ULTRASTRUCTURE OF THE SPERM OF APLYSIA CALIFORNICA COOPER

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Running Head: STRUCTURE OF *APLYSIA* SPERM

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Abstract—The structure of the sperm of *Aplysia californica* was studied by both transmission and scanning electron microscopy. *Aplysia californica,* a species with internal fertilization, has the modified type of molluscan sperm structure. Spermatids had a glycogen helix spiraled about the flagellum, both enclosed by a common microtubular basket. A second vacuole helix was periodically seen only in spermatids and absent in spermatozoa. An additional basket of microtubules appeared to direct the elongation and spiraling of the nucleus about the flagellum/glycogen helix. A flat acrosome was present while the centriolar derivative was embedded in a deep nuclear fossa with strands of heterochromatin arranged nearly perpendicular to its long axis. The mitochondrial derivative consisted of small, frequently electron dense, closely spaced rods but individual mitochondria were also seen surrounding the axoneme of spermatids. The axoneme consisted of dense fibers that appeared to have a "C" shape substructure with a central dense fiber thus providing a 9+1 arrangement of singlet units; the typical 9+2 microtubule arrangement of flagella was absent. Flagella with two axonemes were frequently seen as well as an extra axoneme within the head of immature sperm.

Keywords—Aplysia californica; sperm; ultrastructure

INTRODUCTION

No information is available on the ultrastructure of sperm of *Aplysia californica*, Cooper other than Beeman's (1977) interpretative drawings from micrographs, indirect observations in reports dealing with other species of the genus or that from opisthobranchs in general (Kubo & Ishikawa, 1981; Vita *et al*., 2001). The structure of the sperm of the few members of the Anaspidea that have been studied, however, appears similar to that for members of other orders of this class (Healy & Willan, 1984; Healy, 1988). For example, Vita *et al*. (2001) studying the structure of sperm of *A. depilans* found features that have been recognized as typical for a related order, the Notaspidea; these include the presence of an acrosome, a nucleus that spirals about the flagellum, a mid piece consisting of one or more glycogen helices, a complex mitochondrial derivative, course fibers in the axoneme, and a dense ring structure and glycogen piece near the terminus of the flagellum. With few exceptions, Kubo and Ishikawa (1981) develop a similar picture for *A. kurodai*.

We studied the structure of the sperm of *A. californica* with both transmission and scanning electron microscopy. Our findings not only differed from the only ultrastructural but brief report for this species (Beeman, 1977), but also provided a more complete description which, therefore, allowed comparison with other species of the genus and that of related orders.

MATERIAL AND METHOD

Aplysia californica, 100 to 300 g wet mass, were obtained from the NCRR *Aplysia* Resource Facility, University of Miami. Ovotestes and sperm duct were quickly isolated from two individuals and fixed in a 2.5% glutaraldehyde solution in phosphate buffer, post fixed in 1% osmium tetroxide, dehydrated in a graded alcohol series, enbloch stained with uranyl acetate in 50% ethanol, and embedded in L R White resin. Semi thin (1 µm) and ultrathin (<60nm) sections were cut on a Leica EM UC6 ultramicrotome. Semithin sections were examined by light microcopy (Olympus BX60) with digital camera (Olympus DP71). Stained ultrathin sections (uranyl acetate and lead citrate) were viewed by transmission electron microscopy (TEM) at 60kV (Philips 300 TEM). Image measurements utilized Imaje J 1. 46 for Macintosh (National Institutes of Health). For scanning electron microscopy (SEM), ovotestes were macerated in buffered fixative used for TEM (above), large cellular debris removed by centrifugation, the supernatant post-fixed in osmium (as for TEM), dehydrated in ethanol and finally in acetone, dried by hexamethyldisilazane (HMDS), sputter coated with gold and viewed with a Jeol 5600 LV scanning electron microscope (SEM)

RESULTS

Immature sperm from the ovotestis of *Aplysia californica* have an oblong head, were organized into groups with a parallel orientation and were surrounded by round cells (nurse cells? Fig. 1A, B). Flagella at this stage already bore a helical structure (Fig. 1A-C), probably the glycogen helix (see below). The head of immature sperm contained copious cytoplasm, mitochondria and a nucleus with dispersed heterochromatin (Fig. 1D). A membrane bound array of 9+1 course fibers periodically appeared near the primary flagellum within the cytoplasm of the head (Fig. 1D). During sperm maturation the nucleus invaginated to form a cavity, the nuclear fossa, into which the flagellar base (centriolar derivative) fitted (Fig. 1D). Linear arrays of heterochromatin in the nucleus became oriented perpendicular to the long axis of the nuclear fossa containing the centriolar derivative (Fig. 1G). Arrays of granules (16 $\pm$ 1nm; mean diameter $\pm$ SD, n=11) consisting of 2 to 4 sub particles occurred within the heterochromatin of the nucleus (Fig. 1H).

