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NO. 11

Sperm Ultrastructure of the Protobranchia: Comparison with Other Bivalve Mollusks and Potential Taxonomic and Phylogenetic Significance

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June 9, 2017

Publication 1571

PUBLISHED BY FIELD MUSEUM OF NATURAL HISTORY

Publication Note

Fieldiana: Life and Earth Sciences, ISSN 2158-5520

Formed by the merger of:

Fieldiana: Botany (ISSN 0015-0746);

Fieldiana: Geology (ISSN 0096-2651);

Fieldiana: Zoology (ISSN 0015-0754).

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Cover: Survey micrograph showing spherical sperm nuclei in spermatozoa of *Scaeoleda caloundra* (Bivalvia: Nuculanidae).

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Received December 16, 2016

Accepted January 20, 2017

Published June 9, 2017

Publication 1571

PUBLISHED BY FIELD MUSEUM OF NATURAL HISTORY

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John M. Healy, Paula M. Mikkelsen, Gonzalo Giribet, and Rüdiger Bieler

Abstract

Sperm ultrastructure of nine species of protobranch bivalves, representing three of four extant orders (Solemyida, Nuculida, Nuculanida), is discussed. Greatest diversity occurs in Solemyida (acrosomal vesicle low-conical, tall-conical, or very elongate, with radial plates; nucleus rod-shaped, teardrop-shaped, or very elongate; four, five, or six mitochondria) and the least in Nuculida (acrosomal vesicle low- to tall-conical; lacking radial plates; nucleus rod-shaped, five or six mitochondria) followed by Nuculanida (short, conical acrosomal vesicle with radial plates; spheroidal nucleus; four or five mitochondria). The wide variety of shapes in Solemyidae suggests taxonomic potential, especially in resolution and/or recognition of supraspecific taxa, but no diagnostic family characters were identified. Taxonomic potential exists for Nuculida (acrosomal shape) and Nuculanida (mitochondrial number). Protobranch sperm is highly diverse, and no defining character of the whole group was found. Support was found for the Nuculida and Nuculanida as natural groups but not for their close relationship. Nuculanida and Solemyida exhibit radial plates in the acrosomal vesicle but otherwise share no derived characters. The striking similarity of most sperm features of Nuculanida with certain pteriomorphians, especially Pectinoidea, a relationship also suggested by some mitochondrial DNA sequence data, poses interesting questions concerning their relationships and/or shared functional constraints.

Key Words: Bivalvia, evolution, Mollusca

Introduction

The subclass Protobranchia contains what are generally considered the most ancient extant bivalves (Cox, 1959, 1960; Newell, 1969; Cope, 1996, 1997, 2000; Morton, 1996; Reid, 1998; Zardus, 2002). Although it is likely that protobranchs appeared relatively early in the history of the class (Morton, 1996), this issue and indeed the precise origins of the Bivalvia and affinities of a number of Cambrian bivalved taxa are far from resolved (e.g., see Runnegar & Pojeta, 1992; Cope, 1996, 2000; Jell & Darragh, 1998; Carter et al., 2000; Elicki & Girsu, 2009). In shallow waters, protobranchs are reasonably well represented, but not speciose in comparison with Pteriomorpha and Heterodonta (Reid, 1998). In deep water, however, particularly in abyssal environments, the reverse is true, with protobranchs forming a large percentage of the known bivalve fauna (Allen, 1978; Reid, 1998; Zardus, 2002).

Although the most recent classifications, based on molecular and/or morphological phylogenetic analyses, usually distinguish three main groups of protobranchs—Solemyida (Solemyoidea + Manzanelloidea), Nuculida (Nuculoidea), and Nuculanida (Nuculanoidea) (Sharma et al., 2012, 2013; Bieler et al., 2014; González et al., 2016)—relationships among and within the groups continue to be the subjects of vigorous debate (see Giribet, 2008; Sharma et al., 2012). For example, Solemyida have been seen as worthy of their own subclass (Cryptodonta or Lipodonta; see Cope, 1996) or associated either with the Nuculida (Waller, 1998; an association named as Opponobranchia by Giribet, 2008) or with the Nuculanida (Morton, 1996). Similarly, Nuculida and Nuculanida have traditionally been associated as Palaeotaxodonta (Newell, 1969; Cope, 1996, 1997; Salvini-Plawen & Steiner, 1996), sometimes with the Nuculanida actually incorporated within the Nuculida (Purchon, 1987; Maxwell, 1988; Starobogatov, 1992; Reid,

1998). Carter et al. (2000), however, concluded that nuculanoids were sufficiently different from other protobranchs to warrant their own superorder, Nuculaniformii, leaving Nuculida and Solemyida to form another superorder (Nuculiformii) (both superorders of their revised concept of the Palaeotaxodonta) (see also Carter et al., 2011). Other authors have gone further, arguing that the immediate affinities of Nuculanoidea lay not with Nuculida and Solemyida but with Pteriomorpha (Wilson et al., 2010; Plazzi et al., 2011) or finding a poorly supported relationship to Archiheterodonta (Giribet & Distel, 2003), thereby reopening the question of protobranch monophyly. Giribet (2008) could not determine whether the Nuculanida belong with Nuculida + Solemyida (i.e., his Opponobranchia) to form the Protobranchia or whether the Opponobranchia are the sister clade to the Nuculanida + Autolamellibranchiata. His suggested new classification ranked Opponobranchia, Nuculanida, and Autolamellibranchiata as three equal divisions of the class but left “their relationships as unresolved” (p. 124). However, recent molecular phylogenetic analyses usually support monophyly of Nuculoidea + Nuculanoidea (Sharma et al., 2012, 2013; Combosch et al., 2017; but see Bieler et al., 2014), and phylogenomic analyses now appear to have resolved the monophyly of Protobranchia (Smith et al., 2011; González et al., 2016).

As mentioned previously, the phylogenetic interpretation of many Cambrian “bivalve” taxa varies widely within the literature, and even the universally accepted early Cambrian bivalve genera *Fordilla* and *Pojetaia* have been claimed as isofilibranch and protobranch, respectively (e.g., Runnegar & Bentley, 1983; Runnegar & Pojeta, 1992), or as the earliest “stem-group” bivalves (Runnegar & Pojeta, 1992). Recent classifications (e.g., Bieler et al., 2010; Carter et al., 2011) have placed these earliest—and obviously important—fossil taxa into their own higher grouping at the very base of the Bivalvia, thereby

essentially giving them “stem-group” status but with an understanding that they remain *incertae sedis*, at least for the present. Of the extant protobranchs, genera such as *Nucula* are most often favored in reconstructions of early protobranch morphology and biology (e.g., Yonge, 1939, 1959; Purchon, 1987; Morton, 1996). Waller (1998, pp. 15–16) stressed the importance of the pericalymma (“test-cell”) larva as a unifying protobranch apomorphy but also concluded that “the question of which extant family in the Protobranchia is the “most primitive” is in fact impossible to answer.” The mode of development and feeding mode of protobranchs have also been identified as important characters distinguishing them ecologically from most other bivalves that follow a typical latitudinal diversity gradient (Roy et al., 2000). Clearly, advances in our understanding of all aspects of the biology of protobranchs are still required to ascertain their role both in bivalve evolution and in living marine communities.

The taxonomic and potential phylogenetic utility of sperm morphology in mollusks has long been recognized (e.g., Retzius, 1904, 1906) although tempered by an awareness that environment of fertilization and almost certainly egg (mature oocyte) size, structure, and yolk content all presumably play some role in determining the shape and size of sperm features (Franzén, 1955, 1956, 1983; Afzelius, 1983; Rosati & Focarelli, 1996; Drozdov & Tyurin, 2014). Retzius (1904) appears to have been the first worker to examine spermatozoa in protobranch bivalves, providing detailed light-microscopic observations on *Nucula nucleus* (Linnaeus, 1758) (Nuculoidea) and *Nuculana minuta* (O. F. Müller, 1776) (the latter as *Leda minuta*) (Nuculanoidea). He demonstrated a marked difference in nuclear shape between the two species (rod-shaped in *N. nucleus*, spheroidal in *N. minuta*). Franzén (1955) likewise showed the same differences between *Nucula sulcata* Bronn, 1831, and *Nuculana pernula* (O. F. Müller, 1779) (as *Leda pernula*). More recently, Ockelmann and Warén (1998) illustrated the spermatozoa of *Microgloma pusilla* (Jeffreys, 1879) based on light microscopy of alcohol-preserved material and concluded that this species should be moved from the Nuculoidea to the Nuculanoidea based on, among other things, sperm nuclear shape. They further stated that they had examined eight species of Nuculoidea (from two genera) and 13 species of Nuculanoidea (from seven genera) for sperm morphology and found that the two superfamilies differed consistently from each other in nuclear shape.

It is at the ultrastructural level that sperm features can be studied in the greatest detail, particularly with transmission electron microscopy (TEM). Such work on bivalves, when performed on a comparative basis, has proven of considerable taxonomic use from the genus level upward (e.g., see Popham, 1979; Hodgson & Bernard, 1986; Healy, 1989; Hodgson et al., 1990; Garrido & Gallardo, 1996; Kafanov & Drozdov, 1998; Healy et al., 2000, 2006, 2008a,b, 2015; Keys & Healy, 2000; Le Pennec et al., 2002; Tyurin & Drozdov, 2003). In addition, characters have been used in cladistic analyses of bivalve relationships (Giribet & Wheeler, 2002; Bieler et al., 2014), where they have proven to be among the most informative subsets of characters (Bieler et al., 2014). Given their obvious importance as basal bivalves, it is surprising that very few protobranch species have been examined for sperm ultrastructure (Popham & Marshall, 1977; Franzén, 1983; Gustafson & Reid, 1988; Beninger & Le Pennec, 1997; Morse & Zardus, 1997; Tyurin & Drozdov, 2005) (see Table 1). Apart from Tyurin and Drozdov (2005), who compared spermatozoa of two species of

TABLE 1. Species of Protobranchia studied for sperm ultrastructure.

Species	Source
<i>Acharax alinae</i> Métivier & von Cosel, 1993	Beninger and Le Pennec (1997)
<i>Solemya reidi</i> F. R. Bernard, 1980	Gustafson and Reid (1988)
<i>Solemya velum</i> Say, 1822	present study
<i>Solemya velesiana</i> Iredale, 1931	present study
<i>Nucula sulcata</i> Bronn, 1831	Franzén (1983); present study
<i>Linucula hartvigiana</i> (Dohrn, 1864)	Popham and Marshall (1977) (as <i>Nucula hartvigiana</i>)
<i>Acila castrensis</i> (Hinds, 1843)	Morse and Zardus (1997); present study
<i>Ennucula</i> sp.	present study
<i>Nuculana pernula</i> (O. F. Müller, 1779)	present study
<i>Scaeolea caloundra</i> (Iredale, 1929)	present study
<i>Yoldia limatula</i> (Say, 1831)	Morse and Zardus (1997)
<i>Yoldia keppeliana</i> G. B. Sowerby III, 1904	Tyurin and Drozdov (2005)
<i>Yoldia notabilis</i> Yokoyama, 1922	Tyurin and Drozdov (2005)
<i>Yoldiella philippiana</i> (Nyst, 1845)	present study
<i>Neilonella corpulenta</i> (Dall, 1881)	present study

Yoldia (Nuculanidae), the remaining accounts have focused on sperm features of single species, aspects of gamete size (especially nuclear length) or spermatogenesis, or used micrographs in discussions of bivalve reproduction. Hence, the level of cellular (ultrastructural) detail provided has hitherto been insufficient to properly review relationships among protobranchs from the perspective of comparative sperm ultrastructure.

