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Sperm ultrastructure of the Actinocyclidae (Mollusca, Nudibranchia) and homology of the terminal region of nudibranch sperm

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Summary

Sperm ultrastructure is examined and described for the actinocyclidid nudibranchs *Actinocyclus verrucosus*, *Hallaxa iju* and *Hallaxa indecora*. Although general characteristics were consistent with previously described heterobranch observations, present investigations revealed ultrastructural synapomorphies for the family based on the morphology of the terminal region of the spermatozoon. In actinocyclidids, the axonemal microtubules penetrate for some distance beyond the annulus, and the annular accessory body elongates to completely seal the terminal region. *Chromodoris* also has an annular accessory body that completely seals the axoneme and terminal region, but it does not extend far beyond the annulus, and it is possible that these states were derived independently. Cytochemical staining confirmed that there was no glycogen present in the posterior region of the sperm for *H. indecora* or *Chromodoris kuniei*. However, representatives of other chromodoridid genera (*Noumea*, *Risbecia*) have an axoneme that penetrates through the entire annular complex, after which it is sheathed by a glycogen deposit. Similarities in the acrosomal complex support the proposed sister group relationship between the Actinocyclidae and Chromodorididae.

Key words: Opisthobranchia, spermatozoa, glycogen staining, Chromodorididae

Introduction

The Actinocyclidae consists of two doridoidean genera, *Actinocyclus* Ehrenberg, 1831 and *Hallaxa* Eliot, 1909. These genera were included in the cryptobranch Doridiidae (Eliot, 1910) or the more exclusive Glossodorididae (= Chromodorididae) (Thiele, 1931), but were later removed to a new family Actinocyclidae by Pruvot-Fol (1934). Recent taxonomic reviews of *Actinocyclus* (Valdés, 2002b) and *Hallaxa* (Gosliner and Johnson, 1994) have been useful in understanding the morphological variation within these genera. The latter study suggested both genera are monophyletic, but focused on delineating the two genera, and did not identify any morphological synapomorphies that unite the Actinocyclidae.

Gosliner and Johnson (1994) suggested the Actinocyclidae + Chromodorididae diverged at an early stage from other cryptobranch dorids, and this has been

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supported by a more recent phylogenetic analysis (Valdés, 2002a). As many of the doridinean relationships remain unresolved (Valdés and Gosliner, 2001; Valdés, 2002a), the actinocyclidid–chromodoridid clade plays an important role in reconstructing doridinean phylogeny. Comparative studies of sperm ultrastructure in chromodoridids (Wilson and Healy, 2002a) and related dorids (Healy and Willan, 1991; Wilson and Healy, 2002b; Fahey and Healy, 2003; Wilson, 2003) have uncovered potential characters for phylogenetic analyses. In this study, the sperm ultrastructure of two genera of the Actinocyclidae is described, and reveals morphology in the terminal region not seen elsewhere in the Doridoidea. This necessitated further comparison to other chromodorid terminal regions, using cytochemical staining, to assess the homology and polarity of ultrastructural characters in this region.

Materials and Methods

Actinocyclus verrucosus Ehrenberg, 1831, *Hallaxa iju* Gosliner and Johnson, 1994 and *Hallaxa indecora* (Bergh, 1905) (first record in Australia) were collected from a rocky intertidal platform at Point Cartwright, Mooloolaba (26°41′S, 153°07′E), Queensland, Australia, in 2000 and 2001. These three specimens are deposited at the South Australian Museum, Adelaide and the Australian Museum, Sydney (respectively, SAM D19274, AM C203858 and SAM D19275). Live animals were anaesthetized in chilled seawater. Sperm from the ampulla of *Actinocyclus verrucosus* were fixed directly in chilled 3% glutaraldehyde (prepared in 0.1M sodium phosphate buffer, pH 7.2 and 10% w/v sucrose). Both *Hallaxa* species were placed whole in chilled glutaraldehyde with the dorsum cut open to allow fixative penetration. Subsequently sperm-filled ampullae were dissected out. Initial fixation was carried out at 4°C for at least 12 h.

All tissue was further prepared for transmission electron microscopy (TEM) using a Lynx EL automated processor. This involved buffer rinses, fixation in 4% osmium tetroxide, further buffer rinses and dehydration through an ascending series of ethanols. Exact details of the protocol are given in Wilson and Healy (2002a). Tissues were embedded in Spurr's resin and ultrathin sections were cut using a diamond knife and LKB 2088 Ultratome V. Ultrathin sections were collected on 200-μm uncoated mesh copper grids and stained with the modified lead staining method of Daddow (1986).

