

Comparative Silver Staining of Molluscan Spermatozoa

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ABSTRACT

Mollusc spermatozoa were studied by transmission electron microscopy with the application of the silver nitrate method. Silver stained some components of the acrosomal vesicle and of the cytoskeletal elements of these cells in a pattern specific for each species. The importance of this staining method in relation to phylogenetic relationships is presented and discussed.

RÉSUMÉ

Coloration comparée à l'argent des spermatozoïdes de Mollusques

Des spermatozoïdes de Mollusques ont été étudiés en microscopie électronique à transmission grâce à la méthode au nitrate d'argent. Dans chaque espèce, l'argent colore certains composants de la vésicule acrosomienne et les éléments cytoplasmiques de ces cellules d'une manière spécifique. L'importance de cette méthode de coloration pour les relations phylogéniques est présentée et discutée.

The ultrastructural characteristics of mature spermatozoa have been used to examine phylogenetic relationships and have also been related to the different aspects of reproductive biology of the organism [18, 38, 39, 50, 51, 54]. In this respect, a great deal of work has been done on the ultrastructure of spermatozoa in the Patellogastropoda and Archaeogastropoda (Prosobranchia) as well as in the Heterodonta and Pteriomorphia (Bivalvia) [1-3, 6-37, 39-53, 55, 56, 59, 61-66].

In recent years, we have attempted to show that the silver staining method can be a useful and easy way to better understand specific aspects of spermatogenesis [57, 59, 60], as well as for comparative phylogenetical studies [58, 61-64]. In the present study, we present and critically review the silver staining characteristics of mature molluscan spermatozoa, with emphasis on its importance as a tool for studying phylogenetic relationships. We also provide the detailed technique so as to enable other laboratories to reproduce this staining method.

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MATERIALS AND METHODS

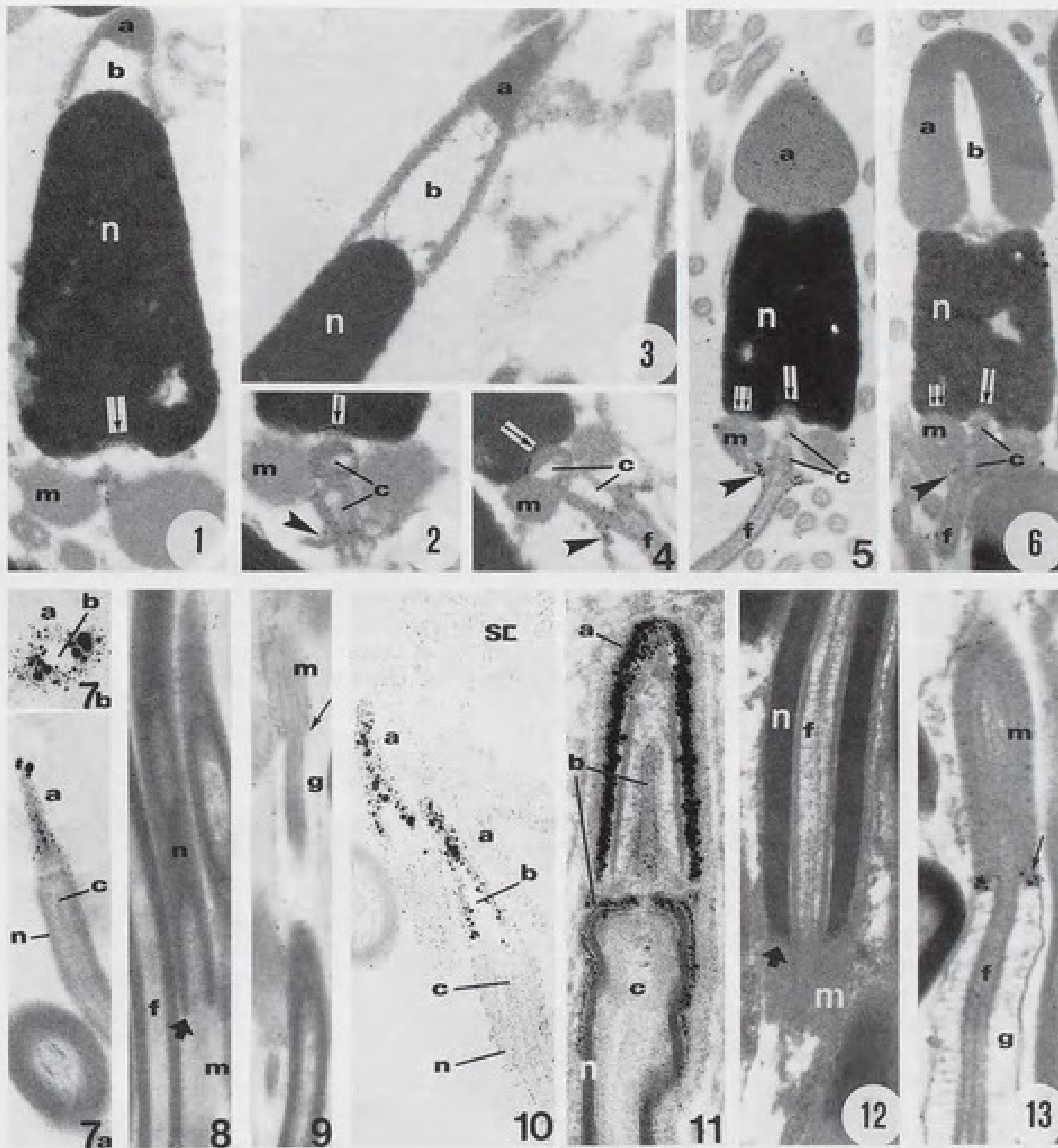
Specimens (Table 1) were collected from sands at the Atlantic coast of Portugal and Spain. Small pieces of testis (< 1 mm) were fixed with 2.5% glutaraldehyde buffered with 0.2 M Na Cacodylate, pH 7.4, for 2 h at 4°C, rinsed in the same buffer for 2 h at 4°C, postfixed in Carnoy (acetic acid:methanol, 1:3) for 5 min at room temperature, gradually rehydrated in ethanol (10 min in each step), and rinsed in distilled water for 30 min. They were then immersed in 50% aqueous silver nitrate (filtered by 0.2 µm) and placed in a water-bath under a Philips lamp (500 W) in such a way as to reach 55°C for 10 min. Finally, the specimens were rinsed in distilled water (3 x 2 min), incubated in an ammoniacal silver-formalin solution (2 g silver nitrate in 2.5 ml distilled water + 2.5 ml 33% ammonia solution + 5 ml 3% aqueous formalin, 0.2 µm filtered) for 3 min, rinsed in distilled water (3 x 2 min), dehydrated in an ethanol graded series, embedded in Epon, and examined by transmission electron microscopy.