The present study aims at filling in this gap and thus examines sperm ultrastructure in nine species of Protobranchia, representing five extant families within Solemyoidea, Nuculoidea, and Nuculanoidea, and compares results obtained with those of previous accounts. In the light of all available sperm data (both light microscopic and ultrastructural), we then discuss relationships between the various groups of protobranchs, with emphasis on the potential taxonomic utility of this evidence, and also discuss possible implications for bivalve phylogeny. In addition, sperm data are here provided for three species of the Pectinidae (subclass Pteriomorpha, Pectinoidea) in order to demonstrate remarkable acrosomal and nuclear similarities to the Nuculanoidea.

Materials and Methods

Nine species of protobranchs were examined for sperm morphology using TEM (see Table 1). With the exception of *Solemya velesiana* Iredale, 1931 (see below), whole specimens or the testes only were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer containing w/v 10% sucrose. Small (2–3 mm³) pieces of testicular tissue were removed and processed using a BioWave microwave oven containing a ColdSpot (Pelco, Ted Pella Inc.), following the manufacturer’s instructions. Tissues were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer with 10% sucrose w/v and then dehydrated through an

ascending series of ethanol and infiltrated with Epon before overnight polymerization in a conventional oven at 60°C.

For *Solemya velesiana*, whole seawater-formalin-fixed specimens were accessed from the wet collection of the Australian Museum (Sydney) (AMC 159651). Small (2–3 mm³) pieces of testicular tissue were removed and further fixed in 3% glutaraldehyde prepared in seawater, then seawater rinsed before being osmicated (again using seawater as buffer). Following a final rinse in seawater, the tissue pieces were taken through a graded series of ethanol (20%–100%) before gradually being embedded in Spurr's Epoxy Resin and polymerized in a 60°C oven overnight.

In addition, three species of Pectinidae (Pectinoidea, Pteriomorpha) were examined for sperm TEM: *Gloripallium pallium* (Linnaeus, 1758), *Volachlamys singaporina* (Sowerby II, 1842), and *Annachlamys kuhnoltzi* (Bernardi, 1860). Processing details are as for *S. velesiana* except the material was live collected and fixed initially in 3.5% phosphate-buffered glutaraldehyde (with phosphate buffer also used for rinses and osmication).

For all protobranch species, ultrathin sections (70–80 nm) were cut using an Ultracut ultramicrotome (Leica EM UC6), stained with lead citrate and uranyl acetate, and photographed following observation on a Jeol 1011 transmission electron microscope (operated at 80 kV) equipped with a digital camera. For pectinid species, an LKB IV ultramicrotome and Hitachi 300 transmission electron microscope (operated at 80 kV) were used.

In the sperm ultrastructure descriptions, acrosomal vesicle and nucleus dimensions are based on five observations (N = 5) and on 10 (N = 10) for mitochondrial dimensions (with standard deviations) unless otherwise stated.

Collection data for the species studied (voucher material for samples with BivAToL numbers are deposited at the Field Museum of Natural History [FMNH], Chicago) and others as indicated follow: *Solemya velum* (BivAToL-358), USA, Massachusetts, Woods Hole, 41°30'00"N, 40°00'00"W; Sta-Biv-90, May 2010, Woods Hole Oceanographic Institution; *Nucula sulcata* (BivAToL-189), Sta-Biv-50, United Kingdom, Scotland, East of Isle of Arran, mud, 103 m, 55°35'06"N, 004°57'10"W, June 14, 2008, beam trawl, J. D. Taylor; *Acila castrensis* (BivAToL-205), Sta-Biv-60, North Pacific, USA, Washington, Lopez Island, shelf, 48°32'54"N, 122°55'36"W, December 5, 2008, D. Duggins; *Ennucula* sp. (BivAToL-89), sta-MB-014, Australia, dredged off Moreton Island, southeastern Queensland, 16.9 m, 27°22'25"N, 153°21'00"W, October 18, 2008; *Scaeoleda caloundra* (BivAToL-100), Sta-MB-037, Australia, dredged off Moreton Island, southeastern Queensland, 24 m, 27°23'40"N, 153°30'41"W, October 2008; *Nucula pernula* (BivAToL-147), Sweden, Tjärnö Marine Biological Laboratory, Strömstad, September 2008, J. Fuchs; *Yoldiella philippiana* (BivAToL-375), Sta-Biv-98, Sweden, near Tjärnö, 58°52'25"N, 011°06'11"E, September 10, 2010, 160 m, mud, J. D. Taylor and E. A. Glover; *Neilonella corpulenta* (BivAToL-218B [split from mixed lot also including *Neilonella whoii*]), sta-Biv-69, Atlantic, Gay Head-Bermuda transect, Etter station 6a,7a, 9a, 17a, 2200–3500 m, June 2008, J. D. Zardus; *Solemya velesiana* Australian Museum, Sydney, AMC 159651, Australia, Quarantine Bay, New South Wales, 37°04'35"N, 149°53'02"W; *Gloripallium pallium* (QMMO 66823), Australia, Queensland Museum, Brisbane, dived, under fallen coral plate, Heron Island reef, 9 m, 23°26'32"N, 151°54'53"W, March 1999; *Volachlamys singaporina* (QMMO 85751), Aus-

FIG. 1. Living specimen of *Ennucula* sp., photographed on October 18, 2008 (Moreton Bay, Queensland, Australia, 27°29'55.68"S, 153°24'8.64"E). Largest shell dimension approximately 12 mm; BivAToL-89.

tralia, dredged off Peel Island, Moreton Bay, southeastern Queensland, 10 m, 27°18'00"N, 1°12'36"W, February 1999; *Annachlamys kuhnoltzi* (QMMO 85752), Australia, dredged off Peel Island, Moreton Bay, southeastern Queensland, 10 m, 27°18'00"N, 153°12'36"W, February 1999.

The taxon here called *Ennucula* sp. (Fig. 1) could not be identified to species level; it was referred to as *Leionucula* cf. *cumingi*[i] by Sharma et al. (2013) because of its similarity with the species currently placed as *Ennucula cumingii* (Hinds, 1843) (e.g., Huber, 2015).

The nominal species here placed as *Scaeoleda caloundra* following Bieler et al. (2014) has variously been assigned to *Nuculana* or *Saccella* in the literature. Its actual affiliation will need further study, but we agree with Huber (2015, p. 360) that it “does neither represent a *Nuculana* and likely not a *Saccella*.”

Results

Spermatozoa of all species investigated exhibit (in antero-posterior sequence) the following components: an acrosomal complex (positioned at the nuclear apex), a highly electron-dense nucleus, a short midpiece (mitochondria surrounding two centrioles), and a single flagellum (“tail”) projecting posteriorly. Although a number of authors use the term “head” as a collective term for acrosomal complex + nucleus, we prefer to treat the acrosomal complex and nucleus as discrete components of the spermatozoon.

Order Solemyida (Solemyidae)

Solemya (Petrasma) velum Say, 1822

The acrosomal complex consists of a broad-conical, membrane-bound acrosomal vesicle (length $0.48 \pm 0.02 \mu\text{m}$, maximum diameter $0.5 \pm 0.02 \mu\text{m}$), rounded apically and deeply and widely invaginated posteriorly, and an extensive deposit of granular subacrosomal material filling the vesicle invagination (Fig. 2A–G). The complex is positioned on a short (length approximately $0.2 \mu\text{m}$), peg-shaped exten-

FIG. 2. Spermatozoa of *Solemya velum* (Solemyidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note examples of nuclear lacunae at periphery of nucleus. **B.** Transverse section (TS) near apex of acrosomal vesicle showing flattened profile of both vesicle and vesicle invagination (the latter filled with subacrosomal material) and radial plates within vesicle. **C–F.** TS acrosomal complex at successively more posterior levels than in **A**. Note initially flattened vesicle invagination (**C**) becomes circular (**D**) and then is intruded by apical extension of nucleus (**E**, **F**). Radial plates are visible at all levels. **G.** LS acrosomal complex and nuclear apex, showing plates within acrosome vesicle and peg-shaped extension of nucleus. **H–J.** LS (**H**) and TS (**I**, **J**) showing apparent malformations of the acrosomal vesicle and its invagination. **K.** TS nuclei showing scattered nuclear lacunae. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; c, centriolar pair; m, mitochondrion; n, nucleus; nl, nuclear lacuna; p, peg-shaped extension of nucleus; pm, plasma membrane; rp, radial plates within acrosomal vesicle; sm, subacrosomal material.

sion of the nucleus that projects into the posterior third of the vesicle invagination (Fig. 2E–G). Transverse sections reveal the presence of 43–47 angularly oriented, dense plates within the markedly less dense contents of the acrosomal vesicle (Fig. 2B–F). Such sections also show that the anterior region of the acrosomal vesicle is laterally compressed (Fig. 2B). Figure 2C shows that the invagination of the vesicle retains this compressed profile for a distance posteriorly even after the acrosomal vesicle profile has become circular. In some spermatozoa, the acrosomal vesicle showed clear evidence of malformation (Fig. 2H–J), with a projecting limb developed from the vesicle wall.

The nucleus is $4.5 \pm 0.5 \mu\text{m}$ long widest ($1.1 \pm 0.1 \mu\text{m}$) basally and tapers toward the acrosomal complex (Fig. 2A, G–K). Whereas the apex features a peg-shaped extension, the base of the nucleus has five shallow depressions associated with the midpiece mitochondria in addition to a small, centrally positioned depression associated with the proximal centriole (Fig. 3A, B). Nuclear contents are conspicuously granular in appearance, with the exception of occasional electron-lucent lacunae, especially laterally.

The midpiece region is composed of five (or less commonly four) mitochondria (maximum diameter $0.65 \pm 0.05 \mu\text{m}$, each showing irregular cristae), packed tightly around the orthogonally arranged proximal and distal centrioles (Figs. 2A, 3A–G). Although the proximal centriole is positioned outside the centriolar fossa, a deposit of pericentriolar material connects this centriole to the fossa (Fig. 3A, B). Although the mitochondria appear ovoid in longitudinal section (Fig. 3A, B), in transverse section they are angular in profile due to flattening of their surfaces where contacting each other (Fig. 3C, E–G). An array of nine, club-shaped satellite fibers attaches the distal centriole and the annulus associated with the plasma membrane (Fig. 3D). The flagellum, which is continuous with the distal centriole (Fig. 3A, B), consists of an axoneme (composed of nine doublet microtubules surrounding a central pair of singlet microtubules) sheathed by the plasma membrane (Fig. 3B, F).

Solemya (Solemyarina) velesiana Iredale, 1931

The acrosomal complex consists of a tall-conical, deeply invaginated, membrane-bound acrosomal vesicle (length $0.5 \pm 0.02 \mu\text{m}$, maximum width at base $0.3 \pm 0.02 \mu\text{m}$) and granular subacrosomal material within the vesicle invagination (Fig. 4A, B, D, E). The acrosomal vesicle (and its invagination) is circular in transverse section throughout its entire length and clearly shows the presence of short radial plates (40–45 in number) (Fig. 4D, E). In longitudinal section, the plates appear as a continuous dense layer within the vesicle, which basally is recurved (Fig. 4B). The apex of the nucleus, on which the acrosomal complex sits, is either flat or slightly convex (Fig. 4).

Longitudinal sections reveal that the nucleus has a teardrop shape and is $6.2 \pm 0.2 \mu\text{m}$ long (Fig. 4A, C). It is widest posteriorly (maximum diameter $1.65 \pm 0.05 \mu\text{m}$) and tapers anteriorly to approximately $0.25 \mu\text{m}$ in diameter near the apex (Fig. 4A, C, D). Occasional electron-lucent lacunae, of rounded shape, are seen with the nucleus, most commonly near the nuclear surface (Fig. 4A, F). Basally, the nucleus exhibits five (or more rarely six) shallow depressions that fit the anterior portion of each of the midpiece mitochondria as well as a centrally positioned, centriolar fossa that is partially occupied by pericentriolar material and the anterior half of the proximal centriole (Fig. 4F–I).