As the morphology of the actinocyclids examined here differed considerably from other nudibranchs, cytochemical staining for glycogen was carried out on *H. indecora*, and the chromodoridids *Risbecia pulchella* (Rüppell and Leuckart, 1828) (Natal Museum V8251), *Noumea haliclona* (Burn, 1957) (SAM D19269) and *Chromodoris kuniei* Pruvot-Fol, 1930 (SAM D19261). These additional chromodoridids were fixed and sectioned as above, and full descriptions of their sperm ultrastructure will be published elsewhere in a more comprehensive review of chromodoridid sperm. To assess the distribution of glycogen, the thiocarbohydrazide-silver proteinate method of Thiery (1967) was used. Ultrathin sections were collected on uncoated gold grids and treated with 1% aqueous periodic acid for 30 min, followed by distilled water washes. The grids were then floated on 2% thiocarbohydrazide in 20% acetic acid for 2 h. This was followed by a 20% acetic acid wash, and a graduated series of more dilute washes, finishing with distilled water. They were then stained by a 1% aqueous silver proteinate solution for 30 min in the dark, and washed with distilled water. All grids were examined on a JEOL 1010 TEM operated at 80 kV. Measurements were taken from digitized images in the Matrox Inspector 4. Mean midpiece length was determined from light microscopy by subtracting the mean TEM measurements for the acrosomal complex, nucleus and terminal region from whole sperm measurements. The variation in organelle measurements within an individual is represented by standard deviation when five or more representative organelles were measured.

Results

Sperm ultrastructure

Actinocyclus verrucosus *Ehrenberg, 1831*

Acrosomal complex: The acrosomal vesicle is slightly ovoid, measuring 0.11 μm (±0.01, n = 5) in length (Fig. 1A). The vesicle rests in a shallow depression at the anterior end of the acrosomal pedestal. The acrosomal pedestal is short and conical and measures 0.22 μm in length (±0.03, n = 5). Longitudinal sections reveal fine, parallel striations oriented parallel to the transverse plane (Fig. 1A). There is only a shallow overlap of 0.02 μm (n = 2) between the nucleus and the pedestal.

Nucleus: The nucleus of *A. verrucosus* measures 4.63 μm in length (n = 4) and is usually electron-dense. Some nuclei were partly decondensed, showing chromatin fibres (Fig. 1B). The nucleus is helical in shape (Fig. 1B,C). At the base of the nucleus, an invagination is filled by a bell-shaped centriolar derivative that is continuous with the axoneme/coarse fibre complex (Fig. 1E).

