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Sperm ultrastructure of *Microhedyle remanei*, an interstitial acochlidian gastropod with dermal fertilization

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The ultrastructure of sperm of an acochlidian opisthobranch is described for the first time in detail, in the tiny, gonochoristic *Microhedyle remanei* (Microhedylidae) from Bermuda. Transmission electron microscopy (TEM) shows the spermatozoa of *M. remanei* sharing many plesiomorphic features with opisthobranch gastropods, such as having an apical spiral nucleus with a basal invagination filled with a bell-shaped centriolar derivative; there is a single glycogen helix embedded into matrix and paracrystalline material. Sperm of *M. remanei* are characterized by possessing a strongly helically coiled nucleus with up to five keels terminating into one very prominent and intertwined keel. The sperm midpiece shows the glycogen helix which is very densely arranged. This clearly differs from *Hedylopsis ballantinei*, a member of the related acochlidian family Hedylopsidae, where three glycogen helices with different lengths and a probably much shorter nucleus are present. This variation among acochlidian sperm may be phylogenetically relevant and may be related to special acochlidian reproductive features such as sperm transfer via hypodermic injection or dermal fertilization via spermatophores.

INTRODUCTION

The Acochlidia are extraordinary and poorly known opisthobranch gastropods. Most of the 27 acochlidian species known worldwide are marine mesopsammic, living in the interstices of sand. Uniquely among opisthobranchs, a few acochlidian species secondarily succeeded in inhabiting brackish or freshwater systems. Likely evolutionary adaptations to mesopsammic conditions refer to miniaturization, worm-like body shapes, loss of shell and reductions of many organs (Swedmark, 1971; Arnaud et al., 1986; Westheide, 1987). The most intriguing morphological discrepancies compared to typical opisthobranchs, however, concern the reproductive system. The Acochlidia combine a variety of special reproductive features. Several Hedylopsidae possess penial papilla with apical hollow stylets which in Acochlidiidae may be modified into giant papillae armed with several rows of cuticular spines. At least in some species, sperm is transferred by hypodermic impregnation rather than by (reciprocal) copulation, which is the usual method among the generally hermaphroditic opisthobranchs (Swedmark, 1968; Wawra, 1992). Acochlidian species of the families Asperspinidae, Ganitidae and Microhedylidae including *Microhedyle remanei* Marcus, 1953 have completely lost the copulatory organs; here, sperm transfer occurs via spermatophores placed anywhere on the body wall ('dermal' fertilization; see e.g. Wawra, 1992; Morse, 1994); members of the latter two families secondarily show separate sexes (Sommerfeldt & Schrödl, 2005; Neusser et al., 2006). The development of such unique reproductive features and modes of sperm transfer played a key role in acochlidian evolution (Schrödl & Neusser, unpublished) and are likely to be reflected also in sperm structure showing special adaptations.

Some light microscopic information on sperm morphology suggests that all gonochoristic acochlidians plus the hermaphroditic Asperspinidae, possess slender sperm with an elongate spiral head, while all other hermaphroditic acochlidians, have sperm with short, pear-shaped heads (see review by Wawra, 1987). Sommerfeldt & Schrödl (2005) reported that sperm of two *Hedylopsis* species show short but slender heads; their first transmission electron microscopical (TEM) data on spermatozoa of the acochlidian *Hedylopsis ballantinei* was, however, limited to the sperm midpiece.

Up to now the origin and phylogeny of the Acochlidia could not be resolved by morphological or molecular data (see Vonnemann et al., 2005). Sperm ultrastructure might offer an additional and powerful data set, which has generally proved to be useful to study molluscan relationships (Healy, 1996). According to Thompson (1973), Healy (1983) and Healy & Willan (1984), euthyneuran spermatozoa are characterized by the morphology of the acrosomal complex, the almost always helically coiled nucleus and a complex mitochondrial derivative consisting of paracrystalline and matrix materials surrounding a central axoneme, periaxonemal coarse fibres and one or more glycogen-filled helices. All relevant authors have emphasized the high variability within opisthobranch spermatozoa and the importance of sperm ultrastructure as a useful indicator of systematic affinities. Nevertheless, beside some approaches on for example the retusiid bullomorphs (Healy, 1982; Berry et al., 1992) and on the anaspidean genus *Aplysia* (see Kubo & Ishikawa, 1981; Vita et al., 2001), recent comparative studies with detailed