The midpiece consists of five (or less commonly six) ovoid mitochondria (diameter $0.55 \pm 0.05 \mu\text{m}$) grouped around the proximal and distal centrioles (arranged at right angles to each other) (Fig. 4F–J). Each mitochondrion shows an ovoid profile in longitudinal section (Fig. 4F, G) but in transverse section has flattened surfaces wherever pressed against each other (Fig. 4J). The flagellum is continuous with the distal centriole.

Order Nuculida (Nuculidae)

Acila castrensis (Hinds, 1843)

The acrosomal complex consists of a low, broadly conical, membrane-bound acrosomal vesicle that is convex apically and deeply and widely invaginated posteriorly and also an extensive deposit of granular subacrosomal material filling the vesicle invagination (Fig. 5A–E). The acrosomal vesicle is $0.44 \pm 0.02 \mu\text{m}$ long and has a maximum diameter (near the base) of $0.63 \pm 0.02 \mu\text{m}$. The subacrosomal material both separates the acrosomal vesicle from the nuclear apex (a gap of approximately $0.14 \mu\text{m}$) and is attached to it (Fig. 5B, C). Transverse sections show that the acrosomal vesicle (and its basal invagination) is almost circular in profile throughout its length (Fig. 5D, E). Contents of the acrosomal vesicle are finely granular and moderately electron-dense, with the exception of a thin, comparatively electron-lucent peripheral layer (Fig. 5B, C).

The nucleus is rod-shaped, $9.4 \pm 0.2 \mu\text{m}$ long, widest ($2.0 \pm 0.01 \mu\text{m}$) posteriorly, and tapered anteriorly (Fig. 5A). The nuclear apex is flat or very slightly convex (Fig. 5B, C). Basally, the nucleus shows five (or rarely four) curved depressions, each associated with one of the midpiece mitochondria, and also a small centrally positioned depression filled with pericentriolar material (Fig. 5A, F, G). Nuclear contents are conspicuously granular in their appearance, with the exception of occasional, spheroidal, electron-lucent lacunae (Fig. 5A, G).

The midpiece region is composed of five (or more rarely four) spheroidal mitochondria (maximum diameter $1.0 \pm 0.01 \mu\text{m}$, each showing irregular cristae) grouped around the orthogonally arranged proximal and distal centrioles (Fig. 5A, F–I). Transverse sections show that the mitochondria are almost circular in profile, with only slight lateral compression at points of contact (Fig. 5H, I). Both centrioles show the typical arrangement of triplet microtubular elements, although this is often masked by pericentriolar material. Pericentriolar material attaches the proximal centriole to the nucleus, as well as connecting the centrioles to each other, and also forms a distinct mass associated with the distal centriole (Fig. 5F). Posteriorly, the triplets of the distal centriole give way to doublets as part of the transition to the axonemal microtubular arrangement of the flagellum (Fig. 5F). An array of nine satellite fibers attaches the distal centriole to the plasma membrane (Fig. 5F, J). These fibers are thickest anteriorly, but posteriorly they narrow and are terminally forked where attached to the annulus and plasma membrane (Fig. 5J). The flagellum consists of an axoneme (composed of nine doublet microtubules surrounding a central pair of singlet microtubules) sheathed by the plasma membrane (Fig. 5K). The axonemal doublets are continuous with the triplet microtubules of the distal centriole, whereas the central pair of microtubules begins immediately posterior to the distal centriole (Fig. 5F).

FIG. 3. Spermatozoa of *Solemya velum* (Solemyidae). **A.** Longitudinal section (LS) through base of nucleus, midpiece, and initial portion of flagellum. Proximal and distal centrioles surrounded by ovate mitochondria. Note shallow nuclear fossa above proximal centriole filled with granular material. **B.** LS showing satellite fibers attaching distal centriole to annulus lining plasma membrane. **C.** Transverse section (TS) through midpiece (showing five mitochondria) at level of distal centriole. **D.** TS satellite fiber complex. Each fiber is associated with a triplet of the distal centriole and has a splayed end attaching to the annulus. **E.** TS midpiece at level of proximal centriole. **F.** TS midpiece and flagellum. **G.** TS midpiece (at level of proximal centriole) showing only four mitochondria. Abbreviations: an, annulus; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; pc, proximal centriole; sf, satellite fibers.

FIG. 4. Spermatozoa of *Solemya velesiana* (Solemyidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note nuclear lacunae at periphery of nucleus. **B.** LS acrosomal complex and nuclear apex. **C.** Survey micrograph showing teardrop shape of nucleus and rounded transverse profiles of nuclei. **D.** Transverse sections (TS) nuclear apex and acrosomal complex at anterior and posterior regions. **E.** TS showing detail of radiating plates within acrosomal vesicle. **F.** Longitudinal section (LS) through base of nucleus, entire midpiece, and initial portion of flagellum. Proximal and distal centrioles surrounded by ovate mitochondria. **G.** Longitudinal section (LS) through base of nucleus, midpiece and initial portion of flagellum. Proximal and distal centrioles surrounded by ovate mitochondria. **H.** TS junction of nucleus and midpiece. Note five depressions for mitochondria and central nuclear fossa. **I.** TS posterior to level shown in **H.** **J.** TS midpiece at level of distal centriole showing five mitochondria. Abbreviations: a, acrosomal complex; an, annulus; av, acrosomal vesicle; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; nl, nuclear lacunae; pc, proximal centriole; rp, radial plates within acrosomal vesicle; sf, satellite fibers; sm, subacrosomal material.

FIG. 5. Spermatozoa of *Acila castrensis* (Nuculidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note nuclear lacunae at periphery of nucleus. **B, C.** LS acrosomal complex and nuclear apex. **D, E.** Transverse sections (TS) at anterior and posterior regions of acrosomal complex. **F.** LS through base of nucleus, entire midpiece, and initial portion of flagellum. Proximal and distal centrioles surrounded by spherical mitochondria. **G.** TS junction of nucleus and midpiece. Note five depressions for mitochondria and central nuclear fossa. **H.** TS midpiece at level of proximal centriole showing five spherical mitochondria. **I.** TS midpiece showing rarer four-mitochondria condition. **J.** TS satellite fiber complex. Each fiber is associated with a triplet of the distal centriole and has a splayed end attaching to the annulus. **K.** TS flagella showing 9 + 2 microtubular arrangement of axoneme. Abbreviations: a, acrosomal complex; an, annulus; av, acrosomal vesicle; c, centriolar pair; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; pc, proximal centriole; sf, satellite fibers; sm, subacrosomal material.

FIG. 6. Spermatozoa of *Nucula sulcata* (Nuculidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note nuclear lacunae at periphery of nucleus. **B.** LS acrosomal complex and nuclear apex. Note electron-lucent spaces at tip of acrosomal vesicle. **C.** LS acrosomal complex and nuclear apex. Electron-lucent spaces absent from acrosomal vesicle. **D, E.** Transverse sections (TS) at anterior and posterior regions of acrosomal complex. **F.** TS nuclear apex. **G.** LS through base of nucleus, entire midpiece, and initial portion of flagellum. Proximal and distal centrioles surrounded by spherical mitochondria. **H.** TS midpiece showing slight angular disposition of centrioles relative to each other and satellite fibers projecting into annulus. **I.** TS detail of mitochondria surrounding proximal centriole near base of nucleus. **J.** TS midpiece at level of proximal centriole showing five mitochondria. **K.** Oblique junction of nucleus and midpiece showing four of the nine satellite fibers associated with distal centriole. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; nl, nuclear lacuna; pc, proximal centriole; sf, satellite fibers; sm, subacrosomal material.

The acrosomal complex consists of a low, broadly conical, membrane-bound acrosomal vesicle (length $0.34 \pm 0.2 \mu\text{m}$, maximum diameter $0.48 \pm 0.1 \mu\text{m}$) and an extensive deposit of finely granular subacrosomal material that fills the invagination of the vesicle and the gap between the vesicle and nuclear apex (Fig. 6A–E). Transverse sections show that the acrosomal vesicle (and its basal invagination) is almost circular in profile throughout its length (Fig. 6D, E). Contents of the acrosomal vesicle are finely granular and moderately electron-dense, with the exception of a thin, relatively electron-lucent peripheral layer (Fig. 6B–E). Occasional electron-lucent lacunae were observed near the apical region of the vesicle (Fig. 6B), although it is possible these might have been the result of imperfect fixation or even partial onset of an acrosomal reaction.

The nucleus is rod-shaped, $8.0 \pm 0.2 \mu\text{m}$ long, widest posteriorly ($1.84 \pm 0.2 \mu\text{m}$), and tapers noticeably toward the apex (Fig. 6A, F). Apically, the nucleus is slightly convex and basally shows five (or occasionally six) shallow, curved depressions (associated with the midpiece mitochondria) in addition to a small (approximately $0.14 \mu\text{m}$ deep) fossa associated with pericentriolar material (Fig. 6A, G). Nuclear contents are conspicuously granular in appearance (Fig. 6B, C, F, G), with the exception of occasional spherical electron-lucent lacunae (Fig. 6A).

The midpiece consists of five spheroidal mitochondria (diameter $0.82 \pm 0.2 \mu\text{m}$, each showing irregular cristae) grouped around the orthogonally arranged proximal and distal centrioles (Fig. 6A, G–K). Where mitochondria contact each other, their surfaces are flat, indicating a certain degree of compression (Fig. 6I, J). Due to the presence of fine pericentriolar material, the microtubular composition of the two centrioles (triplets) was masked and impossible to properly demonstrate. Posteriorly, the triplets of the distal centriole give way to doublets as part of the transition to the axonemal microtubular arrangement. An array of nine club-shaped satellite fibers attaches the distal centriole to the plasma membrane and associated annulus (Fig. 6G, H, K). The flagellar axonemal doublets are continuous with the triplet microtubules of the distal centriole, whereas the central pair of microtubules begins immediately posterior to the distal centriole (Fig. 6G, H).

Ennucula sp.

The acrosomal complex consists of a very low, broadly conical, membrane-bound acrosomal vesicle (length $0.27 \pm 0.2 \mu\text{m}$, maximum diameter $0.5 \pm 0.2 \mu\text{m}$), which is convex apically and deeply and widely invaginated posteriorly, and also a diffuse deposit of granular subacrosomal material within the vesicle invagination (Fig. 7A–D). Transverse sections show that the acrosomal vesicle (and its basal invagination) is almost circular in profile throughout its length (Fig. 7C, D). Contents of the acrosomal vesicle are finely granular and moderately electron-dense, with the exception of a thin, relatively electron-lucent peripheral layer (Fig. 7B, D). A small bulge in the plasma membrane is often observed at the apex of the acrosomal vesicle, and it is possible that this represents a first stage of the acrosome reaction (Fig. 7B).

The nucleus is rod-shaped, $4.0 \pm 0.2 \mu\text{m}$ long, widest ($1.25 \pm 0.1 \mu\text{m}$) centrally, and tapered slightly posteriorly and markedly so anteriorly (Fig. 7A). The nuclear apex is slightly convex (Fig. 7B). The base of the nucleus has five shallow de-

pressions associated with the midpiece mitochondria in addition to a shallow centriolar fossa that is filled with granular material associated with the proximal centriole (Fig. 7A, E, H). Nuclear contents are very electron-dense, granular in fiber profile, and also exhibit numerous, oblong to elongate, jagged lacunae (Fig. 7A, B, E).

The midpiece consists of five spheroidal mitochondria (diameter $0.8 \pm 0.1 \mu\text{m}$), each showing irregular cristae) grouped around the orthogonally arranged proximal and distal centrioles (Fig. 7E, G). Slight compression of the mitochondrial surfaces occurs where they contact each other. The proximal and distal centrioles show the typical arrangement of triplet microtubular elements. Posteriorly, the triplets of the distal centriole give way to doublets as part of the transition to the axonemal microtubular arrangement. An array of nine satellite fibers attaches the distal centriole to the annulus and plasma membrane (Fig. 7E–G). These fibers are thickest anteriorly, and posteriorly they narrow and terminally fork in the region of the annulus (Fig. 7F, G). Putative glycogen granules occur around the centrioles and satellite fibers (Fig. 7G). The flagellum shows a standard $9 + 2$ axoneme, sheathed by the plasma membrane (Fig. 7J). The axonemal doublets are continuous with the triplet microtubules of the distal centriole, whereas the central pair of microtubules begins immediately posterior to the distal centriole (Fig. 7E).

