vampyroteuthis* produces an almost identical fibrous plug to that of *Octopus* spp. without loss of a centriole (see Fig. 5), suggesting that the plug is probably some form of centriolar adjunct, possibly a modified rootlet. This idea is further supported by the fact that the plug is essentially constructed around a framework of microtubules originating in the centrioles (see Figs 3B, D, E, 4F, 5I). The spermatid centrioles in *Vampyroteuthis*, apart from producing the flagellum, almost certainly give rise to the microtubular sheath surrounding acrosomal and nuclear regions of mid- and late spermatids (see Figs 3D, E, 4F, 5).

Midpiece formation takes place relatively late in spermiogenesis in all cephalopod species that have been examined ultrastructurally. Rather than forming a mitochondrial spur (as in Sepiida and Teuthida – Maxwell, 1975; Healy, 1989b, 1990), a periflagellar mitochondrial sleeve (*Spirula* – Healy, 1990) or a periaxonemal sheath (octopods – Galangau & Tuzet, 1968b; Maxwell, 1974; Healy, 1989, 1990), mitochondria in *Vampyroteuthis* spermatids cluster around the centriolar pair at the base of the nucleus (see Fig. 5). The same pattern of midpiece formation occurs in molluscs which fertilize externally such as scaphopods, bivalves and archaeogastropods (Longo & Dornfeld, 1967; Koike, 1985; Healy unpublished data). It would be interesting to determine the environment of fertilization of *Vampyroteuthis* bearing in mind that it, like other cephalopods, produces spermatophores.

Systematic considerations

Retention of several primitive features in *Vampyroteuthis* spermatozoa (spherical acrosomal vesicle, short nucleus, pericentriolar mitochondria, two triplet substructure centrioles; Healy, 1989) suggests that the Vampyromorpha developed early in the evolution of the Coleoida (see also Bandel & Leich, 1986 for a discussion of fossil vampyromorphs). The presence of a large, initially fibrous plug in the nuclear invagination of spermatids and spermatozoa of *Vampyroteuthis* and *Octopus* (absent in other coleoids and in *Nautilus*) is here considered strong supporting evidence for a close phylogenetic link between the Vampyromorpha and the Octopoda. However, sperm morphology, backed by general anatomy, also indicates a need to continue recognition of