A flattened acrosome (300 x 100 nm) was found at the tip of the nucleus (Fig. 1E; Table 1). The nucleus appeared slightly pointed at its junction

with the acrosome. No extra vesicular structures appeared to be associated with the acrosome.

The nucleus became enclosed by a basket of microtubules (35 ± 4, mean diameter ± SD, n=16) spaced 69 ± 8nm (mean spacing ± SD, n=17) apart (Fig. 1H). The nucleus with the microtubular basket spiraled about the flagellum thereby resulting in the loss of the round shape of the immature nucleus (Fig. 2G).

The flagellum already bore a helical structure, the glycogen helix, and this and the axoneme were enclosed in a common microtubular basket (Figs 1C; 2C, E). The spacing of microtubules about the glycogen helix (56 ± 9 nm, mean spacing ± SD, n=77) was closer than that for microtubules about the nucleus (mean = 69 nm, see above). The wavelength (TEM) of the nuclear spiral (3.63 ± 1.09µm, mean wavelength ± SD, n=12) was approximately half the wavelength of the glycogen spiral (8.05 ± 0.91µm, mean wavelength ± SD, n=5. Table 1). SEM showed the mean flagellar diameter was 0.56µm (±0.05, ± SD, n=19) while the mean diameter of the glycogen helix spiraled about the flagellum was 0.41µm ±0.05, (± SD, n=16. Fig. 1C; Table 1).

A secondary vacuole or helical structure (besides the glycogen helix) was periodically seen only in spermatids and was not present in spermatozoa (Fig. 2B). No microtubules appeared to surround this secondary helix (Fig. 2B).

The axoneme of a mature flagellum consisted of 9 singlet outer units (course fibers, Healy, 1990, 1991, 1996) with 1 central course fiber; no paired microtubules were seen in the axoneme of *A. californica* sperm (Figs 1F, 2A, B, C, E). The electron dense material composing the coarse fibers (peripheral or central) might have obscured their microtubule doublets. Each peripheral course fiber had a dense, "C" shaped subunit (Fig. 2C, F). The C shaped units appeared to be arranged in a regular pattern in the nine peripheral course fibers; this was particularly apparent for the axoneme within the nuclear fossa (Fig. 2F). The central fiber appeared connected to the peripheral fibers by dynein arms (Fig. E) but consisted of two zones of electron dense material connected by less dense material. Unlike the peripheral course fibers there was no polarity to the organization of the material making up the central course fiber (Fig. C, E, F). Cilia on adjacent cells and on cells from other tissues of this species had the 9+2 microtubular arrangement (Coelho et al. 1998. Prince, 2003). Flagella of *A. californica* sperm frequently contained two separate axonemes (Figs 1F, 2A, G). The tip of the flagellum lacked a glycogen helix as well as its surrounding microtubular basket (Fig. 2H).

Mitochondria were found within the cytoplasm of the immature sperm head but localized near the flagellum as it started to become surrounded by the nucleus (see above, Fig. 1D). More distally in the flagellum, mitochondria (49 ± 8nm; 187 ± 023nm, mean diameter and width respectively ± SD, n=6, 11 respectively) surrounded the axoneme (Fig. 2 E). Mitochondria in spermatids became organized into a mitochondrial derivative, a paracrystaline structure consisting of rods (12.8 ± 2.0 nm, mean diameter ± SD, n=46). The rods were frequently electron dense, of undetermined length and often appeared to be arranged length wise in the cross sectional plane (Fig.2A, C, D). This paracrystaline structure encircled both the flagellum as well as the glycogen helix (Fig. 2A, C). The flagellum microtubular basket, therefore, contained the axoneme, mitochondrial derivative and glycogen helix (Fig. 2C).