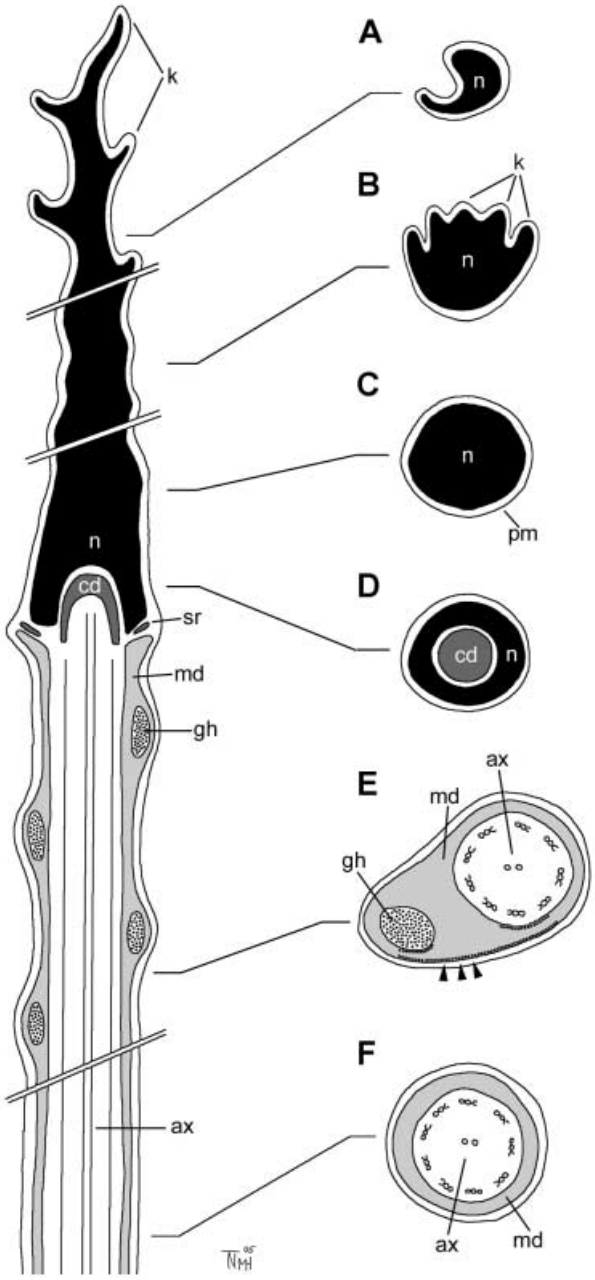

Figure 1. Schematic drawing of spermatozoa of *Microhedyle remanei*; longitudinal section (left), cross sections (right). (A) Apical part of the nucleus with helical keel; (B) mid-region of the nucleus; (C) basal region of the nucleus; (D) basal region of the nucleus invaginated by the centriolar derivative; (E) midpiece with 2 × 9+2 axoneme and one glycogen helix; and (F) midpiece posterior to glycogen helix. ax, axoneme; cd, centriolar derivative; gh, glycogen helix; k, keel; md, mitochondrial derivative; n, nucleus; pm, plasma membrane; sr, subnuclear ring; arrowheads, paracrystalline material.