TABLE 1. — Silver staining of mature spermatozoa in the Mollusca.

AV, acrosomal vesicle; SAM, subacrosomal material; CF/MF, centriolar fossa/mitochondrial fossa; PCS, material that links the proximal centriole to the nuclear base; Ce, centrioles; PCC/An, pericentriolar complex/annulus.

Taxa	AV	SAM	CF/MF	PCS	Ce	PCC/An	Reference
Gastropoda							
Patellogastropoda							
<i>Helcion pellucidus</i>	-	-	+	-	±	±	[63]
<i>Patella rustica</i>	-	-	+	-	±	+	[63]
Archaeogastropoda							
<i>Gibbula umbilicalis</i>	+	-	+	-	±	+	present study
<i>Gibbula cineraria</i>	-	-	±	-	±	±	present study
Caenogastropoda							
<i>Littorina saxatilis</i>	+	-	-	-	-	-	[58]
<i>Littorina littorea</i>	+	-	-	-	+	-	[58]
<i>Nucella lapillus</i>	+	+	+	-	-	+	[4, 5]
Bivalvia							
Heterodonta							
Veneroidea							
<i>Donax trunculus</i>	+	-	+	+	+	+	[61]
<i>Spisula solidissima</i>	+	+	+	-	±	+	[61]
<i>Cerastoderma edule</i>	+	-	+	+	-	-	[58]
<i>Scrobicularia plana</i>	-	-	+	-	-	+	[61]
Pteriomorpha							
Mytiloidea							
<i>Mytilus edulis</i>	+	-	-	-	-	-	[58]
Ostreoidea							
<i>Ostrea edulis</i>	+	-	+	+	-	-	[62]
<i>Crassostrea angulata</i>	+	+	+	+	+	+	[62]