DISCUSSION

The structure of molluscan sperm appears to depend on whether fertilization is external or internal (Anderson & Personne, 1976; Hodgson *et al*., 1990; Healy, 1996). Sperm of mollusks using external fertilization (the primitive type, Anderson & Personne, 1976; Bozzo *et al*. 1993) have a regular morphology; the head containing a terminal acrosome and spherical nucleus, the midpiece with several round mitochondria with apparent cristae and the tail or axoneme with the classical 9+2 microtubular arrangement. Mollusks using internal

fertilization (most of the Gastropods and Pulmonates, Anderson & Personne, 1976) have the modified type of sperm structure, this becomes apparent during maturation. The nucleus elongates concurrent with its spiraling about the flagellum thus forming a helix (Beeman, 1977). The flagellar base (centriolar derivative or analog; Healy, 1988, 1990; Vita *et al*., 2001) inserts into a posteriordepression in the nucleus (the nuclear fossa, Hodgson *et al*., 1990). The midpiece mitochondria become highly modified into a paracrystaline structure, the mitochondrial derivative (Healy, 1996), with the loss of the appearance of individual mitochondria and their cristae. Anderson and Personne, (1976) found that these paracrystaline structures have a variable morphology from rods to an open lattice, and appear to contain repeating units of the electron transport chain and/or other components of the energy transformation system. Another vacuole develops either concurrent with the mitochondrial derivative, directly from it or just distal to its termination and it can overlap with the spiraled nucleus. This vacuole generally contains glycogen, is spiraled about the flagellum and is termed the glycogen helix (Healy, 1988, 1991). In this paper, the spiraled nucleus and glycogen vacuole are termed nuclear and glycogen helices respectively. In modified sperm the 9+2 microtubule pairs of the axoneme frequently have a single dense structure, the course fiber, associated with them. These course fibers can obscure the microtubules of the flagellum.

Sperm of *Aplysia californica*, a hermaphrodite with internal fertilization, have the modified structure. Beeman's (1977) illustrative drawings and few micrographs of *A. californica* sperm provide only cursory ultrastructural information for this neurophysiologically important species (Kandel, 1979). Our more detailed description of this species differed from Beeman's (1977) brief account as well as reports for other species of the genus and for gastropods in general. We detail these differences below. Immature sperm of *A. californica* occurred in parallel, organized bundles. A similar orientation has been reported for spermatids of *Aplysia* spp. (Thompson & Bebbington, 1969). Spermatids of *A. californica* were similar to the structure of primitive sperm (an oblong head containing a spherical nucleus and mitochondria about the base of the axoneme) and had a flattened acrosome. Small acrosomes have been reported for *A. depilans* (Vita *et al*. 2001), and a variable presence in *A. punctata* (Vita *et al*. 2001) but reported to be absent generally in aplysiids (Thompson & Bebbington, 1969) and specifically in *A. californica* (Table 1; Beeman, 1977). Kubo & Ishikawa (1981) found in *A. kurodai* an acrosome at the tip of the nucleus of the sperm that was approximately six times smaller than the one we recorded for *A. californica*. *Aplysia Kurodai* had additional vesicular structures about the acrosome while *A. californica* did not. *Aplysia californica* had only one type of sperm; non-functional, supernumerary sperm (oligopyrene sperm [Franzen, 1955; Anderson & Personne, 1976]) each often bearing several flagella was never observed but is frequently recorded for other gastropods (Franzen, 1955; Hodgson *et al*., 1990)

Spermatids of *A. californica* had a glycogen helix wrapped around the flagellum; Beeman (1977) did not examine this stage. The flagellum and glycogen helix were enclosed by a common microtubular basket. Healy (1988) describes, however, that the glycogen helix in several pyramidellid gastropods developed concurrent with or just distal to the termination of the mitochondrial derivative. Healy (1991) reports the absence of a glycogen helix in the sperm of the gastropod, *Architectonica perspectiva*, while Beeman (1977) for *A. californica* and Kubo and Ishikawa (1981) for *A. kurodai* report not only the presence of a glycogen helix but also the presence of a second helical structure. Neither of the latter accounts shows a cross section of this secondary structure nor whether it is enclosed by microtubules, as is the case for the glycogen helix. We found a second vacuole outside the axoneme-glycogen helix basket but it was not surrounded by microtubules and appeared transitory.

Maturation of *A. californica* sperm began with elongation of the nucleus and the development of a

deep nuclear fossa into which the centriolar derivative (Healy, 1988; Vita *et al*., 2001) inserts. The heterochromatin of the nucleus became arranged in linear arrays which became organized nearly perpendicular to the long axis of the nuclear fossa. In *A. kurodai* the chromatin strands were arranged in a helical pattern and apparently assisted in the spiraling of the nucleus (Kubo & Ishikawa, 1981). Granules, 25 nm in diameter, composed of 5-6 sub particles, appear among the chromatin in *A. kurodai* (Kubo & Ishikawa, 1981) but in *A. californica* these were smaller (16 nm) and consisted of only 2 to 4 sub particles.