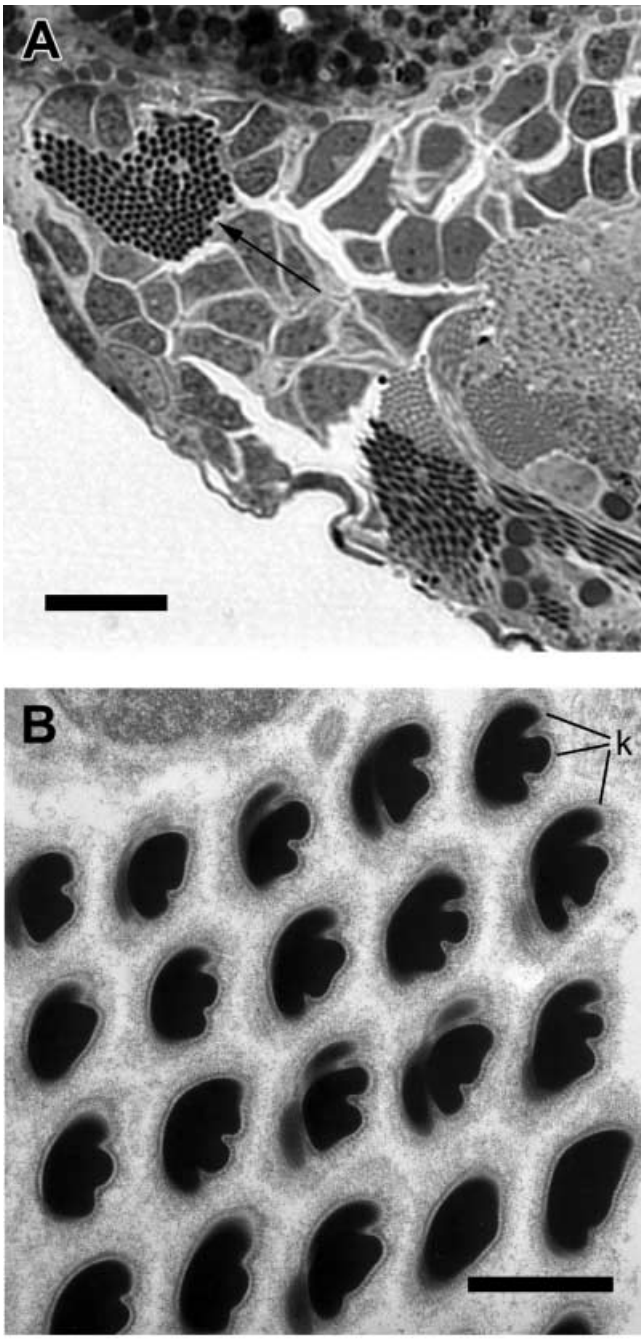

Figure 2. Densely packed sperm cells in the testis of *Microhedyle remanei*. (A) light micrograph (cross section), the arrow indicates a group of sperm cells in parallel orientation; (B) electron micrograph (cross section), mid-region of keeled nuclei. k, keel. Scale bars: A, 10 μm; B, 1 μm.

ultrastructural data clearly are concentrated on spermatozoa of Notaspidea (Healy & Willan, 1984) and Nudibranchia (e.g. Eckelbarger & Eyster, 1981; Healy & Willan, 1984; Medina et al., 1986; Healy & Willan, 1991; Fahey & Healy, 2003; Wilson, 2005; Wilson & Healy, 2002a,b, 2006). Up to now, the sperm ultrastructure and morphology of other opisthobranch groups, among them the Acochlidia, remain essentially unstudied.

This study gives the first detailed TEM description of spermatozoa of an acochlidian species. Sperm of the gonochoristic *Microhedyle remanei* (Microhedylidae *sensu* Wawra, 1987) is compared to that of the hermaphrodite *Hedylopsis ballantinei* (Hedylopsidae *sensu* Wawra, 1987) in order to obtain more detailed data whether or not acochlidian sperm variation may be phylogenetically informative, and how sperm structure is related to an array of special reproductive features displayed by acochlidian taxa.