FIGS 1-13. — Prosobranchia. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; f, flagellum; g, glycogen sheath. 1, 2: *Helcion pellucidus*. Silver stains the centriolar fossa (arrow), and lightly stains the centrioles (c) and the pericentriolar complex (arrowhead). x 29 000. 3, 4: *Patella rustica*. Silver stains the centriolar fossa (arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 24 600. 5: *Gibbula umbilicalis*. Silver stains the acrosomal vesicle (a), the centriolar fossa (arrow), the mitochondrial fossa (double arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 17 900. 6: *Gibbula cineraria*. Silver lightly stains the centriolar fossa (arrow), the mitochondrial fossa (double arrow), the centrioles (c), and the pericentriolar complex (arrowhead). x 17 900. 7-9: *Littorina saxatilis*.



Silver stains the acrosomal vesicle (a). Large arrow, mitochondrial fossa; small arrow, annulus. x 32 000; x 18 600; x 18 600. **10:** *Littorina littorea*. Silver stains the acrosomal vesicle (a) and the centriole (c). Results for the mitochondrial fossa and the annulus are as for *L. saxatilis*. SC, sertoli cell. x 41 000. **11-13:** *Nucella lapillus*. Silver stains the acrosomal vesicle (a), the subacrosomal material (b), the mitochondrial fossa (large arrow), and the annulus (small arrow). x 51 600; x 30 000; x 33 000.

RESULTS AND DISCUSSION

Silver staining has been used to demonstrate the argyrophilia of acidic phosphoproteins contained in nucleolar components and their metaphase counterparts [60]. However, silver staining has also been shown to stain, in invertebrate species, other nuclear and cytoplasmic structures of germ cells and thereafter used to show different staining characteristics in different sperm species [57-64].

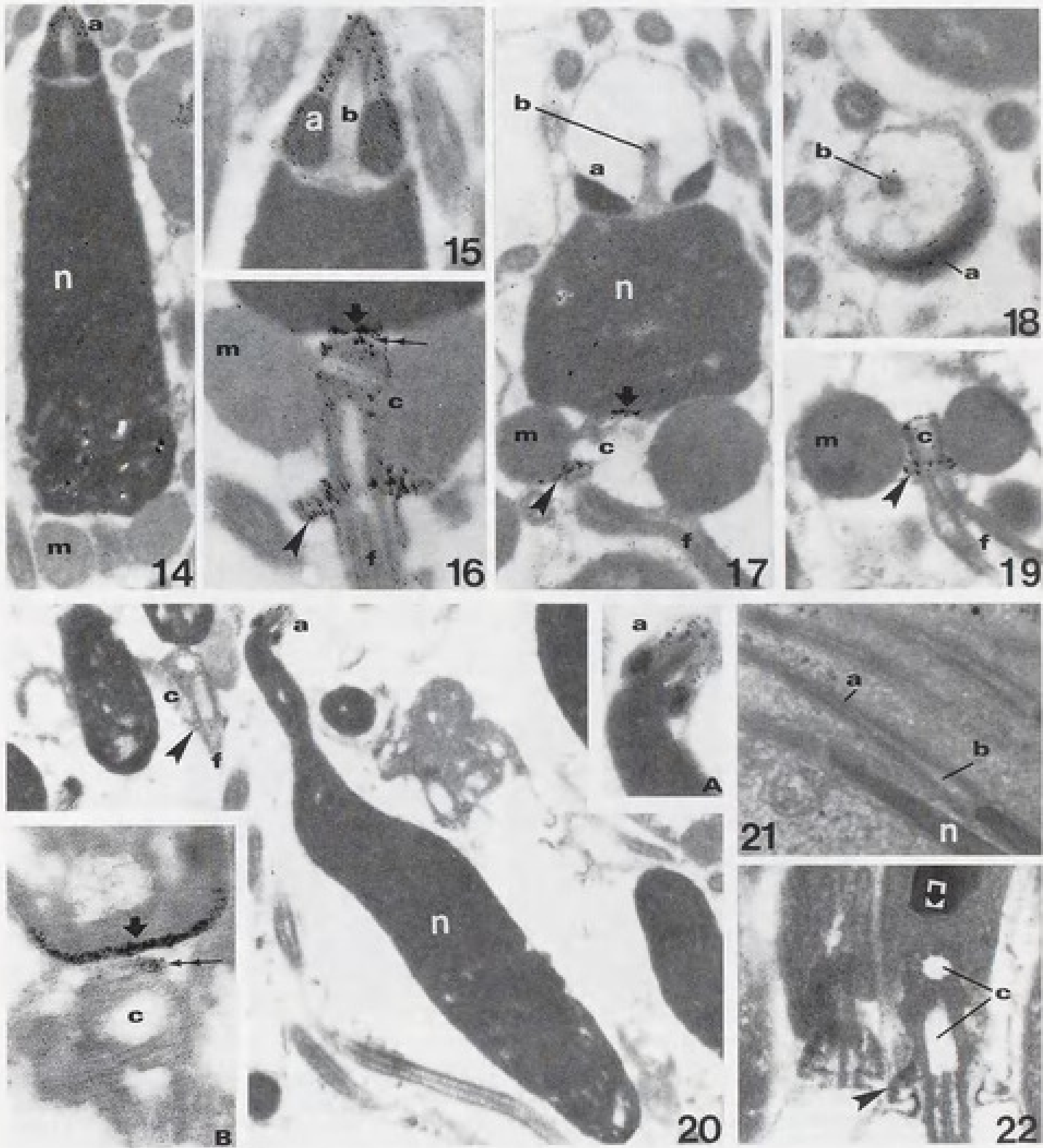
In the present work, we update all results obtained in the Mollusca with this silver staining technique, and discuss its relative importance as a phylogenetical sperm marker (Table 1). In the Patellogastropoda, *Helcion pellucidus* and *Patella rustica*, which are morphologically different, exhibit a similar pattern of silver staining (Figs 1-4); in the Archaeogastropoda, *Gibbula umbilicalis* and *G. cineraria* differ in nuclear and acrosomal vesicle dimensions as also in their silver staining characteristics (Figs 5, 6); in the Caenogastropoda, despite the morphological similarity between *Littorina saxatilis*, *L. littorea* and *Nucella lapillus*, their silver staining characteristics are very dissimilar (Figs 7-13). In the Veneroidea, *Donax trunculus*, *Spisula solidissima*, *Cerastoderma edulis* and *Scrobicularia plana* are all very different in their morphological aspects, and also present very distinctive silver staining characteristics (Figs 14-22). In the Mytiloidea (Pteriomorpha), only *Mytilus edulis* has been studied by silver staining (Figs 23, 24); but in the Ostreoidea, despite the morphological similarity between *Ostrea edulis* and *Crassostrea angulata*, both species can be easily and completely differentiated by silver staining (Figs 25-27).