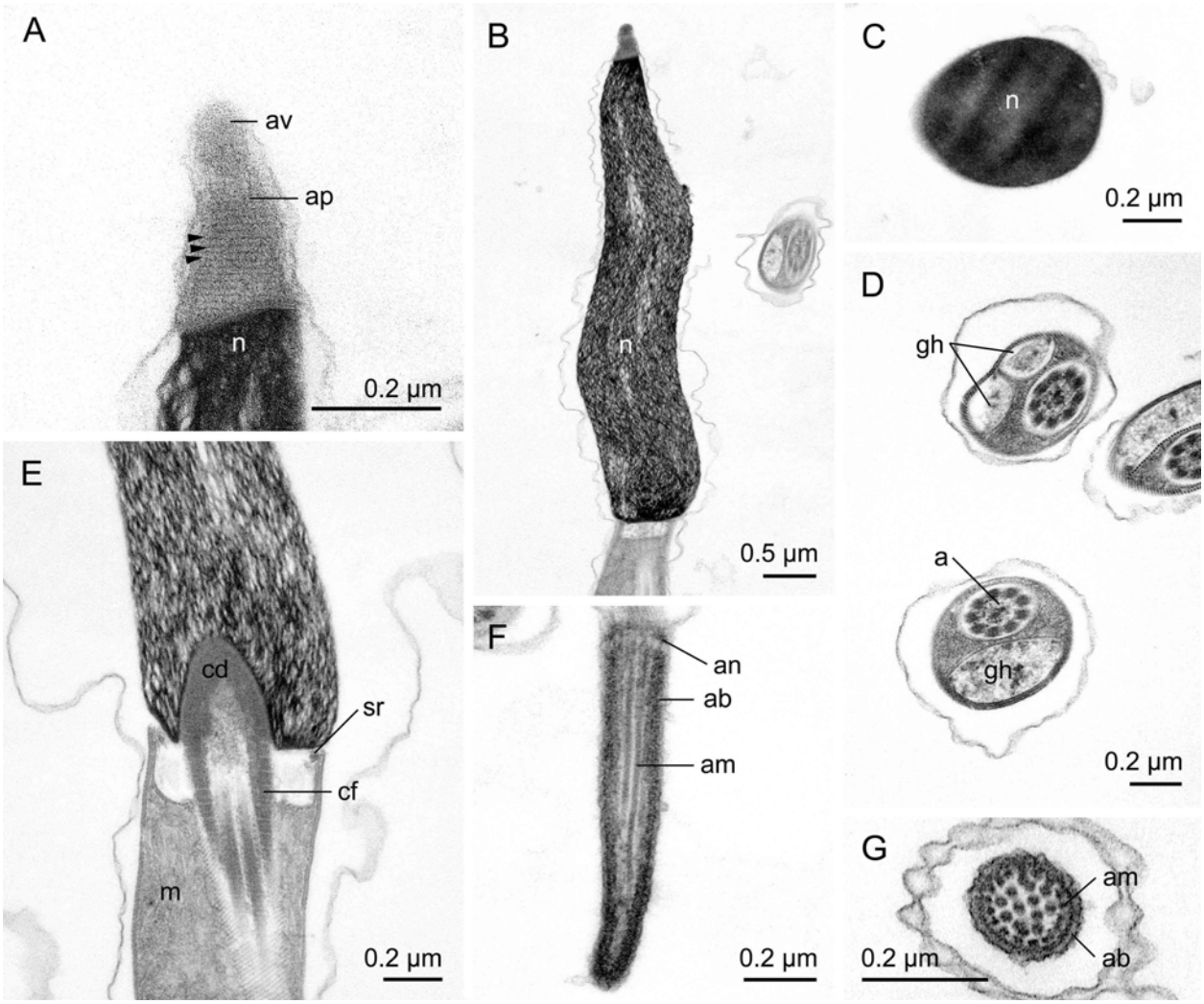

Fig. 1. Sperm ultrastructure of *Actinocyclus verrucosus*. A. Longitudinal section (LS) through acrosomal complex (acrosomal vesicle + acrosomal pedestal) and nuclear apex. Note fine striations of pedestal. B. LS acrosomal complex, nucleus and anterior extremity of midpiece. C. Transverse section (TS) through nucleus. D. TS midpieces showing both one and two glycogen helices within mitochondrial derivative. E. LS of the nucleus/midpiece junction (neck region) showing the centriolar derivative (in nuclear invagination), subnuclear ring, striated coarse fibres and anterior extremity of mitochondrial derivative. F. LS posterior extremity of midpiece and entire terminal region. Note annular complex (annulus + annular accessory body) and deep penetration of axonemal microtubules into terminal region. G. TS terminal region, axoneme with an additional circlet of microtubules. a, axoneme; ab, annular accessory body; am, axonemal microtubules; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; das, distal accessory sheath; gh, glycogen helix; m, mitochondrial derivative; n, nucleus; sr, subnuclear ring.

Midpiece: The midpiece consists of the axoneme/coarse fibre complex enveloped by a mitochondrial derivative (Fig. 1E). The coarse fibres that surround the axoneme are transversely striated, repeating at 43 nm (n = 1). The midpiece is approximately 207 µm in length (±6.16, n = 10). There is a subnuclear ring present where the nucleus and the anterior extremity of the mitochondrial derivative meet (Fig. 1E). Similarly, there is some material present where the axoneme/coarse fibre complex meets the most anterior part of the mitochondrial derivative. There was no evidence of secondary helices, formed by lateral extension of the mitochondrial derivative. Two glycogen helices were present, although single glycogen helices were more abundant (Fig. 1D).

Terminal region: Posterior to the midpiece is the annulus, below which the axoneme continues for some distance (Fig. 1F). The annular accessory body extends for 1.11 µm (n = 3), enveloping and sealing off the axonemal microtubules. The 9+2 pattern of micro-