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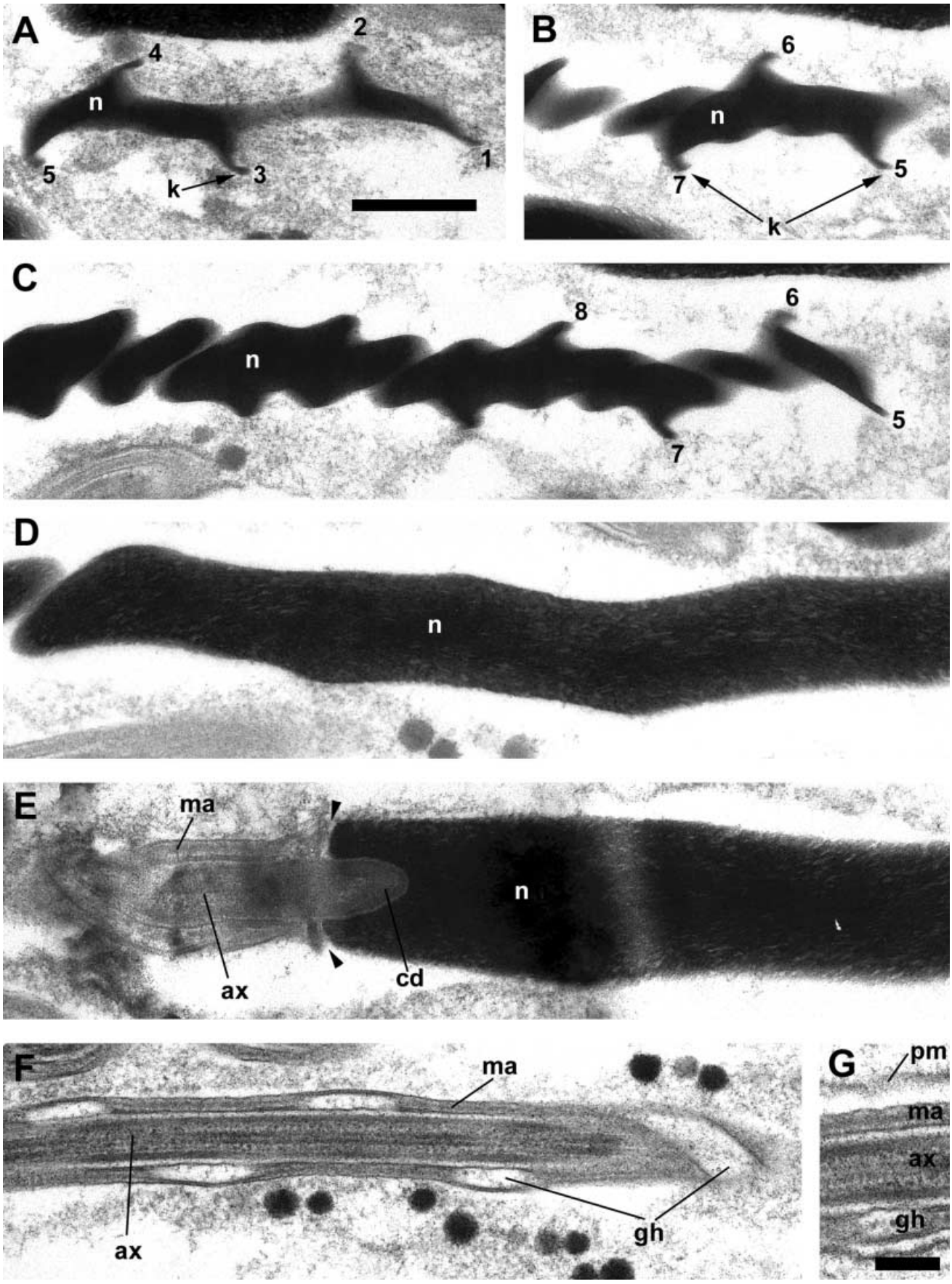

Figure 3. Spermatozoid ultrastructure of *Microhedyle remanei* (longitudinal sections, TEM). (A–C) Apical part of the nucleus with helical keels shown in three neighbouring slices, same numbers indicate same position in nucleus; (D) mid-region of the elongate nucleus; (E) neck-region of the spermatozoid; (F) midpiece with 2 × 9+2 axoneme and one glycogen helix, (G) midpiece, plasma membrane. ax, axoneme; cd, centriolar derivative; gh, glycogen helix; k, keel; ma, matrix; n, nucleus; pm, plasma membrane; arrowheads, subnuclear ring. Scale bars: A (for A–F), 500 nm; G, 200 nm.