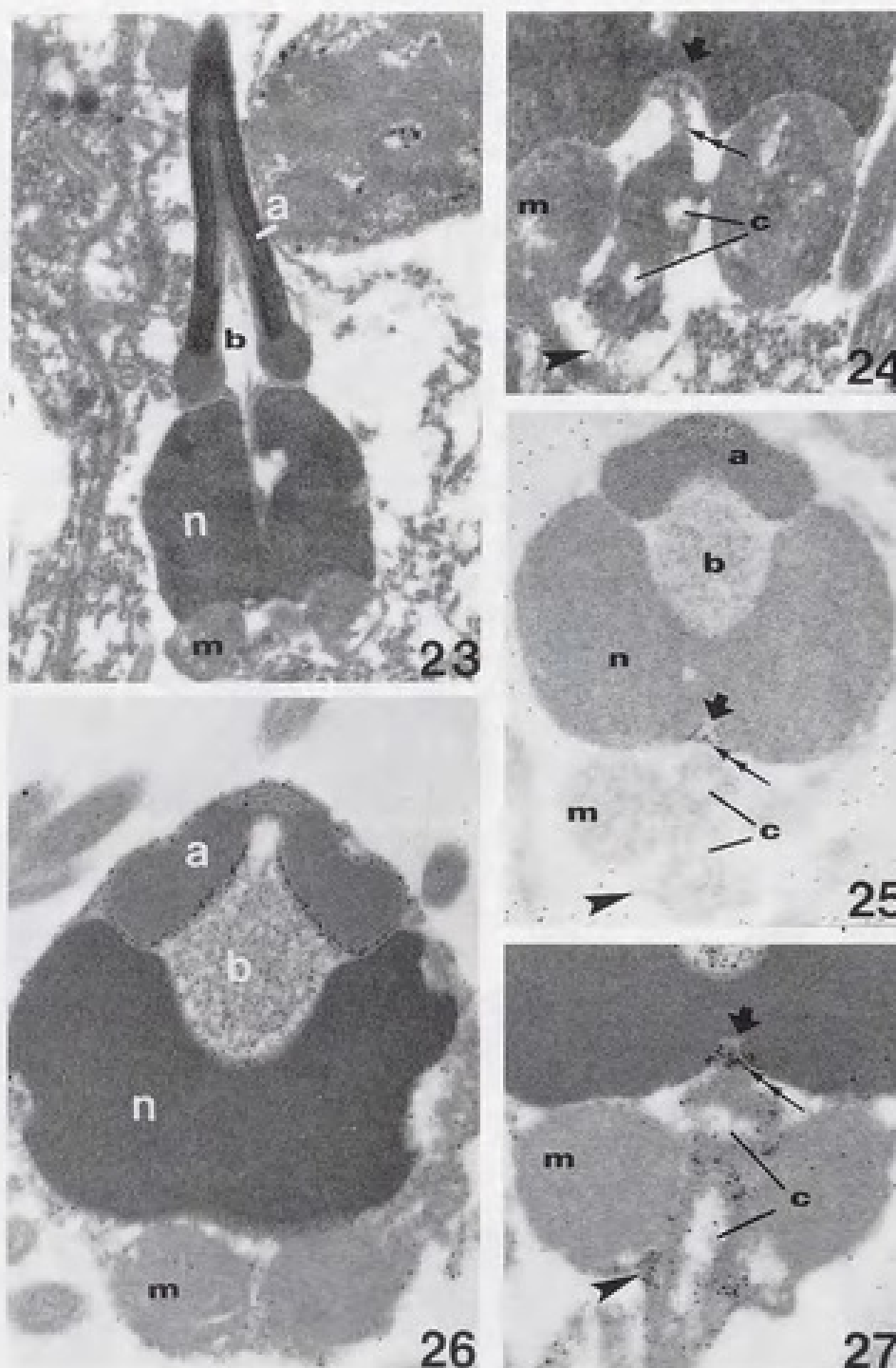
In conclusion, the silver staining of mature spermatozoa can enable us to distinguish different sperm species which present identical ultrastructural characteristics, and it can also help in phylogenetical studies since spermatozoa of the same group frequently share several silver staining characteristics (Table 1). However, the validation of this cytochemical marker as a useful tool in phylogenetical studies awaits further studies on many other molluscan species.