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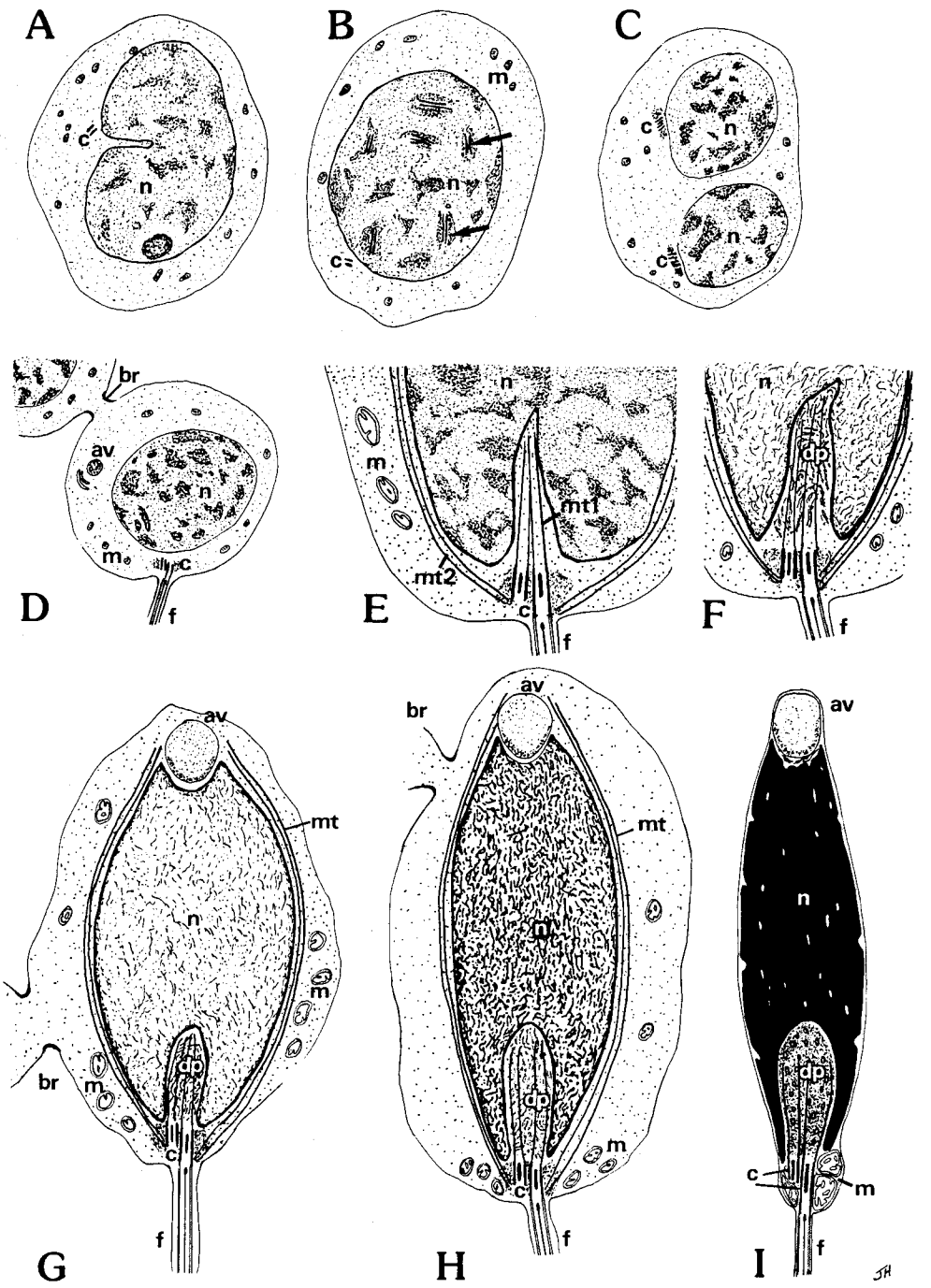
Figs 5A–I. *Vampyroteuthis infernalis*. Summary of spermiogenesis in *Vampyroteuthis* (semi-diagrammatic). A: Spermatogonium showing lobulate nucleus, nucleolus and centriole (× 2700). B: Spermatocyte showing chromosome cores (arrows) (× 2700). C: Two spermatid nuclei in common cytoplasm. Further development of spermatids without complete cytokinesis is commonly observed in *Vampyroteuthis* (× 2700). D: Early spermatid showing flagellum and cytoplasmic bridge (× 2700). F: Development of nuclear invagination and dense plug within the invagination. Microtubules originating from the centrioles penetrate the nuclear invagination (1) and also form a perinuclear sheath (2). Fibrous material gradually fills and inflates the nuclear invagination to form the dense plug (× 13 500). G, H: The head region of advanced spermatids showing position of centrioles, acrosomal vesicle, mitochondria and developing dense plug. Cytoplasmic bridges persist late into spermiogenesis (× 7000). I: Mature spermatozoon (after Healy, 1989). Nucleus and dense plug both show cavities. Note pericentriolar midpiece (× 7000). Abbreviations: av, acrosomal vesicle; br, cytoplasmic bridge(s); c, centriole(s); dp, dense plug; f, flagellum; m, mitochondria; mt, microtubules (1: associated with dense plug. 2: perinuclear); n, nucleus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the Vampyromorpha as a distinct coleoid order and not, as proposed by Fioroni (1981), as a close affiliate of the Sepiida, Teuthida and Sepiolida.

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