In *Aplysia californica* the nucleus becomes enclosed by its own basket of microtubules. Kubo and Ishikawa (1981) suggest that the basket of microtubules about the nucleus directs the spiraling of the nucleus in *A. kurodai*. In *A. californica* the spiraling of the nucleus may be guided also by the glycogen helix that had already been formed. In several pyramidellid gastropods (Healy, 1988) the basket of microtubules about the nucleus continued to enclose the mitochondrial derivative. In *A. californica* a separate microtubular basket encloses the axoneme, glycogen helix and eventually the mitochondrial derivative. The wavelength of the nucleus spiraled about the flagellum ranged by about 3∝m among the various species of *Aplysia* while the diameter of the flagellum and glycogen helix of *A. californica* were disparate to that recorded for other *Aplysia* species (Table 1).

The spacing of microtubules in the nuclear basket in *A. kurodai* (60nm; Kubo & Ishikawa, 1981) was approximately similar to that for *A. californica* (69 $\pm$ 8nm). No microtubular basket associated with the nucleus was mentioned for either *A. depilans* (Vita *et al*., 2001) *A. californica* (Beeman, 1977) or *Aplysia. spp* (Thompson, 1973) but Thompson and Bebbington (1969) observed a membrane of microtubules surrounding the two mitochondrial strands in three species of *Aplysia* (*A. depilans*, *A. fasciata* and *A. punctata*). Kubo and Ishikawa (1981) found that the microtubular basket disappeared once the nucleus was spiraled about the flagellum; we found a similar situation, the glycogen helix and microtubular baskets were absent at the ends of mature flagella. The posterior of the nucleus invaginated to form a deep depression (nuclear fossa) into which the centriolar derivative fits, "like a cork in a bottle". The nuclear fossa in *A. kurodai* (Kubo and Ishikawa, 1981) appeared similar to *A. californica*, but is only a shallow depression in five notaspidean opisthobranchs (Healy & Willan, 1984). In *Cornirostra* Healy (1990) shows the centriolar derivative and its fossa to almost penetrate through the entire nucleus. The centriolar derivative in *A. californica* had neither microtubules nor microfilaments (the latter attaching the centriolar derivative to the cell membrane) as is found in the primitive sperm of four species of bivalve molluscs (Gwo *et al*., 2002).

The mitochondrial derivative in *A. californica* like that reported by Vita *et al*. (2001) for *A. depilans* and by Healy (1993) for *Omalogyra atomus* consisted of small, often electron dense, closely spaced rods. It was not a lateral sheath of regularly arranged, parallel, hollow rods or plates as has been reported for *A. californica* and *Phyllapysia taylori* (Beeman, 1977), *A. depilans* (Thompson & Bebbington, 1969), *Bursatella leachii* (Thomas (1975), several species of notaspidean gastropods (Healy & Willan, 1984), and for many terrestrial gastropods (Healy, 2001). Two mitochondrial derivatives or strands were not observed for *A. californica* contrary to reports for several other species of *Aplysia* (Thompson & Bebbington, 1969; Thompson, 1973).

The axoneme of *A. californica* consisted of nine peripheral and one central course fiber, but they were not associated with microtubule doublets. In addition, the nine peripheral course fibers in *A. californica* had an electron dense "C" shaped substructure that appeared to be arranged in a regular pattern. Thompson (1973) pictures the axoneme of *Aplysia* spp. with the 9 + 2 microtubule doublets but lacking course fibers while Kubo & Ishikawa (1981) for *A. kurodai* and Healy & Willan (1984) for several notaspidean opisthobranchs picture axonemes with microtubules and course fibers. Vita *et al*. (2001) report that the size of the

course fibers decreased concurrent with the appearance of the microtubule doublets in *A. depilans*.

We frequently noted two complete axonemes within the same flagellum of *A. californica* as well as an apparent vestigial axoneme within the cytoplasm of spermatids. Sperm with two axonemes per flagellum are occasionally noted for primitive sperm of several species of bivalve mollusks (Gwo *et al*. 2004).

Healy (1996) states in his review of the structure of opisthobranch sperm that the modified type of sperm generally has an acrosome, the mitochondrial derivative is a continuous sheath, the spermatid nucleus has an anterior and posterior plaque, and course fibers are associated with axonemal doublets. Our study of the sperm of *A. californica* found an architecture that differed considerably from this report; the mitochondrial derivative consisted of small, often electron dense rods, no anterior plaque was observed for spermatid nuclei, the electron dense material composing the coarse fibers appeared to obscure axonemal, microtubular doublets and the peripheral coarse fibers had a "C" shaped substructure. In addition, the glycogen vacuole was spiraled about the spermatid flagellum, a common microtubular basket enclosed the axoneme, glycogen helix and mitochondria and eventually the mitochondrial derivative, while an additional microtubular basket appeared to enclose the nucleus and possibly assisted in its elongation and spiraling.