Order Nuculanida (Nuculanidae)

Scaeolea caloundra (Iredale, 1929)

The acrosomal complex consists of a conical, membrane-bound acrosomal vesicle (apically and deeply and widely invaginated posteriorly) and a loose deposit of coarsely granular subacrosomal material that fills both the vesicle invagination and the curved shallow depression of the nuclear apex (Fig. 8A, C). The acrosomal vesicle is $0.38 \pm 0.2 \mu\text{m}$ long and has a maximum diameter (basally) of $0.56 \pm 0.1 \mu\text{m}$. Dense radial plates, visibly longitudinally as a recurved layer, are present within the acrosomal vesicle and number 33–36 (Fig. 8C, D).

The nucleus is spheroidal but wider ($2.3 \pm 0.2 \mu\text{m}$) than long ($1.85 \pm 0.2 \mu\text{m}$), with a shallow (approximately $0.25 \mu\text{m}$ deep) but still conspicuous anterior depression filled with coarse granular, subacrosomal material (Fig. 8A, B). Nuclear contents are fibrillar but appear granular in section, with occasional spherical lacunae also visible (Fig. 8B, C). Basally, there are four shallow, curved depressions to accommodate the anterior portion of the midpiece mitochondria in addition to a small but discrete centriolar fossa positioned at the center of the larger depressions (Fig. 8A, E, G).

Four spheroidal mitochondria (maximum diameter $0.95 \pm 0.05 \mu\text{m}$; containing irregular, slightly inflated cristae) surround the proximal and distal centrioles to collectively form the midpiece (Fig. 8A, E–G). The large gap between the centrioles and the base of the nucleus (Fig. 8E) is almost certainly an artifact of fixation (osmotic stress). Satellite fibers are associated with the distal centriole (Fig. 8E) but were not observed in transverse section. The flagellum progresses from and is continuous with the distal centriole (Fig. 8A, E).

Nuculana pernula (O. F. Müller, 1779)

The acrosomal complex consists of a squat-conical acrosomal vesicle (length $0.24 \pm 0.05 \mu\text{m}$; maximum width $0.65 \pm$

FIG. 7. Spermatozoa of *Ennucula* sp. (Nuculidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note large, ragged nuclear lacunae throughout nucleus. **B.** LS acrosomal complex and nuclear apex. **C, D.** Transverse sections (TS) at anterior and posterior regions of acrosomal complex. **E.** LS through base of nucleus, entire midpiece, and initial portion of flagellum. **F.** TS satellite fiber complex. Each fiber is associated with a triplet of the distal centriole and has a splayed end attaching to the annulus. **G.** Oblique junction of nucleus and midpiece showing four of the nine satellite fibers associated with distal centriole. **H.** TS junction of nucleus and midpiece. Note depressions for mitochondria and central nuclear fossa. **I.** TS midpiece at level of proximal centriole showing five spherical mitochondria. **J.** TS flagella showing 9 + 2 microtubular arrangement of axoneme. Abbreviations: a, acrosomal complex; an, annulus; av, acrosomal vesicle; dc, distal centriole; f, flagellum; g, putative glycogen granules; m, mitochondrion; n, nucleus; nl, nuclear lacuna; pc, proximal centriole; sf, satellite fibers; sm, subacrosomal material.

FIG. 8. Spermatozoa of *Scaeoleda caloundra* (Nuculanidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note spherical nuclear lacunae. **B.** Survey micrograph showing spherical sperm nuclei. **C.** LS acrosomal complex and nuclear apex. Note shallow nuclear depression filled with subacrosomal material. **D.** Transverse section (TS) through middle region of acrosome complex showing radial plates associated with acrosomal vesicle. **E.** LS nuclear base, entire midpiece, and proximal portion of flagellum. The centriolar pair have probably been displaced through suboptimal fixation. **F.** TS midpiece with four spherical mitochondria (apparent absence of centriole probably due to displacement). **G.** Oblique TS showing four mitochondria lodged in dish-shaped depressions of the nuclear base. Nuclear fossa and nuclear lacuna also visible. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; c, centriolar pair; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; nl, nuclear lacuna; pc, proximal centriole; pm, plasma membrane; rp, radial plates within acrosomal vesicle; sf, satellite fibers; sm, subacrosomal material.

0.05 μm) and a diffuse, coarsely granular deposit of subacrosomal material, the latter associated mainly with the anterior depression of the nucleus (Fig. 9A–D). As Figure 9B–D shows, the vesicle is strongly overlapped by the anterior depression of the nucleus. Dense radial plates are very well developed within the acrosomal vesicle and number 33–36 (Fig. 9B–D). These plates collectively form a recurved dense layer in longitudinal sections (Fig. 9B).

The nucleus is spheroidal but markedly wider ($2.6 \pm 0.2 \mu\text{m}$) than long ($2.2 \pm 0.2 \mu\text{m}$), with a moderately deep (approximately 0.25 μm) anterior depression filled with coarse, granular subacrosomal material (Fig. 9A, B). Nuclear contents are fibrillar but appear granular in section, with occasional, small, rounded lacunae also visible (Fig. 9A–E). Basally, there are curved depressions to accommodate the anterior portion of each of the four midpiece mitochondria (Fig. 9A) in addition to a small but discrete centriolar fossa (Fig. 9E).

Four spheroidal mitochondria (maximum diameter $1.0 \pm 0.1 \mu\text{m}$) surround the proximal and distal centrioles to collectively form the midpiece (Fig. 9A, F, G). Cristae appear to be irregularly arranged. The flagellum progresses from and is continuous with the distal centriole.

Order Nuculanida (Yoldiidae)

Yoldiella philippiana (Nyst, 1845)

The acrosomal complex consists of a short-conical acrosomal vesicle (length $0.34 \pm 0.02 \mu\text{m}$; maximum width $0.5 \pm 0.02 \mu\text{m}$) and an extensive, finely granular deposit of subacrosomal material that occupies the vesicle invagination and the 0.17- μm -deep anterior depression of the nucleus (Fig. 10A, B, E). Contents of the acrosomal vesicle exhibit a dense recurved layer and a relatively electron-dense layer peripherally (Fig. 10B–E). Radial plates are very faintly visible within the acrosomal vesicle, but their number could not be ascertained (Fig. 10E).

The nucleus is spheroidal but wider ($2.5 \pm 0.1 \mu\text{m}$) than long ($2.0 \pm 0.1 \mu\text{m}$). Anteriorly, it exhibits a curved depression (depth approximately 0.17 μm) filled with subacrosomal material (Fig. 10A, B). Posteriorly, the nucleus shows four shallow, curved depressions that accommodate the anterior portions of the midpiece mitochondria in addition to a small centriolar fossa positioned at the center of the larger depressions (Fig. 10A, F). Nuclear contents are fibrillar but appear granular in section, with occasional small, spherical or oblong, electron-lucent lacunae (Fig. 10A, B).

The midpiece consists of four spheroidal mitochondria (maximum diameter $1.0 \pm 0.1 \mu\text{m}$, each with irregular cristae) surrounding the proximal and distal centrioles (Fig. 10A, F, G). Pericentriolar material satellite fibers connect the distal centriole to the annulus and plasma membrane (Fig. 10A, F), but, unfortunately, no favorable sections showing them in transverse section were observed. The flagellum progresses from and is continuous with the distal centriole (Fig. 10A, F).

Order Nuculanida (Neilonellidae)

Neilonella corpulenta (Dall, 1881)

Few spermatozoa were encountered in the testicular tissue available for this species, and the only two micrographs taken

(Fig. 11A, B) allow only an incomplete description and reconstruction.

The acrosomal complex consists of a short-conical acrosomal vesicle (length 0.43 μm ; maximum width 0.43 μm) ($N = 1$) and an extensive, coarsely granular deposit of subacrosomal material associated with the vesicle invagination and especially the anterior depression (depth approximately 0.19 μm) of the nucleus (Fig. 11A). The highly electron-dense layer visible within the acrosomal vesicle (Fig. 11A) indicates the likely presence of radiating plates, but no corresponding transverse sections were obtained to confirm this.

The nucleus is spheroidal, approximately 2.5 μm long and 3.0 μm wide at its widest diameter ($N = 2$). The anterior depression is occupied by subacrosomal material. Posteriorly, the nucleus shows shallow, curved depressions that accommodate the anterior portion of the midpiece mitochondria (number not determined), and presumably a small centriolar fossa is also present although not verifiable here (Fig. 11B). Nuclear contents are fibrillar but appear granular in section, with occasional small, spherical, electron-lucent lacunae (Fig. 11A, B).

The midpiece exhibits spheroidal mitochondria at the base of the nucleus, but, unfortunately, no favorable sections were obtained to establish the exact number (three are visible in Fig. 11B), the arrangement of centrioles, and/or the presence of a centriolar fossa or satellite fiber complex.

Although a flagellum is present (Fig. 11B), its structure was not determined; presumably, it features a standard $9 + 2$ axoneme as per other species of protobranchs examined herein.

Pectinoidean Spermatozoa

Figure 11C–I (combined key results for the pectinids *Gloripallium pallium*, *Volachlamys singaporina*, and *Annachlamys kuhnholtzi*) is presented to demonstrate the striking similarities between the Pectinoidea and Nuculanoidea, most notably in relation to the morphology of the acrosomal complex and the nuclear apex.

The conical acrosomal vesicle of pectinoids exhibits radial plates within the dense layer (usually recurved), and the extensive deposit of granular, subacrosomal material (always lacking an axial rod) fills both the invagination of the acrosomal vesicle and a wide anterior depression of the nuclear apex (Fig. 11E–I). The number of radiating plates within the acrosomal vesicle ranges from 40–45 (Fig. 11E, H). Although the nucleus of pectinoid sperm is known to vary widely between taxa (barrel-shaped, conical [Fig. 11C, D], spheroidal, and almost rod-shaped; see Le Pennec et al., 2002), the anterior depression (filled with subacrosomal material) is always retained. Mitochondrial number (not illustrated here, but see Dorange & Le Pennec, 1989; Healy et al., 2001; Tyurin & Drozdov, 2003) in the Pectinoidea is almost always four, whereas in Nuculanoidea the number varies between four and five, depending on the taxon investigated.

Discussion

Protobranchs exhibit wide diversity in sperm morphology, matched elsewhere in the Bivalvia only by mytiloidean Pteriomorpha (see Kafanov & Drozdov, 1998) and heterodont groups, such as the Galeommatoidea, Tellinoidea, Cardioidea, and Veneroidea (e.g., Eckelbarger et al., 1990; Healy, 1995a;

FIG. 9. Spermatozoa of *Nuculana pernula* (Nuculanidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note spherical nuclear lacunae. **B.** LS acrosomal complex and nuclear apex. Note wide, deep nuclear depression filled with subacrosomal material. **C, D.** Oblique transverse sections (TS) through acrosomal complex showing radial plates associated with acrosomal vesicle. Two small nuclear lacunae are visible in side of nucleus shown in **D.** **E.** TS nucleus at very base of anterior depression (= central electron-lucent space). **F.** TS midpiece showing part of nuclear base. Four spherical mitochondria surround the proximal centriole. **G.** TS midpiece probably at apex of distal centriole. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; f, flagellum; m, mitochondrion; n, nucleus; nl, nuclear lacuna; pc, proximal centriole; rp, radial plates within acrosomal vesicle; sm, subacrosomal material.

