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Sperm ultrastructure of *Mytella* (Bivalvia) populations from distinct habitats along the northern coast of São Paulo State, Brazil

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ABSTRACT: Ultrastructural analyses of bivalve spermatozoa are relevant in studies that aim to identify taxonomic traits for the purposes of discriminating species and conducting phylogenetic studies. In the present work, spermatozoa of mussel specimens of the genus *Mytella*, collected from two populations living in distinct habitats, were examined by electron microscopy. The objective was to identify sperm ultrastructural taxonomic traits that could be used to differentiate *Mytella* species. The specimens were from populations that live in intertidal zones on the southeast coast of Brazil, either buried in muddy-sand sediment or anchored to rocky substrates. The acrosomal vesicle was conical and long, the axial rod extended from the nucleus to the acrosome, the nucleus was an oblate spheroid with a condensed chromatin, the intermediate portion contained mitochondria encircling a pair of centrioles, and there was a single flagellum. The sperm was of a primitive type. The spermatozoon ultrastructure did not distinguish the specimens buried in muddy-sand sediment from those anchored to rocky substrates. The data suggest that the specimens analyzed, despite living in distinct habitats, belong to the same species, which conchological analyses identified as *M. charruana*. The presence of an axial rod in their sperm cells supports the inclusion of *M. charruana* in the subfamily Mytilinae.

Introduction

The genus *Mytella* is represented by the species *M. strigata*, *M. speciosa*, *M. charruana* and *M. guyanensis* (Villarroel and Stuardo, 1995). Shells of the *Mytella* species are elongate, mytiliform to modioliform and have a subterminal umbo. The frontal edge is either smooth or exhibits 3 to 4 dents and there are two scars of frontal retractor muscles that are either smooth or concentrically striated. The posterior part of the mantle contains ramified tentacles (Rios, 1994). *M. charruana* and *M. guyanensis* occur along the coastline of Brazil. In the northeastern region, *M. charruana* is appreciated as an edible mussel and is popularly known as "Sururu" (Rios, 1994).

Mytella charruana is distributed along the Pacific coastline from Mexico to Ecuador and the Galapagos Islands and along the Atlantic coastline from Venezuela to Argentina. It has also been reported to have been

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recently introduced to Florida, U.S.A., most likely by a boat's ballast water. In Florida, *M. charruana* is popularly known as the "charrua mussel" and has been considered to represent a potential menace to local species, including commercially important native oysters (Boudreaux and Walters, 2006).

Specimens of *Mytella charruana* Orbigny, 1842, have been assigned several names, including *Mytella falcata* Orbigny, 1846, *Modiola falcata* Von Ihering, 1897, *Mytilus strigatus* Von Ihering, 1900, *M. arciformis* Dall, 1909, *M. mundahuensis* Duarte, 1926 and *Modiolus falcatus* Morretes, 1949. *M. charruana* specimens measure between 22 and 50 mm and are highly variable in their morphologies. The front shell edge is short and rounded, the dorsal angle is frequently prominent, the color varies from yellow/brown on the anterior-ventral region to green on the dorsal region, and the inner face of the shell is dark purple. Thousands of *M. charruana* specimens are found in intertidal zones, living at high population densities in muddy-sand sediment banks and in shallow estuarine waters.

The species *Mytella guyanensis* is found in intertidal zones of mangrove and estuarine regions. *M. guyanensis* is distributed from western Mexico to Peru and from Venezuela to Brazil (Santa Catarina). In this species, the shell size varies from 33 to 70 mm (Rios, 1994). Because *Mytella guyanensis* and *Mytella charruana* share substantial conchological similarities, it has frequently been difficult to unequivocally distinguish the identities of these two species.