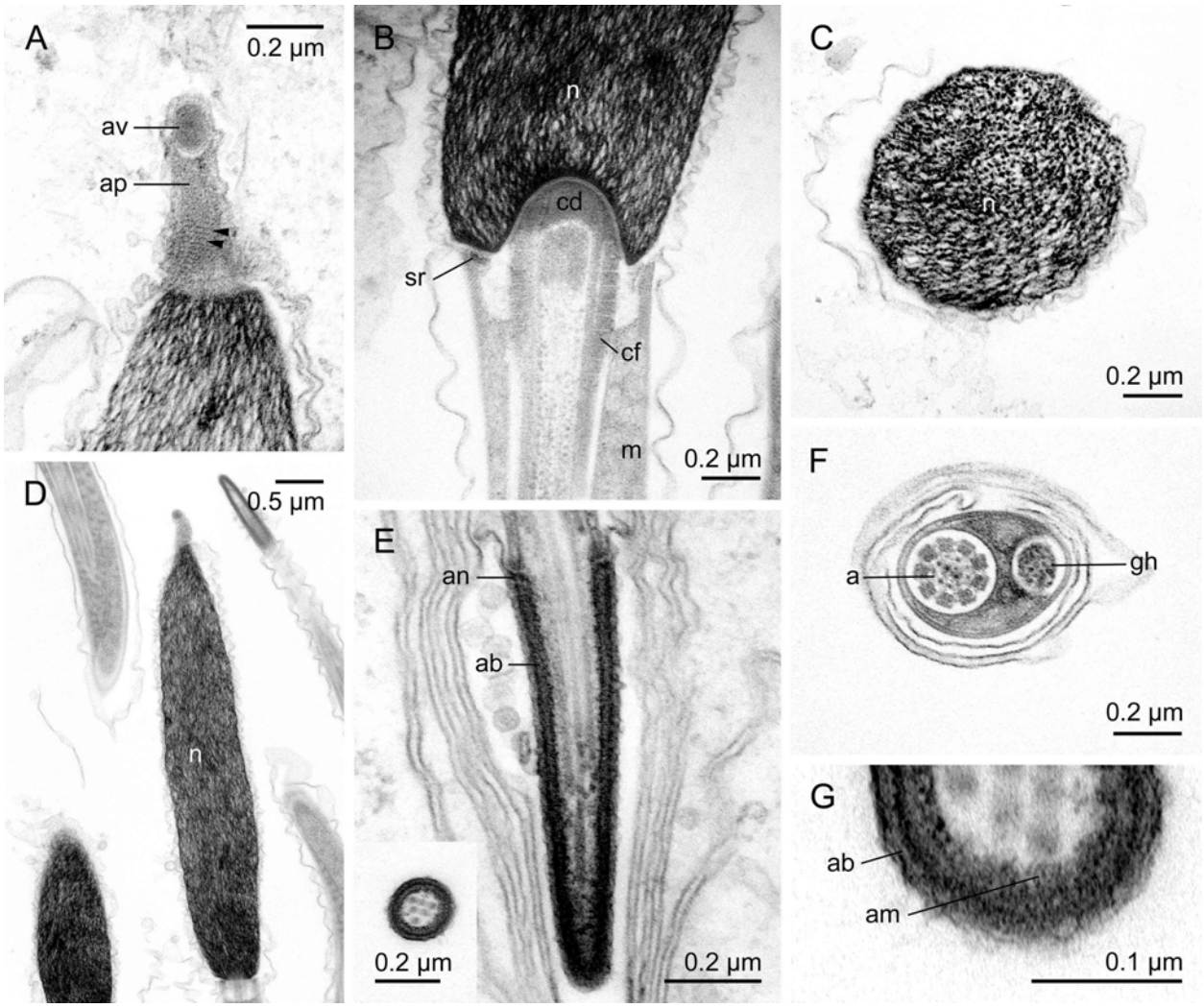

Fig. 2. Sperm ultrastructure of *Hallaxa iju*. A. Longitudinal section (LS) through the acrosomal complex and nuclear apex. Note fine striations of pedestal. B. LS of the nucleus/midpiece junction (neck region). C. Transverse section (TS) through nucleus. D. LS acrosomal complex, nucleus and anterior extremity of midpiece. E. LS posterior extremity of midpiece and entire terminal region showing annular complex (annulus + annular accessory body). Inset: TS terminal region showing axonemal microtubules. F. TS midpiece showing single glycogen helix. G. TS detail of the wall of the terminal region, showing additional circlet of axonemal microtubules on the innermost side of the annular accessory body. a, axoneme; ab, annular accessory body; am, axonemal microtubules; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; das, distal accessory sheath; gh, glycogen helix; m, mitochondrial derivative; n, nucleus; sr, subnuclear ring.

tubules is still visible, although an extra circlet of microtubules is also visible in transverse section (Fig. 1G).

Hallaxa iju *Gosliner and Johnson, 1994*

Acrosomal complex: The acrosomal vesicle in *H. iju* is ovoid and measures 0.12 µm in length (±0.01, $n = 9$). It rests in a shallow depression at the anterior end of the short, conical pedestal (Fig. 2A). The pedestal measures 0.34 µm in length (±0.05, $n = 10$) and is slightly bent anteriorly (not shown). Longitudinal sections reveal fine, angular, parallel striations through the pedestal (Fig. 2A). There is a shallow overlap of 0.02 µm ($n = 3$) between the nucleus and the pedestal.