FIG. 10. Spermatozoa of *Yoldiella philippiana* (Yoldiellidae). **A.** Longitudinal section (LS) through acrosomal complex, nucleus, and midpiece. Note nuclear lacunae at periphery of nucleus. **B.** LS acrosomal complex and nuclear apex. Note wide, moderately deep nuclear depression filled with subacrosomal material. **C–E.** Transverse sections (TS) at anterior, middle, and posterior regions of acrosomal complex (**E** showing partial overlap with nucleus). **F.** LS nuclear base, midpiece, and proximal portion of flagellum. Satellite fibers connect the distal centriole to annulus and plasma membrane. Four flagella are also visible in TS. **G.** TS midpiece showing four spherical mitochondria surrounding probable anterior edge of distal centriole. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; c, centriolar pair; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; pc, proximal centriole; pm, plasma membrane; rp, radial plates within acrosomal vesicle; sf, satellite fibers; sm, subacrosomal material.

FIG. 11. Spermatozoa of *Neilonella corpulenta* (Neilonellidae) (A, B) and representative Pectindae (C–I). A. Longitudinal section through acrosomal complex and nuclear apex. Note subacrosomal material diffuse within invagination of vesicle but well developed within apical depression of nucleus. B. LS nucleus and portion of midpiece. Note spherical nuclear lacunae. C. *Gloripallium pallium*. LS acrosomal complex, nucleus, midpiece, and proximal portion of flagellum. D–F. *Volachlamys singaporina*. D. LS acrosomal complex, nucleus, and midpiece. E. LS acrosomal complex and nuclear apex. F. Transverse section (TS) acrosomal complex. G, H. *Annachlamys kuhnoltzi*. G. LS acrosomal complex and nuclear apex. H. TS acrosomal complex. I. *Gloripallium pallium*. TS acrosomal complex. Abbreviations: a, acrosomal apex; av, acrosomal vesicle; dc, distal centriole; f, flagellum; m, mitochondrion; n, nucleus; nl, nuclear lacuna; pc, proximal centriole; rp, radial plates within acrosomal vesicle; sm, subacrosomal material.

Jespersen et al., 2001, 2002, 2009; Healy et al., 2006, 2008a). It seems likely that such diversity is associated with reproductive flexibility given the fact that it occurs most often in groups that are highly speciose and widespread. Sperm morphology for all animal species appears to have three main determining factors: phylogenetic affiliation, corresponding egg (mature oocyte) morphology, and the environment of fertilization (Franzén, 1955, 1956, 1977, 1983; Afzelius, 1972, 1983; Anderson & Personne, 1976; Baccetti & Afzelius, 1976; Rouse & Jamieson, 1987; Jamieson & Rouse, 1989; Beninger & Le Pennec, 1997; Drozdov & Tyurin, 2014). Hence, ideally, sperm ultrastructure is most useful for addressing taxonomic and/or phylogenetic questions in which egg morphology (size, yolk content, and ultrastructure) and the environment of fertilization are the same or similar in all studied taxa. Unfortunately, information on the environment of fertilization, egg size, and/or ultrastructure in protobranchs is limited, and hence it is still uncertain how these factors have impacted sperm morphology in the group. Other than the work of Drew (1899), it appears that direct observational data on the environment of fertilization in protobranchs are lacking, although in known brooders it can be inferred that fertilization takes place within the mantle cavity rather than externally. Popham (1974a) concluded that the acrosomal vesicle in externally fertilizing shipworms (Teredinidae) is larger than in those that fertilize “internally” (i.e., within the mantle cavity). Although this “rule” might hold for teredinids and might apply more widely in the Bivalvia, the evidence for it in protobranchs is either still needed or not supported (e.g., the huge [7.0 μm] acrosome in the brooding solemyid *Acharax alinae* Métivier & Cosel, 1993; see Beninger & Le Pennec, 1997).

No large-scale, comparative ultrastructural (TEM) studies of bivalve eggs have yet been published, and detailed knowledge of sperm–egg interaction is still fragmentary (for summaries and literature, see Hylander & Summers, 1977; Kyo-zuka & Osanai, 1985; Hosokawa & Noda, 1994). Even information on egg size is widely scattered in the literature and variable in quality (e.g., authors often do not differentiate between mature oocyte diameter and released egg diameter, and cited figures might or might not include a jelly-layer component). Egg diameter varies widely among protobranchs and, as noted by Zardus (2002), is not always a reliable indicator of developmental mode. Morse and Zardus (1997) gave a diameter size range of 90–270 μm for the subclass, although it is now known that some species have eggs up to 600 μm (the solemyoid *Acharax alinae* Métivier & von Cosel, 1993; Beninger & Le Pennec, 1997) or as small as 50 μm (the nukuloid *Nucula pusilla* Angas, 1877; Morton, 2012). Franzén (1983) was the first to attempt some comparison of sperm component size and egg size in Bivalvia, noting that there appears to be a positive correlation of increased egg diameter (and yolk content) and length of the nucleus. Beninger and Le Pennec (1997), using primarily information from the literature on bivalve egg diameters and sperm head + midpiece lengths, concluded that Franzén’s view had some support, at least for taxa with large eggs, such as the solemyoidean that they investigated, *A. alinae*, which has not only a very long acrosome (7.0 μm) but possibly the longest sperm nucleus (19.6 μm) and largest egg (600 μm) of any bivalve. They argued that “the unusually large size of the spermatozoon in *A. alinae* is thus probably a co-evolutionary consequence of the extremely large oocyte size” (Beninger & Le Pennec, 1997, p. 204). Although it could be generally true that broadcast spawning bivalves tend to have smaller eggs than

those of brooders (Strathman, 1987; Morse & Zardus, 1997), there is a sizable “gray area” of egg size (approximately 70–120- μm egg diameter range) in which many exceptions to the rule exist, and it is clear that much remains to be learned about the exact influence that egg structure and environment of fertilization have on sperm ultrastructure in the class. It would, for example, be interesting to determine sperm ultrastructure and fertilization biology in *N. pusilla* given that it has the smallest egg size of all protobranchs that have been examined to date (50 μm ; Morton, 2012) and is a known brooder (Bergmans, 1978).

Table 2 summarizes information drawn from published sources on egg sizes in Protobranchia and (where available) corresponding sperm acrosomal and nuclear lengths (new data from the present study). For most species with sperm and egg data available (9 of 22 species listed), the table reveals no compelling correlation between acrosomal vesicle length or nuclear length and egg diameter.

Structural Comparisons

ACROSOMAL COMPLEX—The shape and length of the acrosomal vesicle varies widely among bivalve taxa, although, as noted by several authors (e.g., Hylander & Summers, 1977; Popham, 1979; Baccetti, 1979; Franzén, 1983; Rosati & Focarelli, 1996), it is highly likely that these sperm features are in part dependent on the morphology of the egg with which it must interact. In the Protobranchia, it is interesting to observe that the Solemyida exhibits one of the most extreme ranges of acrosomal vesicle shapes within the Bivalvia, ranging from low-conical in *Solemya reidi* F. R. Bernard, 1980,¹ to tall-conical in *S. velum* and *S. velesiana* and ultimately to the very elongate (7.0 μm) conical vesicle in the vent species *Acharax alinae* (Gustafson & Reid, 1988; Beninger & Le Pennec, 1997; present study [see summary in Fig. 12]). Only the venerid *Nutricula tantilla* (Gould, 1853) has a longer acrosomal vesicle than *A. alinae*, with a total length of 15.0 μm (Thompson, 1973). In all investigated protobranch species, the acrosomal vesicle and its invagination are both oval to circular in transverse profile, as is typical of other Bivalvia, but exceptionally in *S. velum*, the anterior region of the vesicle and vesicle invagination are flattened in profile, whereas posteriorly both are circular. Nothing comparable to this has previously been observed in other bivalves, although in a number of caenogastropods, flattening of almost the entire acrosomal vesicle and vesicle invagination is well known but of uncertain significance (Giusti, 1971; Healy, 1983; Kohnert & Storch, 1984; Koike, 1985; Hodgson & Heller, 2000). Certainly, a comparative ultrastructural study of the egg morphology in the Solemyidae will be required to assess the degree to which egg envelope structure might have influenced the highly varied acrosomal shape observed in this family and hopefully shed light on the unusual acrosomal complex of *S. velum*.

Malformation of the acrosomal complex in some spermatozoa of *S. velum* (see Fig. 2H–J) is presumably due to faulty development during spermiogenesis, specifically the functioning of the Golgi apparatus (we did not observe any other structural problems in this species). Apical protrusion of the plasma

¹*Solemya reidi* was synonymized under *Solemya pervernica* Kuroda, 1948, by Kamenev (2009) but considered distinct by Huber (2015, p. 358).

TABLE 2. Comparison of egg (mature oocyte) diameter and available sperm data for Protobranchia.

Taxon	Higher taxon	Maximum egg diameter (µm)	Sperm acrosomal vesicle length (µm)	Sperm nuclear length (µm)	Reference (egg data)	Reference (sperm data)
<i>Acharax alinae</i> Métivier & von Cosel, 1993	Solemyida	600	7.0	19.6	Beninger and Le Pennec (1997)	Beninger and Le Pennec (1997)
<i>Solenya reidi</i> F. R. Bernard, 1980	Solemyida	271	0.4	4.2	Gustafson and Reid (1986)	Gustafson and Reid (1988)
<i>Ennucula delphinodonta</i> (Mighels & C. B. Adams, 1842)	Nuculida	210	—	—	Drew (1899) (as <i>Nucula delphinodonta</i>)	—
<i>Solenya velum</i> Say, 1822	Solemyida	200	0.48	4.5	Gustafson and Reid (1986)	present study
<i>Pristigloma nitens</i> (Jeffreys, 1876)	Nuculanida	190	—	—	Sanders and Allen (1973)	—
<i>Yoldiella sabrina</i> (Hedley, 1916)	Nuculanida	187.9	—	—	Reed et al. (2014)	—
<i>Nucula nucleus</i> (Linnaeus, 1758)	Nuculida	180	—	7.0	Franzén (1983)	Franzén (1983)
<i>Nucula sulcata</i> Bronn, 1831	Nuculida	160	0.34	7.9–8.2	Franzén (1983)	Franzén (1983); present study
<i>Yoldia limatula</i> (Say, 1831)	Nuculanida	150	0.6	2.1	Drew (1899)	Morse and Zardus (1997)
<i>Yoldia notabilis</i> Yokoyama, 1922	Nuculanida	133–172	—	—	Nakaoka (1994)	—
<i>Solenya velexiana</i> (Iredale, 1931)	Solemyida	131–157	0.3	6.2	Taylor et al. (2008) (here estimated from micrograph)	present study
<i>Ledella ecaudata</i> (Pelseneer, 1903)	Nuculanida	130	—	—	Reed et al. (2014) (as <i>Yoldiella ecaudata</i>)	—
<i>Nuculana</i> spp.	Nuculanida	120–140	0.24	2.2	marLIN (2006)	<i>N. perna</i> in present study
<i>Yoldiella valettei</i> (Lamy, 1906)	Nuculanida	120.6	—	—	Reed et al. (2014)	—
<i>Nucula nuditosa</i> Winckworth, 1930	Nuculida	90–150	—	—	marLIN (2006)	—
<i>Microgloma yongei</i> Sanders & Allen, 1973	? Nuculanida	120	—	—	Sanders and Allen (1973)	—
<i>Pristigloma alba</i> Sanders & Allen, 1973	Nuculanida	115	—	—	Sanders and Allen (1973)	—
<i>Ledella messanensis</i> (Jeffreys, 1870)	Nuculanida	109	—	—	Lightfoot et al. (1979)	—
<i>Acila castrensis</i> (Hinds, 1843)	Nuculida	90	0.44	9.4	Zardus and Morse (1998)	Morse and Zardus (1997); present study
<i>Nucula proxima</i> Say, 1822	Nuculida	90	—	—	Drew (1899)	—
<i>Microgloma pusilla</i> (Jeffreys, 1879)	? Nuculanida	85–90	2.4	3.0	Ockelmann and Warén (1998)	Ockelmann and Warén (1998)
<i>Nucula pusilla</i> Angas, 1877	Nuculida	50	—	—	—	Morton (1996)