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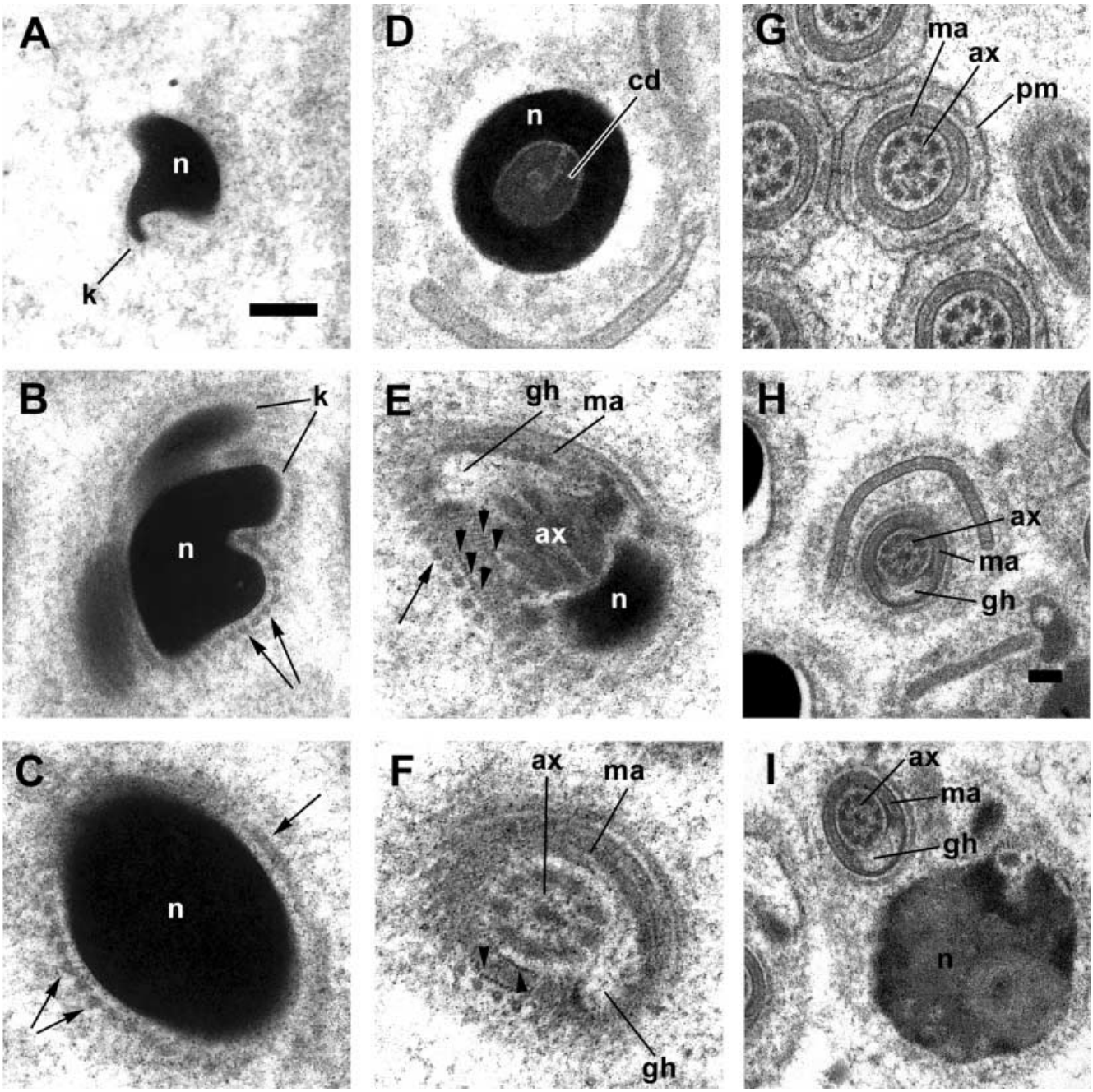

Figure 4. Spermatozoid ultrastructure of *Microhedyle remanei* (cross sections, TEM). (A) Apical part of the nucleus with helical keel; (B) mid-region of the nucleus, note microtubules around the nucleus; (C) basal region of the nucleus of a late spermatid; (D) basal region of the nucleus invaginated by the centriolar derivative that itself is hollow-cone-shaped; (E) oblique section through the neck region of a late spermatid; (F) midpiece with $2 \times 9+2$ axoneme and one glycogen helix; (G) midpiece posterior to glycogen helix; and (H, I) midpieces surrounded by accessory cells. ax, axoneme; cd, centriolar derivative; gh, glycogene helix; k, keel; ma, matrix; n, nucleus; pm, plasma membrane; arrows, microtubular manchette; arrowheads, paracrystalline material. Scale bars: A (for A–G), 200 nm; H (for H, I), 200 nm.