The axoneme ultrastructure of *A. californica* in particular and for many mollusks in general is atypical yet they still function. Primary ciliary dyskinesia (PCD or immotile-cilia syndrome) is a world wide chronic disease caused by the abnormal function and structure of the airway's cilia (Leigh *et al*., 2009). Study of abnormal but functional axoneme structure in such organisms as *A. californica* might shed light onto human diseases involving ciliary dysfunction.

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FIGURE DESCRIPTIONS

Figure 1. Sperm of *Aplysia californica*. Scanning electron microscopy of a sperm clump surrounded by nurse cells (**A**), without nurse cells (**B**), and sperm flagella with glycogen helices (arrows **C**). Transmission electron microscopy of a spermatid with an extra axoneme (arrow) in the head (**D**), flattened acrosome on the opposite side of the nucleus from the flagellum (**E**), two axonemes in a flagellum surrounded by a basket of microtubules (**F**), orientation of heterochromatin about the centriolar derivative (**G**) and the microtubular basket about the nucleus which contains granules (**H**). Abbreviations: AC, acrosome; CD, centriolar derivative; G, granule; M, mitochondrion; MT, microtubule; NC, nurse cell; NF, nuclear fossa. Scale bars: A & B 10 μm; C 1 μm; D-G 0.5 μm; H 100 nm.

Figure 2. Transmission electron microscopy of the sperm of *Aplysia californica*. Flagella frequently had two axonemes (**A** & arrows in **G**). **B.** A second vacuole (arrows) periodically occurred. The mitochondrial derivative of dense rods surrounds the axoneme and glycogen helix (**A**, **C** & **D**). A microtubular basket surrounds both the axoneme mitochondrial derivative and glycogen helix (**C**). Small mitochondria surround the axoneme (**E**) before they are modified into the mitochondrial derivative. Coarse fibers of the axoneme have a "C" shaped substructure (**F).** Longitudinal section of spermatid showing 2 flagella, composing the axoneme, the glycogen helix, mitochondrial derivative and elongated nucleus in a microtubular basket (**G**). The flagella tip (**H** arrows) lacks a dense ring structure and glycogen helix and external microtubular basket. Abbreviations: CF, course fiber; GH, glycogen helix; M, mitochondrion; MD, mitochondrial derivative; MT, microtubule; N, nucleus. Scale bars: A- H 100 nm; E & F 0.5 μm.

Table 1. Sperm characteristics for various species of *Aplysia* and related Gastropods.

Trait	Dimensions	Species	Citation
Acrosome			
	300 x 100 nm	*A. californica*	This paper
	50 nm	*A. kurodai*	Kubo & Ishikawa, 1981
	present	*A. depilans, A.* punctata	Vita et al., 2001
	absent	*A. californica*	Beeman, 1977
	generally absent	aplysiids	Thompson & Bebbington, 1969
Nuclear Helix Wavelength (∝m)			
	3.6 ± 1.1,12 (a)	*A. californica*	This paper
	@3.8	*A. depilans*	Corral & Azevedo, 2001
	6-7	*A. kurodai*	Kubo & Ishikawa, 1981
	3.7 - 4.6 (b)	*A*. spp.	Thompson, 1973
	4.4 (b)	*A*. spp. (c)	Thompson & Bebbington, 1969
	2-2.7 (b)	*Pleurobranchia maculata*	Healy & Willan, 1984
	3.2	*Umbraculum sinicum*	Healy & Willan, 1984
Flagellum Diameter			
	0.56 ± 0.05, 19(a)	*A. californica*	This paper
	@0.4	*A. depilans*	Corral & Azevedo, 2001
	0.22	*A*. spp.(c)	Thompson & Bebbington, 1969
	0.22 (b)	*A*. spp.	Thompson, 1973
Glycogen Helix			
Wavelength (∝m)	8.1 ± 0.9, 12 (a)	*A. californica*	This paper
	@3.4 - 4.6	*A. depilans*	Corral & Azevedo, 2001
	4.2 (b)	*A*. spp	Thompson, 1973
	5 (b)	*Cantareus aspersus*	Healy, 2001
Diameter	0.41 ± 0.05, 16 (a)	*A. californica*	This paper
	@1	*A. depilans*	Corral & Azevedo, 2001

a = mean ± SD, N ; b = measurements from micrographs or drawings; c= *Aplysia depilans*, *A. fasciata* & *A. punctata* not distinguished.