FIG. 12. Comparison of sperm ultrastructure in Protobranchia (semidiagrammatic; based on all available sources; see Table 1). Some details of species from the literature not demonstrated by original authors have here been reconstructed based on the more detailed evidence for other species shown herein but await formal demonstration. Sperm of all species shown in idealized longitudinal section. **A.** *Acharax alinae*. **B.** *Solemya velesiana*. **C.** *S. velum*. **D.** *S. reidi*. **E, F.** Detail of acrosomal complex in *S. velesiana* in longitudinal and transverse section. Note radial plates within acrosomal vesicle. **G.** *Nucula sulcata*. **H.** *Acila castrensis*. **I.** *Linucula hartvigiana*. **J.** *Ennucula* sp. **K, L.** Detail of acrosomal complex in *N. sulcata* in longitudinal and transverse section. Note absence of radial plates within acrosomal vesicle. **M.** *Scaeoleda caloundra*. **N.** *Nuculana pernula*. **O.** *Neilonella corpulenta*. **P.** *Yoldiella philippiana*. **Q.** *Yoldia limatula*. **R.** *Y. keppeliana*. **S.** *Y. notabilis*. **T, U.** Detail of acrosomal complex in *Scaeoleda caloundra* in longitudinal and transverse section. Note radial plates within acrosomal vesicle. For sources of data, see Table 1. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; c, centriolar pair; f, flagellum; m, mitochondrion; n, nucleus; rp, radial plates of acrosomal vesicle; sm, subacrosomal material. Scale bars: (1) E, F, K, L, T, U; (2) all other illustrations.

and acrosomal vesicle membranes observed by us in some spermatozoa of nukulids (e.g., *Acila castrensis*) is here interpreted as the initial stage of the acrosomal reaction (in which the acrosomal vesicle membrane and plasma membrane fuse) rather than as an abnormality (see Nijima & Dan, 1965a,b; Popham, 1974b; Hylander & Summers, 1977; Hosokawa & Noda, 1994). The reported incidences of acrosomal abnormality in bivalves (and mollusks in general) are low (e.g., Dorange & Le Pennec, 1989; Healy & Harasewych, 1992), although this could be due to a dismissal of aberrancy as an artifact of fixation or the phenomenon has simply been overlooked. Duplication of the acrosomal complex was observed by Komaru et al. (1995) in a small percentage (maximum 3%) of spermatozoa in tetraploid *Mytilus galloprovincialis* Lamarck, 1819, from Japan.

As important as acrosomal vesicle shape and length undoubtedly are as sperm features, it is the organization of the vesicle contents that has often proven of even greater value in terms of potential taxonomic/phylogenetic use. Most notably, among protobranch sperm, the presence of radial plates within the acrosomal vesicle of both our investigated species of Solemyidae (*Solemya velum* and *S. velesiana*) and Nuculanoidea (*Scaeolea caloundra*, *Nuculana pernula*, and *Yoldiella philippiana*) contrasts with an apparent absence of these structures in the four species of Nuculoidea examined to date (*Nucula sulcata*—Franzén, 1983, and present study; *Acila castrensis*—Morse & Zardus, 1997, and present study; *Linucula hartvigiana* (Dohrn, 1864)—Popham & Marshall, 1977; *Ennucula* sp.—present study). As noted for *Y. philippiana*, the presence of the plates can be masked to some extent by other acrosomal vesicle contents, and this could perhaps explain why they have not hitherto been described in previously studied Nuculanoidea (*Yoldia limatula* (Say, 1831)—Morse & Zardus, 1997; *Y. notabilis* Yokoyama, 1922, and *Y. keppeliana* Dall, 1871—Tyurin & Drozdov, 2005; unfortunately, neither of these studies showed the acrosomal vesicle in transverse section). Absence of radial plates in the Nuculoidea, however, appears to be real because we examined many transverse-sectioned acrosomes in *N. sulcata*, *A. castrensis*, and *Ennucula* sp. without observing such structures. Neither Franzén (1983) nor Popham and Marshall (1977) demonstrated in transverse section the acrosomal vesicle of nukuloids they studied (*N. sulcata* and *L. hartvigiana*, respectively), but both described the vesicle contents as being homogeneous. Although Beninger and Le Pennec (1997) did not report radial plates for *A. alinae*, their micrographs clearly show a highly electron-dense layer running the entire length of the acrosomal vesicle. This layer was interpreted by Beninger and Le Pennec (1997, figs. 3, 4) as the entire acrosomal vesicle, but we believe it to be only the radial plate component. Careful scrutiny of transverse sections of this species would resolve this issue. Similarly, Gustafson and Reid (1988) presented only a single micrograph of *S. reidi* showing the acrosomal complex in longitudinal profile and at a relatively low magnification, and hence the presence of plates in that species has yet to be confirmed (although we predict that they will be present in this and other species of *Solemya*). Outside of the Protobranchia, radial plates have also been observed in a number of pteriomorphian groups, most notably the Pectinoidea (extremely similar to Nuculanida, see Dorange & Le Pennec, 1989; Healy et al., 2000, 2001; Fig. 12), Pterioidea (Thielley et al., 1993; Healy et al., 2000), Pinnoidea (De Gaulejac et al., 1995; Healy et al., 2000), and Limoidea (Healy et al., 2000). However, the precise shape of the plates layer

within the acrosomal vesicle differs among groups (for comparative figures and summary, see Healy et al., 2000) perhaps enough to require further confirmation of homology through spermiogenic and cytochemical sources of information (see Sousa & Azevedo, 1988; Sousa et al., 1998). Interestingly, elsewhere among the Mollusca, radial plates are also routinely observed in the acrosomal vesicles of caenogastropods (Giusti, 1971; Healy, 1983; Koike, 1985), and presumably whatever function that they serve in these gastropods is similar to that of the plates in protobranchs and other bivalves that exhibit them.

The subacrosomal material associated with the acrosomal vesicle invagination in all investigated protobranchs is granular and loose and occupies the bulk of the acrosomal vesicle invagination (as observed in most other Bivalvia). An axial rod, such as that seen in Ostreioidea and many Mytiloidea (e.g., Nijima & Dan, 1965a,b; Daniels et al., 1971; Gutiérrez et al., 1978; Hodgson & Bernard, 1986; Healy & Lester, 1991; Bozzo et al., 1993; Sousa & Oliveira, 1994; Garrido & Gallardo, 1996; Gwo et al., 1996; Kafanov & Drozdov, 1998; Healy et al., 2000, 2015), is absent in the Protobranchia, as is the case in most other Bivalvia. In the Nuculanida, alone among protobranchs, subacrosomal material also fills an anterior depression of the nucleus (Morse & Zardus, 1997; Tyurin & Drozdov, 2005; present study), as is the case in many Pteriomorphia (for review and comparative figures, see Healy et al., 2000).

Of considerable interest is the similarity in acrosomal (and nuclear; see below) morphology between the Nuculanoidea (Fig. 12) and several groups of pteriomorphians, such as the Pterioidea, Pinnoidea, Limoidea, and Pectinoidea (acrosomal vesicle contents featuring numerous radial plates; nucleus short, rounded with an anterior depression containing a significant proportion of the subacrosomal material) (for comparative figures, see Healy et al., 2000). The similarity is most striking when comparing Nuculanoidea and the Pectinoidea (Figs. 11, 12) in which the radial plates show a pronounced recurved longitudinal profile. Because both Nuculanoidea and Pectinoidea have been shown to be derived within their respective clades in recent molecular phylogenetic studies (Sharma et al., 2013; Lemer et al., 2016), such similarities would appear to be due to convergence. However, in the Solemyidae examined by us (*S. velum*, *S. velesiana*), the basal portion of the radial plates is also slightly recurved, indicating that any convergence explanation would have to extend to the Solemyoidea in addition to the Nuculanoidea and Pectinoidea. It appears that additional work on pteriomorphian and nuculanoidean species is required to properly explore the significance of the radial plates and their precise homology (recurved versus nonrecurved), ideally with the aid of staining techniques (Sousa et al., 1998) and more detailed spermiogenic (acrosomal developmental) data.

NUCLEUS—The range of nuclear morphologies observed within the Protobranchia is extremely wide (Fig. 12), and it is interesting to note that in both the Solemyidae and the Nuculidae, the prevailing shape is rod-shaped to elongate (extremely extended in *A. alinae* to 19 μm length—Beninger & Le Pennec, 1997) with a flat or convex apex, whereas all investigated members of the Nuculanoidea have a short spheroidal nucleus with a wide and sometimes marked anterior depression filled by subacrosomal material. A spheroidal nucleus similar in shape to those of the Nuculanoidea is observed in many Pteriomorphia, notably the Ostreioidea, Pinnoidea, Pterioidea, some Pectinoidea, and a few Mytiloidea (Daniels

et al., 1971; Gutiérrez et al., 1978; Dorange & Le Pennec, 1989; Healy & Lester, 1991; Bozzo et al., 1993; Thielley et al., 1993; De Gaulejac et al., 1995; Gwo et al., 1996; Kafanov & Drozdov, 1998; Healy et al., 2000, 2001, 2015; Le Pennec et al., 2002; Beninger & Le Pennec, 2006; Drozdov et al., 2009; Espinosa et al., 2010) and also occurs in certain Heterodonta such as the Mactroidea and some Veneridae (Longo & Anderson, 1969; Guerra et al., 2003; Drozdov et al., 2009). Although Drozdov and Tyurin (2014) argued that a spheroidal nucleus is a feature of the “sperm prototype” of bivalves, Bieler et al. (2014) considered it one of a number of derived conditions, and that a barrel-shaped nucleus was most likely the ancestral condition not only in bivalves but also in mollusks in general. Hence, in our opinion, the remarkable similarity of nuculanoid sperm nuclei to those of the pteriomorphian groups here indicated (in addition to equally striking acrosomal similarities) could be of importance. Aside from the Protobranchia, a number of other groups of bivalves (e.g., Mytiloidea, Veneroidea, Cardioidea, and Galeommatoidea) are known to exhibit large variation in nuclear length and/or shape between species (Healy, 1995a; Garrido & Gallardo, 1996; Kafanov & Drozdov, 1998; Keys & Healy, 2000; Jespersen et al., 2001, 2002, 2009; Healy et al., 2006, 2008a), and it has been argued that increased nuclear length could correlate with increased egg diameter (see Franzén, 1983; Beninger & Le Pennec, 1997). As shown in Table 2, there does not appear to be any firm correlation between egg size and sperm nuclear length among examined species of Protobranchia, although data for many more species are required to fully investigate this issue.

The peg-shaped anterior extension of the nucleus exhibited in *S. velum* is not shared (apparently) with other investigated solemyoids (see Gustafson & Reid, 1988; Beninger & Le Pennec, 1997; Fig. 12), but elsewhere in the Bivalvia, such an elaboration of the nuclear apex has been observed in certain Cardiidae (*Fulvia tenuicostata* (Lamarck, 1819) Popham, 1979; *Cerastoderma* spp.—Sousa & Azevedo, 1988; Drozdov et al., 2001; *Tridacna* spp.—Keys & Healy, 2000; for comparative figures, see Healy et al., 2008a). Presumably, this feature serves to further secure the acrosomal complex to the nuclear apex, but more work is required to determine why it only occurs in certain taxa and is not more widely distributed. Interestingly, the same structure has been noted in a few cerithioidean gastropods (Healy, 1983) and certain Annelida (see Franzén, 1975; Jamieson & Rouse, 1989). A similar puzzle is presented by the “teardrop-shaped” nucleus of *S. velesiana*; such a shape has not previously been observed in any other examined bivalve species but is reminiscent of that seen in Polyplacophora, with the closest comparison occurring in the primitive *Lepidopleurina* (Hodgson et al., 1988; Healy et al., 1995; Pashchenko & Drozdov, 1998; Healy, 2000). Once again, only further study will reveal the frequency of this nuclear shape and its possible function compared to the other types seen within the Solemyida.