Nucleus: All nuclei were slightly bloated and fibrous, showing little sign of ornamentation (Fig. 2C, D). The nucleus measured 4.67 µm (±0.23, $n = 7$). At the base of the nucleus, an invagination is filled by a rounded centriolar derivative that is continuous with the axoneme/coarse fibre complex (Fig. 2B).

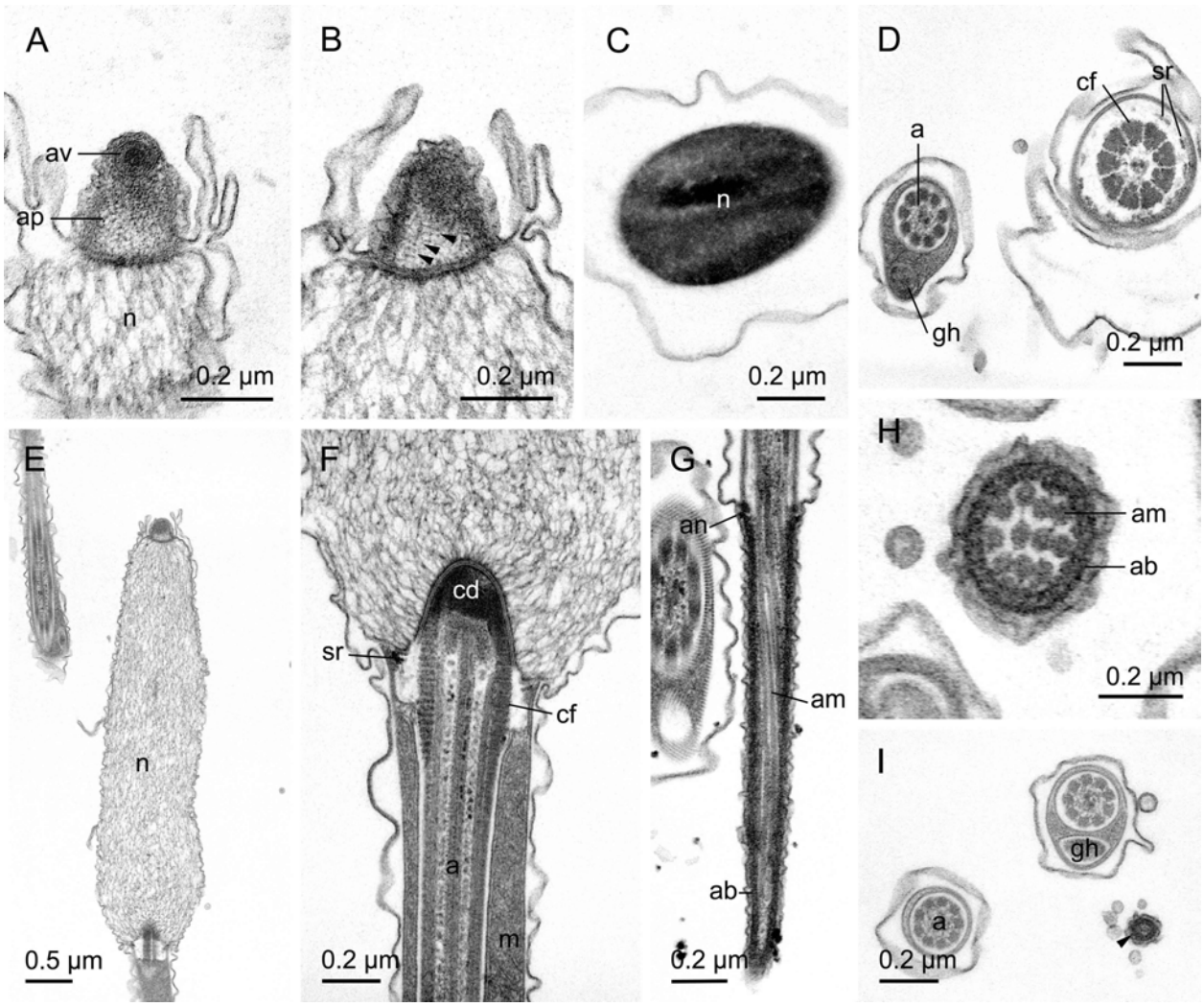

Fig. 3. Sperm ultrastructure of *Hallaxa indecora.* A,B. Longitudinal sections (LS) through the acrosomal complex and nuclear apex. Note fine striations (see arrows) of pedestal. C. Transverse section (TS) through nucleus. D. TS anteriormost region of midpiece (at left) showing nine coarse fibres associated with axonemal doublets, continuous structure of subnuclear ring lining anterior extremity of mitochondrial derivative; (at right) TS through midpiece showing single glycogen helix. E. LS acrosomal complex, nucleus and anterior extremity of midpiece. F. LS of the nucleus/midpiece junction (neck region). G. LS posterior extremity of midpiece and entire terminal region showing annular complex. H. TS terminal region. I. TS of midpieces and tip of terminal region. a, axoneme; ab, annular accessory body; am, axonemal microtubules; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; gh, glycogen helix; m, mitochondrial derivative; n, nucleus; sr, subnuclear ring.