MATERIALS AND METHODS

Several specimens of *Microhedyle remanei* were collected off the south-west Castle Roads (Bermuda Islands) in July 1999. They were extracted from sediment samples (coarse sand from 6.5 m depth) and relaxed in a solution of 7% $MgCl_2$. The specimens were fixed in 4% glutardialdehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2), rinsed in the same buffer, followed by post-fixation in buffered 1% OsO_4 for 1.5 h. Dehydration was effected by a graded acetone series. The fixed specimens were embedded overnight in Spurr's low viscosity epoxy resin (Spurr, 1969) for semi- and ultrathin sectioning. Due to limited material of male specimens with mature spermatozoa in the ampulla, spermatozoa and late spermatids of the testis were examined (Figure 2A). First, one male mature specimen was semithin (1.5 μm) sectioned with Ralph glass knives from the anterior up to the beginning of the testis. Then, for analysis of the sperm ultrastructure by TEM, thin sections (65 nm) were prepared with a diamond knife (microtome MT XL, RMC, USA) from autosperm lying in the testis of

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M. remanei. The ultrathin sections were transferred on formvar-covered single-slot copper grids and, finally, hand-stained with 8% uranyl acetate and lead citrate according to Reynolds (1963). These were analysed with a Philips CM 10 TEM (80kV). The sections were deposited in the ZSM Mollusca section (Zoologische Staatssammlung München, Germany).

RESULTS

The terminology applied for the description of sperm ultrastructure follows Thompson (1973), Medina et al. (1988) and Healy & Willan (1991). The spermatozoa are subdivided into the head, the midpiece and the tail.

Acrosomal complex and nucleus

An acrosomal complex could not be detected on any of the slices. The length of the nucleus is at least 11 μm. It is helically coiled over the entire length (Figures 1&3), and its content is highly electron dense. The basal region of the nucleus is circular in cross section (Figures 1C&4D). The nucleus shows a maximum of five keels that are diminishing to a single, very prominent keel near the nuclear apex (Figures 1A,B, 2B, 3A–C & 4A,B). Thus, the nucleus diameter decreases from the basal to the apical region. The 'wavelength' of the keel is almost 1 μm (Figure 3A) in the apical region. The cross and longitudinal sections show a shallow (about 0.3 μm) basal invagination of the nucleus (implantation fossa *sensu* Medina et al., 1988) (Figures 1D, 3E & 4D). Spermatids in the late stage of spermiogenesis have already a well developed nucleus with highly condensed chromatin, but still show a row of microtubules (microtubular manchette *sensu* Medina et al., 1986) surrounding the nucleus (and the midpiece) helically (Figures 2B & 4B,C,E,F) that disappears at the end of spermiogenesis (Figure 3).

Neck region and midpiece

The basal invagination of the nucleus is occupied by a bell-shaped centriolar derivative (Figures 1D, 3E & 4D). The latter contacts the axonemal microtubules and interconnects them with the nucleus. Coarse fibres attached to the microtubules could not be found. A subnuclear ring is present (Figures 1 & 3E).

The midpiece is characterized by a single glycogen helix which is helically coiled around the central axoneme ($2 \times 9+2$ microtubuli) (Figures 1, 3F,G & 4E,F). The 'wavelength' of the glycogen helix is approximately 1–1.25 μm. Both central axoneme and glycogen helix are surrounded by the mitochondrial derivative consisting of the matrix and paracrystalline material (Figures 1E & 4E,F). Towards the distal part of the midpiece the glycogen helix ends and transverse sections show only the axoneme embedded in the mitochondrial derivative (Figures 1F & 4G). Secondary helices are absent.

Glycogen piece and annulus

Due to the absence of sections through the terminal region of the sperm cell, we cannot confirm the presence or absence of a glycogen piece or an annulus.

DISCUSSION

Structure comparison

Acrosomal complex and nucleus

An acrosomal complex was not detectable in *Microhedyle remanei*. Thompson (1973) described some opisthobranch taxa such as *Umbraculum* and *Aplysia* as lacking detectable acrosomal complex. Nevertheless, acrosomal pedestals and vesicles were found later by Healy & Willan (1984) and Vita et al. (2001). Thus all opisthobranchs previously studied in sufficient detail possess an acrosomal complex, however, in very variable sizes and structures. Probably, the absence of an acrosomal complex in *M. remanei* was due to the limited material available. In *M. remanei*, the nuclear apex is thin and keeled and shows a homogeneous electron density. Therefore, the presence of a conical acrosomal complex, as reported for many nudipleurans, e.g. for *Chromodoris annae* by Healy & Willan (1991), can be excluded. If an acrosomal complex is present, it is likely a very small acrosomal vesicle on a thin pedestal which is intertwined with the nuclear keel apically; a similar condition was found in some aeolid nudibranch species such as *Pteraeolidia ianthina* by Healy & Willan (1991).