MIDPIECE AND FLAGELLUM—Most bivalves exhibit either the same or a very similar arrangement of mitochondria in the midpiece to that observed in the Protobranchia (i.e., spherical mitochondria surrounding an orthogonally arranged pair of centrioles and nine terminally forked, satellite fibers connecting the distal centriole to the annulus and plasma membrane [Popham, 1979; Franzén, 1983; Healy, 1996]). Such an arrangement is typical of most “primitive sperm” (Franzén, 1983) or

“aquasperm” (Rouse & Jamieson, 1987); hence, there are no features of the midpiece that distinguish protobranchs from most other Bivalvia. The mitochondria of *S. velesiana* are noticeably smaller than in other investigated *Solemya* (*S. velum*, this study; *S. reidi*, Gustafson & Reid, 1988), and those of nuculoids and nuculanoids (present study; also see Popham & Marshall, 1977; Morse & Zardus, 1997; Tyurin & Drozdov, 2005) and in fact most other (nonprotobranch) bivalves (Popham, 1979). In our reconstruction of the spermatozoa of *A. alinae*, based on the TEM work of Beninger & Le Pennec (1997) using formalin-alcohol-fixed material, the mitochondria appear to be as small as those of *S. velesiana* (their given length of 5 μm for *A. alinae* appears to be a typographical error for 0.5 μm). Although our material of *S. velesiana* was also formalin fixed (seawater-formalin), we have not observed any link between use of formalin and marked shrinkage of sperm mitochondria in other bivalves examined by us (e.g., see Healy et al., 2008b, *Cuspidaria latesulcata* (Tenison-Woods, 1878). Hence, we conclude that the small size of the mitochondria in *S. velesiana* (and apparently also *A. alinae*) is real and will be an interesting character for comparison when other *Solemya* species are investigated.

The most important variation in midpiece morphology among protobranchs is the number of mitochondria. Mitochondrial number in bivalves varies both between species and often within species, but it is almost always possible to determine a “prevailing number” of mitochondria (for further discussion, see Bieler et al., 2014). There is conservatism in the prevailing mitochondrial number within many higher taxa (four in most Ostreoida [see Healy et al., 2000], seven or eight in Crassatelloidea and Carditoidea [Healy, 1995b]) but also considerable group variation in many others (e.g., Mytiloidea four to 14 depending on genus; Kafanov & Drozdov, 1998). Among the Protobranchia, the prevailing number of mitochondria in the Solemyoidea is four in *S. reidi* and *A. alinae* (Gustafson & Reid, 1988; Beninger & Le Pennec, 1997) but five in *S. velum* and *S. velesiana* (present study) and also five in the Nuculoidea (Popham & Marshall, 1977; Franzén, 1983; Morse & Zardus, 1997; present study), whereas in the Nuculanoidea, it is four in *Yoldiella phillipinarum*, *N. pernula*, and *S. caloundra* (present study) and five in all three examined species of *Yoldia* (Morse & Zardus, 1997; Drozdov & Tyurin, 2005). Hence, there appears some taxonomic potential for sperm mitochondrial number within families of the Protobranchia, particularly in the Solemyoidea and Nuculanoidea, although its significance in a higher-level phylogenetic discussion seems limited.

The morphology of the flagellum in all species examined (composed of a 9 + 2 microtubular axoneme sheathed by the plasma membrane) is no different from those of the vast majority of other bivalves or indeed other invertebrates with aquatic fertilization (Baccetti & Afzelius, 1976).

Taxonomic and Phylogenetic Considerations

Our results combined with those of previous studies (Popham & Marshall, 1977; Franzén, 1983; Gustafson & Reid, 1988; Morse & Zardus, 1997; Giribet & Wheeler, 2002; Tyurin & Drozdov, 2005; Bieler et al., 2014) indicate that there is significant potential for sperm ultrastructure as a source of taxonomically and possibly phylogenetically useful characters within the subclass. At the broadest level, sperm ultrastructural results are mostly consistent with widespread recogni-

tion of at least three major divisions of the Protobranchia (Nuculida, Nuculanida, and, less convincingly, Solemyida) (e.g., Sharma et al., 2012, 2013; Bieler et al., 2014; González et al., 2016). Unfortunately, sperm differences between these groups (and lack of unifying sperm similarities) are so marked that they could potentially be cited as evidence against protobranch monophyly, although, as we have indicated, firsthand data on fertilization biology and egg morphology—both important to understanding sperm morphological features—are still mostly wanting (see Discussion below under each of the three groups). Of the 19 sperm characters used by Bieler et al. (2014) in their total evidence phylogenetic analyses of the Bivalvia, investigated Protobranchia scored as plesiomorphic for 14–18 depending on the taxon (Nuculida scoring 17–18, Solemyida and Nuculanida scoring 14–15), a not unexpected result given the phylogenetic placement of the subclass. This unfortunately does greatly limit the scope of any sperm-based phylogenetic discussion of Protobranchia in relation to other bivalve groups, but it is hoped that as further information accumulates (more investigated taxa, selective staining of acrosomal features, and spermiogenic data), an expanded character list and states might be developed. Within the Protobranchia, the molecular study of Sharma et al. (2013) revealed numerous taxonomic/phylogenetic issues, including the need for a fourth clade of protobranchs to accommodate the Nucinellidae, the need for reassessment of the relationships and taxonomic position of several taxa, and, perhaps most alarmingly, the “nonmonophyly of most nuculoid and nuculanoid genera and families” (p. 471). Any additional ultrastructural sperm data are bound to help clarify some of the numerous unresolved taxonomic issues within the subclass.

SOLEMYIDA—Within the order Solemyida, only sperm data for members of Solemyidae are available (Fig. 12) (see also Gustafson & Reid, 1988; Beninger & Le Pennec, 1997; Bieler et al., 2014), leaving the Manzanelloidea (extinct Manzanelidae + extant Nucinellidae) yet to be examined. Despite the limited results at hand, the observed differences in sperm morphology among the four studied species of Solemyidae are marked (Fig. 12), and of all the protobranch families investigated to date, it appears that sperm could be of greatest taxonomic use in this one. The extraordinary sperm of *Acharax alinae* (see Beninger & Le Pennec, 1997; see also our reconstruction shown in Fig. 12) with its extremely elongate acrosomal vesicle and nucleus—if typical of the genus—could provide valuable extra features to differentiate it from other Solemyidae and lend additional support for the distinction at family-group rank (Acharacidae Scarlato & Starobogatov, 1979, currently considered Acharacinae within Solemyidae; Bieler et al., 2010), a result consistent with molecular sequence data (Taylor et al., 2008; Sharma et al., 2013). Beninger and Le Pennec (1997), however, cautioned against the use of sperm features for taxonomic and phylogenetic investigation and attributed the elongation of sperm components in *A. alinae* to the exceptionally large oocytes of this species. Further work, especially work on the type species of *Acharax*, *A. johnsoni* (Dall, 1891), will be necessary to determine whether the extreme sperm features of *A. alinae* are characteristic of the genus.

There has been much discussion of *Solemya* taxonomy, with various authors indicating the need to use anatomical and molecular data to verify, alter, or erect supraspecific taxa (Taylor et al., 2008; Kamenev, 2009; Huber, 2015). Most recently,

Walton (2015) concluded that there is need for a complete reappraisal of the nominal subgenera of *Solemya* and suggested that until this is carried out (with molecular input), the groups recognized by Taylor et al. (2008) and Kamenev (2009) should be used for New Zealand species. Results of our study strongly suggest that comparative sperm ultrastructure could also play a significant role in the reassessment of *Solemya* taxonomy. Within the genus *Solemya*, currently six subgenera are recognized: *Solemya* s.s. (type species *S. mediterranea* Lamarck, 1818 = *S. togata* Poli, 1795), *Pseudacharax* Huber, 2011 (type species *S. japonica* Dunker, 1882); *Petrasma* Dall, 1908 (type species *S. borealis* Totten, 1834); *Zesolemya* Iredale, 1939 (type species *S. parkinsonii* E. A. Smith, 1874); *Austrosolemya* Taylor, Glover, & Williams, 2008 (type species *S. australis* Lamarck, 1818); and *Solemyarina* Iredale, 1931 (type species *S. velesiana*). Of these, only the type species of *Solemyarina* has been examined for sperm ultrastructure (Fig. 12), and no sperm data are available for *Zesolemya*, *Austrosolemya*, or *Solemya* s.s. Within *Petrasma*, *S. velum* and *S. reidi* have been examined for sperm, and the results differ markedly between these two species and also from *S. (Solemyarina) velesiana* (Fig. 12). Much will depend on future studies of the type species *S. togata* and other species of *Petrasma* to determine this taxon's true affinities. Despite being only superficially described by Gustafson and Reid (1988), the sperm of *S. reidi* are known to be the simplest of all four examined solemyoids, and hence a more detailed account of this species is of considerable importance, particularly with reference to the acrosomal complex.

NUCULIDA—Sperm of all investigated species of Nuculida differ from those of Solemyida and Nuculanida in lacking radial plates within the acrosomal vesicle and, in addition, from the Nuculanida in the morphology of the nucleus. If further studies can verify these differences, then it would seem that sperm ultrastructure is at odds with the conclusions of some authors of close ties either between Solemyida and Nuculida (i.e., Opponobranchia; Waller, 1998; Carter et al., 2000; Giribet & Wheeler, 2002; Giribet & Distel, 2003; Giribet, 2008; Wilson et al., 2010; Plazzi et al., 2011) or between the Nuculanida and Nuculida (i.e., Palaeotaxodonta; Smith et al., 2011; Sharma et al., 2012, 2013). At the species and genus levels, our results for *Nucula sulcata* and *Acila castrensis* agree with those previously published for these species (Franzén, 1983; Morse & Zardus, 1997, respectively). Spermatozoa of the two species are very similar in all respects, with the exception that the nucleus of *A. castrensis* is slightly longer. In contrast, the nucleus of *Ennucula* sp. is less than half the length of those of either *N. sulcata* or *A. castrensis*, and the nuclear lacunae are large and elongate, but the acrosomal details are very similar to those of *N. sulcata* and *A. castrensis*, presumably indicating close relationship between *Nucula* and *Acila*—as also suggested in molecular studies in which *N. sulcata* and *A. castrensis* are inferred as closely related and *Nucula* as nonmonophyletic (Sharma et al., 2013; Combosch et al., 2017). Spermatozoa of *Linucula hartvigiana* differ from all other investigated Nuculida in having a tall-conical acrosome and slightly elongate mitochondria (Popham & Marshall, 1977). Like *Ennucula* sp., the nucleus is shorter than those of both *N. sulcata* and *A. castrensis*, and the nuclear lacunae are oblong and jagged. Although it is too early to judge whether sperm data can be of fundamental taxonomic value within the Nuculida, the available evidence is promising and worthy of further comparative study.

NUCULANIDA—All available ultrastructural data indicate a very conserved sperm morphology for the Nuculanida (e.g., small, conical acrosomal vesicle; short but wide spheroidal nucleus featuring broad anterior depression for subacrosomal deposit), which distinguishes this order from both the Nuculida and the Solemyida (Fig. 12). The presence of radial plates (recurved basally) within the acrosomal vesicle appears to link Nuculanida and Solemyida, although the number of plates differs (33–35 in Nuculanida, 43–48 in Solemyida) as does the degree of recurvature of the plates (weak in Solemyida, strong in Nuculanida). In addition, the two groups show markedly different nuclear morphologies. The light-microscopical study of Retzius (1904) for *N. minuta* clearly shows nuculanoid sperm morphology. Ockelmann and Warén (1998) stated that they had examined, with light microscopy, 13 species from the genera *Nuculana*, *Yoldia*, *Megayoldia*, *Yoldiella*, *Portlandia*, *Pseudomalletia*, and *Tindaria* and apparently found these to be of the same morphology (“*Nuculana* type,” i.e., possessing a spheroidal nucleus) and easily distinguished from the “*Nucula* type” (i.e., possessing a rod-shaped nucleus). If these results can be confirmed, this would show that nuculanoid sperm morphology is truly characteristic of the group as a whole, including the Tindariidae, which, according to some authors (e.g., Sharma et al., 2013), could be among the most basal families of the Nuculanida.