Midpiece: The midpiece length was not determined. The coarse fibres that surround the axoneme are transversely striated, with an undetermined periodicity (Fig. 2B). A subnuclear ring is present. There is no evidence of any secondary helices and only a single glycogen helix was observed in the midpiece (Fig. 2F).

Terminal region: The axoneme continues past the termination of the midpiece with the annulus (Fig. 2E). The annular accessory body surrounds and seals off the axonemal microtubules, and measures 0.91 µm in length (±0.06, n = 5). The 9+2 pattern of microtubules is still visible, although an extra circlet of microtubules is also visible in transverse section (Fig. 2G).

Hallaxa indecora *(Bergh 1905)*

Acrosomal complex: The spherical acrosomal vesicle rests in a shallow depression at the anterior end of the short, conical acrosomal pedestal (Fig. 3A). The vesicle is 0.08 µm in diameter (±0.01 µm, n = 12), while the pedestal measures 0.15 µm in length (±0.03, n = 11). Longitudinal sections reveal fine, angular,

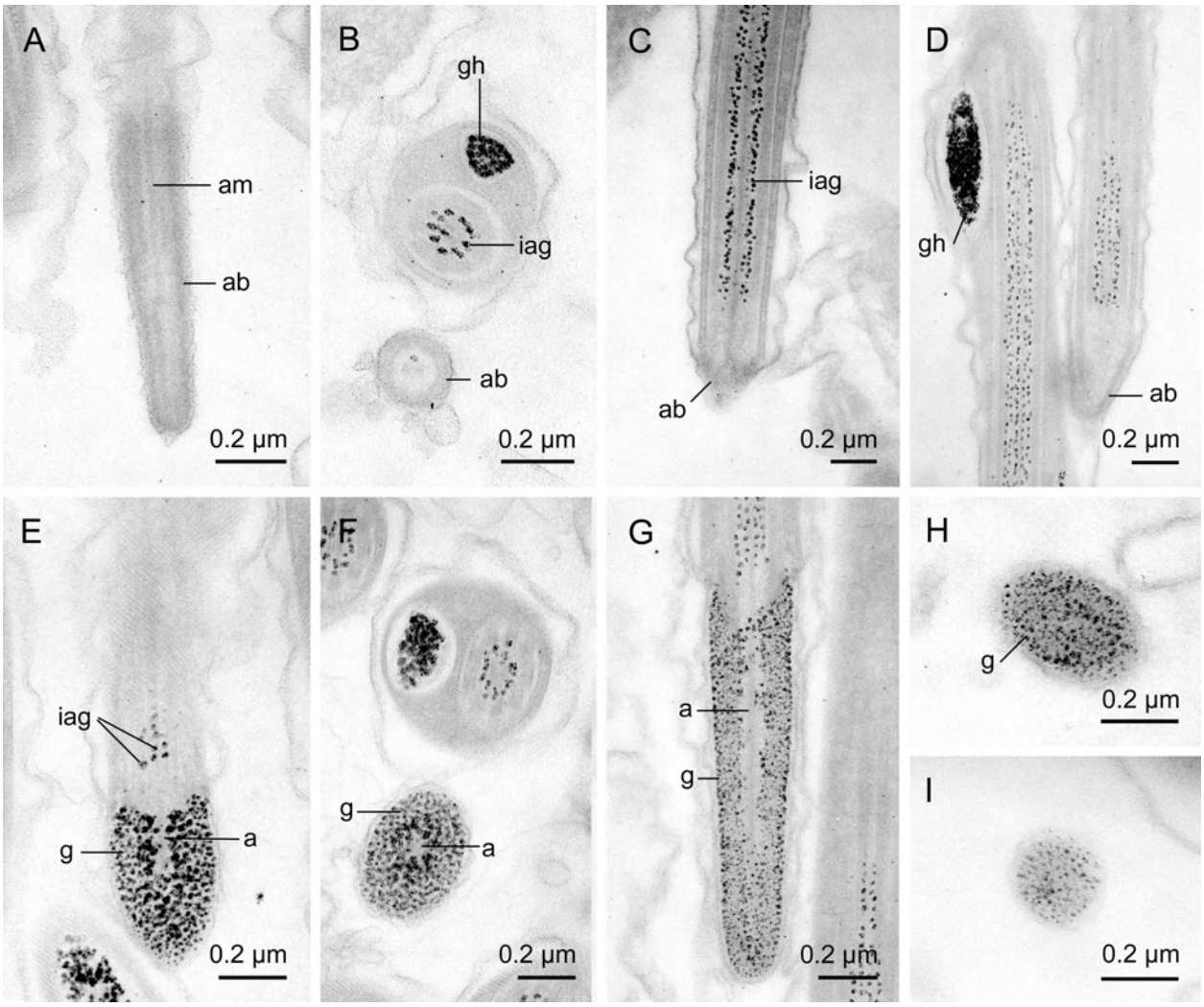

Fig. 4. Distribution of glycogen within the midpiece and the terminal region as determined by thiosemicarbazide silver proteinate. A,B. *Hallaxa indecora*. A. Longitudinal section (LS) entire terminal region, showing absence of glycogen granules. B. Transverse section (TS) through midpiece (upper), note glycogen in the glycogen helix and axoneme; (lower) TS through terminal region (glycogen absent). C,D. *Chromodoris kuniei*. C. LS posterior midpiece (note intra-axonemal glycogen) and entire terminal region (glycogen absent). D. LS midpiece (left) showing glycogen in helix and axoneme; (right) terminal region. E,F. *Noumea haliclona*. E. LS posterior midpiece (note intra-axonemal glycogen) and entire terminal region (thick deposit of glycogen granules around axoneme). F. TS midpiece (upper) showing intra-axonemal glycogen and glycogen within helix and (lower) terminal region of sperm. G–I. *Risbecia pulchella*. G. LS midpiece, both intra-axonemal glycogen and terminal glycogen deposit visible. H,I. TS through anterior and posterior region of terminal region. a, axoneme; ab, annular accessory body; am, axonemal microtubules; g, glycogen; gh, glycogen helix; iag, intra-axonemal glycogen.