The nucleus of *Microhedyle remanei* is remarkably long (minimum length of 11 μm), i.e. nearly twice as long as most nudibranch sperm nuclei studied by Healy & Willan (1991), and also longer than all the apically situated heterobranch sperm nuclei compared by Thompson (1973). Nuclei of comparable length are found in *Phyllidia nobilis* (12–15 μm), *Phyllidiopsis cardinalis* (15 μm), *Doriopsis granulosa* (14 μm) (Healy & Willan, 1991) and *Glossodoris pallida* (11.56 μm) (Wilson & Healy, 2002b). The helical and strongly keeled nucleus of *M. remanei* clearly contrasts to similarly elongated nuclei found in the pleurobranchid *Berthella ornata* with a plainly rounded basal keel, or to smooth nuclei of some phyllidiid nudibranchs lacking any keel (Healy & Willan, 1984, 1991). Sperm nuclei of the pleurobranchid *Pleurobranchus peroni* develop five keels similar to that of *M. remanei*, but in contrast to the latter, the keels arise immediately from the basal part of the nucleus (Healy & Willan, 1984). The apical nucleus structure of *M. remanei* closely resembles the intertwined and very prominently keeled nuclei reported by Healy & Willan (1991) from some aeolidoidean nudibranchs such as *Favorinus japonicus*, *Flabellina rubrolineata* and *Pteraeolidia ianthina*. But the nuclei in the latter species are considerably shorter showing lengths between 4 and 7 μm.

Neck region and midpiece

The complex sperm midpiece of *Microhedyle remanei* originates within a basal nuclear invagination by the centriolar derivative. A subnuclear ring surrounds the axoneme which is spirally encircled by a single glycogen helix embedded into the matrix and paracrystalline material. This resembles the condition found in most opisthobranchs (Healy, 1996), but periaxonemal coarse fibres are not (yet?) detectable in late spermatids and spermatozoans of *M. remanei*.

Within opisthobranchs the number of glycogen helices varies. Most nudipleuran, cephalaspidean and anaspidean opisthobranchs examined have one glycogen helix as present in *Microhedyle remanei*, but several species possess an additional, less elevated secondary helix, which is

lacking in *M. remanei*. In comparison to other opisthobranchs with just a single helix, the glycogen helix of *M. remanei* has a uniquely short wavelength of 1–1.25 μm. Such dense arrangements also occur in certain freshwater pulmonates such as *Planorbarius* or *Lymnaea*, however, with more helices involved (see Thompson, 1973). The architectibranch *Acteon tornatilis* shows up to four densely arranged glycogen helices with different lengths. This condition closely resembles that observed in *Hedylopsis ballantinei*, the second acochlidian species with known midpiece ultrastructure; sperm of the latter species show up to three equally well-developed glycogen helices of different lengths which are densely arranged around the axoneme (Sommerfeldt & Schrödl, 2005). Sperm with up to three glycogen helices were also recorded from at least two chromodoridid species by Wilson & Healy (2006), but these helices are aggregated on one side of the axoneme and show different diameters.

Glycogen piece and annulus

We have no reliable information concerning the terminal portion of acochlidian sperm cells. At least glycogen pieces have not been detected in either *Microhedyle remanei* (see present study) or in *Hedylopsis ballantinei* where, according to Sommerfeldt & Schrödl (2005), many of the cross-sections show a 'naked' axoneme not encircled by any mitochondrial derivative material which seems to refer to the posteriormost sperm portion. However, only sperm from gonadal tissue could be studied and thus may refer to incompletely developed stages.

Sperm morphology of Microhedyle remanei

Kirsteuer (1973) described the anatomy of *Microhedyle remanei* (as *Unela remanei*) from Santa Marta, Colombia. Spermatozoa, studied by him light microscopically, show a 'length of 95 μm and . . . an about 3 μm long, corkscrew shaped anterior region can be distinguished from an approximately 14 μm long portion, which is slightly thicker and optically denser than the remaining tail filament'. He questioned whether the spiral anterior region is the acrosomal complex followed by an elongate nucleus or whether it represents the whole small nucleus. Kirsteuer's 'spiral anterior region' of 3 μm length clearly refers to the apical portion of the nucleus with one very prominent helicoid keel (approximately 2.5 μm long), which is followed by the elongate and electron dense mid-part of the nucleus (at least 8.5 μm long). Slight differences of sperm head lengths may be explained by difficulties referring to both light microscopic measurements of diffusely stained sperm and transmission electron microscopic minimum length estimations across several longitudinal slides.