At the generic and specific levels, there are differences among nuculanoid taxa that could be taxonomically significant once more comparative data are available. For example, the prevailing mitochondrial number is five in *Yoldia* spp. (*Y. limatula*, *Y. notabilis*, *Y. keppeliana*; see Morse & Zardus, 1997; Tyurin & Drozdov, 2005) but four in the other investigated Nuculanida (*Y. philippiana*, *S. caloundra*, and *N. pernula* [herein]). The depth of the anterior invagination of the nucleus and its relationship with the position of the acrosomal vesicle might also provide useful sperm features (in *N. pernula*, the invagination is relatively deep and partly conceals the acrosomal vesicle [present study], a feature also noted by Franzén [1955] using only light microscopy).

The remarkable sperm similarities between Nuculanoidea and the Pectinoidea has already been commented on above (and demonstrated in Fig. 11), but this raises the question of what significance we can attach to this. The same environment of fertilization (“external”) might be a possible or partial explanation, although direct observational knowledge of this aspect of reproduction in nuculanoideans (e.g., Drew, 1899) is mostly lacking. Many other groups of bivalves fertilize externally yet show marked acrosomal and/or nuclear differences to both the Pectinoidea and the Nuculanoidea (for review and comparative figures, (see Healy et al., 2000). Could egg size be significant? Again, this does not adequately explain the sperm similarities because most eggs of the Nuculanoidea fall into a 120–130- μm -diameter size range (see Table 2) in contrast to the generally smaller (45–90 μm , exceptionally 120 μm) eggs of Pectinoidea (see Beninger & Le Pennec, 1997). Selective staining techniques on the acrosomal vesicle, as well as details of the acrosome reaction and events of acrosome development during spermiogenesis, would at least help to confirm the homology of the radial plates in nuculanoideans, solemyoideans, and pectinoideans (and more generally other pteriomorphians showing plates). If the sperm similarities of Nuculanoidea and Pectinoidea are purely due to convergence and not indicative of relationship, then there does not ap-

FIG. 13. Spermatozoa of *Microgloma pusilla*, drawings, based on alcohol-preserved tissues, redrawn from original sketch with permission from Ockelmann and Warén (1998). **A.** Detail of acrosomal complex, nucleus, midpiece, and proximal portion of flagellum. **B.** Paired spermatozoa.

pear to be any convincing cytological explanation for this. Convergence in sperm morphology would, however, correlate with molecular studies showing Nuculanoidea and Pectinoidea to be advanced groups in their respective clades and therefore phylogenetically distant from each other (Sharma et al., 2013; Lemer et al., 2016). It is also conceivable that the Pectinoidea have retained plesiomorphic sperm features of early pteriomorphians and a protobranch stock with nuculanoid sperm.

Microgloma

Finally, some discussion of the light-microscopic results of Ockelmann and Warén (1998) for spermatozoa of *Microgloma pusilla* is warranted given the enigmatic/unresolved position of *Microgloma* within the Protobranchia. Ockelmann and Warén (1998) examined alcohol-preserved material of this species, and their observations were presented in the form of two line drawings, one showing two spermatozoa arranged in an unusual paired state and one showing a more detailed study of the acrosome, nucleus, midpiece, and tail (reproduced herein as Fig. 13). The acrosomal vesicle is large (approximately 2.4 μm long), tall-conical, and broad basally, with a curved, pointed apex. The nucleus (approximately 3.0 μm long and 2.0 μm at mid-length) has a convex anterior surface and is barrel-shaped. The midpiece was said to contain “several small mitochondria, which appear to be more or less fused” (p. 19). If we assume that the drawings are a faithful representation of the spermatozoa of *M. pusilla*, then the results for this species depart from all other protobranchs that have been examined. Ockelmann and Warén (1998, p. 19) argued that the nucleus of *M. pusilla* resembled more closely the spheroidal nuclei seen in Nuculanoidea than the rod-shaped nuclei of Nuculoidea and suggested that “this may hint at a relationship with the Nuculanoidea.” However, given that both the Solemyida and the Nuculida can show relatively short nuclei (e.g., *S. reidi* and *Ennucula* sp.; Fig. 12), and that the spermatozoa of all investigated

Nuculanida have a spheroidal nucleus (Retzius, 1904; Franzén, 1955; Fig. 12), it seems that nuclear morphology might not be the most reliable indicator of affinity for *Microgloma*. More significant, at least in our opinion, is the size and shape of the acrosomal vesicle in *M. pusilla*. Aside from the 7.0- μ m-long acrosomal vesicle of the solemyoid *A. alinae* (see Beninger & Le Pennec, 1997; reconstructed in Fig. 12), nothing comparable with *M. pusilla* is known among the Protobranchia. Ockelmann and Warén (1998, p. 19) attributed the large, anteriorly curved acrosome of *M. pusilla* to a probable “specialised mode of fertilization,” and this seems at least feasible. Their observation that the “ripe spermatozoa are often found in pairs” (p. 17) recalls the phenomenon of sperm conjugation, or sperm pairing, seen in turritellid caenogastropods (Afzelius & Dallai, 1983), diving beetles (Afzelius & Dallai, 1987; Dallai & Afzelius, 1987), and some marsupials (Roger & Bedford, 1982), although in these other animal groups, the sperm are always parallel and facing the same direction and not arranged head to tail as in *M. pusilla*. (Our micrograph [Fig. 5A] showing head-to-tail sperm of *A. castrensis* [Nuculida] appears to be a fortuitous similarity to the arrangement in *M. pusilla*. Close inspection of a figure by Morse and Zardus [1997, fig. 178] reveals no head-to-tail arrangement in *A. castrensis*.)

Clearly, a thorough TEM study of the entire spermatozoon of *M. pusilla* is needed and ideally also of other species of *Microgloma*, with special emphasis on the acrosomal complex and the nuclear apex. For the present, the available data for *M. pusilla* spermatozoa do not indicate any particular placement for *Microgloma* in the light of our current knowledge of sperm morphology in the Protobranchia. Hence, the decision of Ockelmann and Warén (1998) to transfer the sareptid *Microgloma* from Nuculoidea, where it was originally placed by Sanders and Allen (1973), to Nuculanoidea is not well supported by sperm morphology data, although the placement of Sareptidae within Nuculanida was subsequently well supported (based on *Pristigloma*) by molecular data (Sharma et al., 2013).

Monophyly of the Protobranchia?

Monophyly of the Protobranchia and, in particular, the relationship of the Nuculanida to other protobranchs and to other bivalves have long been contentious issues. For example, Giribet and Wheeler (2002) argued for a nonmonophyletic Protobranchia, concluding that the Nuculanida split off from the Solemyida + Nuculida to become sister group to all other bivalves. More recently, Plazzi and Passamonti (2010), Wilson et al. (2010), and Plazzi et al. (2011) have all concluded that although a close relationship exists between the Solemyida and Nuculida, the Nuculanida branch off with the Pteriomorphia. This would be consistent with some of our results but is presumably a bias of the mitochondrial data because most other analyses, including recent and ongoing phylogenomic studies, find strong support for the monophyly of Autolamellibranchiata (e.g., Smith et al., 2011; Sharma et al., 2012; Bieler et al., 2014; González et al., 2016). The most recent phylogenetic analyses (morphological, molecular, and combined molecular + morphological) have again favored a monophyletic Protobranchia, but in general with a closer relationship between Nuculanida and Nuculida (e.g., Sharma et al., 2012, 2013; Bieler et al., 2014; González et al., 2016) than of either to the Solemyida. Also, any discussion of protobranch monophyly will have to take into account the shared occurrence of a complex

pericalymma larva by all branches of the Protobranchia (Drew, 1899; Waller, 1998; Zardus & Morse, 1998).

Future Directions for Protobranch Sperm Ultrastructure Research

This study has shown that sperm ultrastructure in the Protobranchia could be an additional source of taxonomically and phylogenetically useful information, particularly for the Solemyida but also for the two other major groups (Nuculida and Nuculanida). However, profound sperm differences between these three groups raise other questions, the most important being whether the differences are associated with fertilization biology or provide evidence for nonmonophyly of the Protobranchia. Clearly, there is a need for direct observational work on protobranch spawning to better assess any correlation with sperm components, particularly the length of the acrosomal complex and nucleus. It is also evident that many protobranch taxa remain unexamined for sperm ultrastructure, the most important gaps lying within Solemyoidea, Sareptoidea, the enigmatic genus *Microgloma*, and particularly Manzanelloidea that, from a molecular perspective (e.g., Sharma et al., 2013; Combosch et al., 2017; but see Bieler et al., 2014), appear to offer a bridge between the Solemyida (where they are usually placed) and other bivalves.

Summary/Principal Conclusions

- (1) The Protobranchia show substantial diversity in sperm ultrastructure to the point that no unifying sperm features of the subclass can be identified.
- (2) The absence of wide-ranging observational research on protobranch fertilization biology hampers assessment of the influence that this aspect of reproduction might have on sperm morphology in the group.
- (3) The Solemyida (Manzanelloidea not examined to date) are much more diverse in sperm morphology than either the Nuculida or the Nuculanida, indicating considerable taxonomic utility for sperm data within this order.
- (4) The presence of radiating plates in the acrosomal vesicle of *Solemya* spp. (Solemyida) could suggest a link with the Nuculanida rather than Nuculida (which lack such plates), but substantial differences in nuclear morphology of all three groups prevents any conclusions concerning relationships of these three major protobranch groups.
- (5) The Nuculida and Nuculanida each show conserved sperm ultrastructure and also are easily discriminated on this basis. Given the current state of taxonomy within each of these orders, particularly the Nuculanida, sperm could be useful for resolution of some taxonomic problems, such as the placement of Sareptidae.
- (6) Sperm of the Nuculanida are extremely similar to those of some pteriomorphians, especially the Pectinoidea (and most notably in relation to the acrosomal complex and nucleus). Whether this casts doubt on protobranch monophyly or (more likely given recent molecular studies) indicates some functional constraint in these two clades is uncertain.

- (7) Many taxa remain unstudied for sperm ultrastructure, especially the Sareptidae, *Microgloma* (of uncertain family and superfamily affinity), and Nucinelidae. Various genera in the other families likewise still require detailed comparative work.

Acknowledgments

This work was supported by the Bivalve Assembling the Tree-of-Life project (<http://www.bivatol.org>) through the U.S. National Science Foundation Assembling the Bivalve Tree of Life (BivAToL) program (DEB-0732854/0732903/0732860) to R.B., G.G., and P.M. We gratefully acknowledge the support of our BivAToL colleagues and others who assisted with obtaining specimens, especially J. D. Taylor and E. A. Glover (London), J. Zardus (Charleston, South Carolina), and J. Fuchs (Gothenburg). Living *Ennucula* sp. and *S. caloundra* were collected during BivAToL dredging in the Moreton Bay Marine Park under permit QS2005/CVL588 from Queensland Parks. BivAToL EM technician Erica Lovas (Queensland Museum) carried out tissue preparation, sectioning, and photography of sections at the Centre for Microscopy and Microanalysis (an Australian Microscopy and Microanalysis Research Facility) at the University of Queensland (the staff of which is here thanked for access to facilities and technical assistance). BivAToL illustrator Lisa Kanellos (FMNH) assisted with figure redrawing and labeling. Collections staff of FMNH (Jochen Gerber and Janeen Jones) and the Australian Museum, Sydney (Ian Loch, Alison Miller, and Janet Waterhouse), are thanked for support with specimen loans. The constructive input provided by two anonymous reviewers is greatly appreciated.

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