parallel striations throughout the pedestal (Fig. 3B). There is no measurable overlap between the nucleus and the pedestal.

Nucleus: Almost all nuclei were fibrous and inflated and thus revealed no sign of helices (Fig. 3C,E). The nucleus measured 4.35 µm ($n = 4$). At the base of the nucleus, a shallow invagination is filled by a bell-shaped centriolar derivative that is continuous with the axoneme/coarse fibre complex (Fig. 3F).

Midpiece: Light microscopy gives a total length for the midpiece at 191 µm (±2.76, $n = 10$). The coarse fibres that surround the axoneme are transversely striated, repeating at 33–38 nm ($n = 3$). A subnuclear ring is present, and there is some similar material present where the axoneme/coarse fibre complex meets the anterior region of the mitochondrial derivative (Fig. 3F). No secondary helices were observed, and only a single glycogen helix was incorporated into the mitochondrial derivative (Fig. 3D,I).

Terminal region: The axoneme continues past the

termination of the midpiece with the annulus (Fig. 3G). The annular accessory body surrounds and seals off the axonemal microtubules, and measures 1.55 μm in length (n = 3) (Fig. 3G,H,I).

Cytochemical comparisons of the "glycogen piece"

Comparative cytochemical staining was carried out on *H. indecora* (Actinocyclidae) and three species of the Chromodorididae (*C. kuniei*, *N. haliclona* and *R. pulchella*) in order to determine the presence and distribution of glycogen deposits within the midpiece and terminal region. No glycogen was observed in the terminal region of *H. indecora* (Fig. 4A,B) or *C. kuniei* (Fig. 4C,D), and in both of these species the annular accessory body seals off the axoneme. However, intra-axonemal glycogen (between the central singlet tubules and nine peripheral doublets) and glycogen arranged in helices were observed in all species within the midpiece region. Substantial glycogen deposits were observed in the terminal region of *N. haliclona* (Fig. 4E,F) and *R. pulchella* (Fig. 4G,H,I), where the axoneme continued past the annular accessory body.