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FIGS 14-22. — Veneroidea. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; f, flagellum. **14-16: *Donax trunculus***. Silver stains the acrosomal vesicle (a), the centriolar fossa (arrow), the material that links the proximal centriole to the centriolar fossa (double arrow), the centrioles (c), and the pericentriolar complex (arrowhead). x 22 500; x 40 000; x 40 000. **17-19: *Spisula solidissima***. Silver stains the dense component of the acrosomal vesicle (a), the insertion of the tip of the perforatorium into the acrosomal vesicle (b), the centriolar fossa (arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 25 700; x 36 200; x 27 600. **20: *Cerastoderma edule***. Silver stains the acrosomal vesicle (a), the nuclear base (arrow), and the material that links the proximal centriole to the nuclear base (double arrows). Arrowhead, pericentriolar complex. x 20 000; inset A, x 46 000; inset B, x 80 000. **21, 22: *Scrobicularia plana***. Silver stains the nuclear base (arrow) and the pericentriolar complex (arrowhead). x 16 900; x 47 400.





FIGS 23-27. — Pteriomorpha. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; large arrow, centriolar fossa; double arrows, material that links the proximal centriole to the centriolar fossa; arrowhead, pericentriolar complex. 23, 24: *Mytilus edulis*. Silver stains the acrosomal vesicle. x 17 600; x 40 700. 25: *Ostrea edulis*. Silver stains the tip of the acrosomal vesicle (a), the centriolar fossa (large arrow), and the material that links the proximal centriole to the centriolar fossa (double arrows). x 28 900. 26, 27: *Crassostrea angulata*. Silver stains the tip of the acrosomal vesicle (a), the subacrosomal material (b), the centriolar fossa (large arrow), the material that links the proximal centriole to the centriolar fossa (double arrows), the centrioles (c), and the pericentriolar complex (arrowhead). x 30 900; x 39 900.

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