Sperm relevance for classification

Some light microscopic information on acochlidian sperm morphology (Odhner, 1937; Marcus & Marcus, 1954; Kirsteuer, 1973; Westheide & Wawra, 1974; Wawra, 1978) was traditionally used to distinguish two major acochlidian groups. According to Wawra (1987) the Microhedylidae, Asperspinidae and Ganitidae were characterized by 'spiral' sperm of the 'tyrtowii-type', while the Hedylopsidae, Acochlidiidae and Tantulidae were supposed to have 'pear-shaped' sperm of the 'spiculifera-type'. Pear-shaped sperm cells were reported for *Hedylopsis spiculifera* (and its junior synonym *H. suecica*) by several authors (Kowalevsky, 1901; Odhner, 1937; Cobo Gradin, 1984; Wawra, 1989). A light microscopic re-examination of specimens of *H. spiculifera* by Sommerfeldt & Schrödl (2005), however, proved the presence of elongate spiral sperm cells as is usual for euthyneuran gastropods (Healy, 1996). Apically swollen 'pear-shaped' acochlidian sperm cells as those drawn by Kowalevsky (1901) somewhat resemble the nuclear cup-stage of nudibranch spermatids described by Eckelbarger & Eyster (1981) and Medina et al. (1986); they thus may well refer to stages of sperm head development or be due to artefacts of fixation. Some differences between sperm of *Hedylopsis* and *Microhedyle* may, however, truly exist. Spermatozoa (or spermatids) from the gonad of *Hedylopsis ballantinei* were described by Sommerfeldt & Schrödl (2005). The TEM study shows spiral spermatozoa with the midpiece having three well-developed glycogen helices instead of a single thin one. Unfortunately, no nucleus could be detected in *H. ballantinei*, thus no data of the nucleus ultrastructure are available for comparison (Sommerfeldt & Schrödl, 2005).

Acochlidian phylogeny and sperm evolution

The ultrastructural comparison of sperm of *Hedylopsis ballantinei* and *Microhedyle remanei* is still restricted to features related to the midpiece, but the number and structure of glycogen helices vary considerably. Sperm differences thus may serve for supraspecific delineations as well as for inferring acochlidian phylogeny. Present information on acochlidian sperm midpieces, which resembles the condition in *Acteon* and basal Pulmonata (Thompson, 1973), at least do not contradict the placement of Acochlidia within basal Opisthobranchia which was concluded from acochlidian morphology (Sommerfeldt & Schrödl, 2005). The very elongate and strongly keeled nucleus of *M. remanei* clearly refers to derived features which may be correlated with special reproductive conditions. There is no reciprocal copulation in *M. remanei* (and other Microhedylidae, Ganitidae and Asperspinidae), but so-called dermal fertilization (Wawra, 1992; Neusser et al., 2006): similar to the likewise small runcinid bullomorphs (Kress, 1985) and the enigmatic genus *Rhodope* (see Riedl, 1959; Haszprunar & Künz, 1996) spermatophores are attached to the mates' bodies, and allosperm then has to penetrate the hosts' body wall, body cavity and gonad tissue to fertilize the eggs. According to Karlsson & Haase (2002), the nudibranch *Aeolidiella glauca* is known to transfer sperm via spermatophores, too. They observed that only parts of the released sperm penetrated epidermal cells. However, most sperm migrated along the body surface to the genital opening. Unfortunately, there is no ultrastructure of the spermatozoa of *A. glauca* presented. Opisthobranch spiral sperm with well-developed axoneme and glycogen helices and helical apex are thought to be well-adapted to migrate through likewise dense media such as body liquids and penetrate egg walls by spiral progression (Thompson, 1973). In addition, the extremely elongated and keeled sperm heads of *M. remanei* may be necessary to also perforate thicker and more

resistant body tissues in a corkscrew-like manner. More ultrastructural data on spermatozoa of acochlidian species are necessary to prove such adaptations to be potential autapomorphies of spermatophore-transferring acochlidian clades. Comparative ultrastructural sperm data on potentially related opisthobranch taxa such as diaphanoid or basal sacoglossan species is required to clarify the origin of Acochlidia.

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