Discussion

The characteristics of actinocyclidid sperm are consistent with those of generalized chromodoridid sperm as outlined by Wilson and Healy (2002a). The acrosomal complex in the Actinocyclidae consists of a generally ovoid acrosomal vesicle and a short, conical acrosomal pedestal — the latter exhibiting fine internal striations. The presence of fine striations was not altogether unexpected, given their occurrence in the acrosomal pedestals of other investigated chromodoridids (Medina et al., 1985; Healy and Willan, 1991; Wilson and Healy, 2002a) and a number of other dorid genera (e.g., *Rostanga* — see Healy and Willan, 1991; *Aphelodoris* — see Wilson, 2003). It is difficult to assess with certainty the extent to which fine internal striations of the pedestal have been overlooked in nudibranchs (because of their often cryptic nature in standard TEM preparations). In several cases the pedestal material appears to be genuinely homogeneous (Healy and Willan, 1991; Wilson and Healy, 2002b), but in my experience striations may only be observed sporadically, when sectioned at favourable angles, and it is entirely possible that several other doridinean taxa may show some form of internal organization within the pedestal. Unfortunately, nothing is known of the cytochemistry of the acrosomal complex in heterobranch gastropods. However, it is still interesting to note that, despite many studies of heterobranch sperm, transverse striations of the acrosomal pedestal have yet to be observed outside the Nudipleura (= Nudibranchia + Pleurobranchoidea).

In general, the morphology of the mitochondrial derivative is as observed in other nudibranchs (Medina et al., 1988; Healy and Willan, 1991; Wilson and Healy, 2002a, 2002b) and more generally most other heterobranchs (for comparative work, see Anderson and Personne, 1970, 1976; Healy, 1983, 1988, 1993, 1996; Maxwell, 1983; Hodgson and Healy, 1998): that is, a continuous helical sheath for the axoneme/coarse fibre complex, composed of paracrystalline and matrix layers and containing one or more glycogen helices. Chromodoridids often have secondary helices elaborated from the mitochondrial derivative (Healy and Willan, 1991; Wilson and Healy, 2002a), but these structures are absent in all three actinocyclidids examined here. Using the Thiery (1967) cytochemical test for glycogen, our study confirms the presence of glycogen granules in nudibranch sperm (see also Medina et al., 1988), not only within the axoneme but also within the glycogen helix in species tested here.

Wilson and Healy (2002a) drew attention to the complex construction of the annular complex in chromodoridid sperm: the highly electron-dense annulus proper (a simple ring closely bonded to the inner surface of the plasma membrane at the midpiece–glycogen piece junction) and a slightly less electron-dense collar, clearly associated with the annulus but discernible from it. Re-inspection of the considerable literature on heterobranch sperm (for listing, see works cited in Wilson and Healy, 2002a, 2006b, as well as in this account) reveals that the annular complex is probably universal among opisthobranchs and at least the basommatophoran pulmonates (the precise condition in stylommatophorans being uncertain — see Giusti et al., 1991). The most fundamental spermatozoal difference between the Actinocyclidae and Chromodorididae is the morphology of the annular accessory body, at the terminal region of the sperm. Chromodoridids, like other doridineans, tend to have axonemal microtubules that penetrate beyond the annular complex, after which it is sheathed by a coarsely granular glycogen deposit (Healy and Willan, 1991a; Wilson and Healy, 2002a). Published observations on the sperm of two *Chromodoris* species (and unpublished observations on another) indicate that this genus differs from other chromodoridids in having the axoneme terminate at the annulus. This leaves only the annular accessory body and plasma membrane to constitute the terminal region of the spermatozoon, and there is an absence of recognisable glycogen deposits in the post-annular region (Healy and Willan, 1991a; Wilson and Healy, 2002a). As the axonemal micro-

tubules in the terminal region of actinocyclidid sperm are strongly visible, and packed around the inner edge of the annular accessory body, it was not possible in standard TEM sections to determine if a thin layer of tightly packed glycogen granules was also present. Our cytochemical observations reveal that in the actinocyclidid *Hallaxa indecora*, as in *Chromodoris kuniei*, only the annular accessory body and plasma membrane form the terminal region of the spermatozoon (that is, post-annular glycogen deposits are absent). However, actinocyclidids differ conspicuously from *Chromodoris* spp. in the persistence and proliferation of axonemal microtubules beyond the annulus, and in the markedly elongate annular accessory body (to date the longest known in any heterobranch group). In actinocyclidids, the annulus proper occurs above the annular accessory body, while in chromodoridids it is adjacent to the accessory body.

The similarity between the actinocyclidid and chromodoridid acrosomal complexes supports the sister group relationship proposed by Gosliner and Johnson (1994). However, based on our current understanding of cryptobranch phylogeny, it appears that the terminally-acting annular accessory body of actinocyclidids and *Chromodoris* is independently derived. Other genera in the Chromodorididae that are usually considered basal and may help resolve the issue are *Cadlinella*, *Cadlina* and *Tyrinna* (Rudman, 1984; Gosliner and Johnson, 1999). However, *Cadlinella* shows spermatozoal features (a longitudinally inrolled acrosomal pedestal, a possible autapomorphy for this genus — see Wilson and Healy, 2002b) not consistent with other nudibranchs, while *Cadlina* and *Tyrinna* have yet to be investigated spermatologically.

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