Many works have emphasized the relevance of sperm morphology for taxonomy. The spermatozoan ultrastructure has previously been used to successfully investigate phylogenetic relationships among bivalves. Some authors have meticulously analyzed bivalve sperm morphologies, covering undescribed families and providing important phylogenetic discussions (Bernard and Hodgson, 1985; Hodgson *et al*., 1990; Guerra *et al*., 1994; Sousa and Oliveira, 1994; Healy, 1995; Garrido and Gallardo, 1996; Komaru and Konishi, 1996; Healy, 1996; Kafanov and Drozdov, 1998; Healy *et al*., 2000; Healy *et al*., 2001; Erkan and Sousa, 2002; Gwo *et al*., 2002; Introíni *et al*., 2004; Healy *et al*., 2008; Introíni *et al*., 2009). The specificity of sperm features in some families and sub-families of bivalves has been described in the literature. According to Kafanov and Drozdov (1998), based on the absence or presence of an acrosomal rod, the species of the recent Mytiloidea have been grouped into two subfamilies: Modiolinae and Mytilinae. Species lacking an acrosomal rod can be considered members of the subfamily Modiolinae, while the presence of this structure suggests that other species belong to the subfamily Mytilinae. Hence, electron microscopy of bivalve sperm cells has been widely employed as an important tool to solve taxonomic and phylogenetic questions, including those related to the family Mytilidae. Introíni *et al*. (2004 and 2009) have found relevant differences between closely-related species using transmission and scanning electron microscopy. These differences suggested that characteristics of the acrosome could be an important consideration for taxonomic differentiation.

Comparative studies using phosphotungstic acid staining, together with morphology and morphometrics, could contribute to taxonomic and phylogenetic issues because spermatozoa of the same group usually share similar basic cytochemical features (Sousa *et al*., 1998). Rambourg *et al*. (1969) reported that the component in rat cells that reacts with phosphotungstic acid is glycoprotein and that this acid stains the saccharide portions. However, under similar experimental conditions, phosphotungstic acid also markedly stains the lysin (Endo, 1976). Comparing cytochemical acrosomal staining of spermatozoa could distinguish between different sperm species that present identical ultrastructural features but distinct phosphotungstic acid patterns.

In the present work, *Mytella* specimens living either buried in muddy-sand sediment or anchored to rocky substrates were analyzed for the ultrastructure of their spermatozoa, with the objective of comparing these two populations and identifying sperm ultrastructural traits useful for *Mytella* taxonomic studies. The different compartments of the acrosomal vesicle appear to play important roles in the acrosome reaction, suggesting that its chemical properties are of some interest. In consideration of this, the present work also investigated the acrosome of *Mytella* using phosphotungstic acid staining.

Material and Methods

Specimens of *Mytella* from populations living in intertidal zones buried in muddy-sand sediment were sampled from Camaroeiro beach, Caraguatatuba County (23º37'38.5" S; 45º23'51.1" W) and specimens that live anchored to rocky substrates were sampled in the Enseada beach, São Sebastião County (23S 43'29", 45W 24'50"). Both sampling sites are located along the northern coast of São Paulo State, Brazil. The sampled specimens were immediately immersed in seawater and brought to a laboratory at the State University of Campinas, SP, Brazil,

where they were processed for analyses. Voucher specimens were deposited in the Museu de Zoologia "Prof. Dr. Adão José Cardoso" (ZUEC) at the State University of Campinas (UNICAMP), São Paulo, Brazil under de accession numbers 1714-1718 and 1720-1721.

Transmission electron microscopy (TEM)

Small sections (0.1 to 0.3 mm^3) of male gonads were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, containing 3% w/v sucrose, pH 7.2, for 24 h at 4ºC. After fixation, they were washed with 0.1M cacodylate buffer for 3 h at 4ºC, and postfixed with 1% osmium tetroxide (OsO_4), in the same buffer, for 1h at 4ºC. Subsequently, samples were dehydrated in a graded acetone series and embedded in EPON resin. Sections were stained with uranyl acetate and lead citrate and then examined with a Zeiss Leo 906 TEM. Twenty five spermatozoa of five individuals from each population of bivalves (totaling fifty sperm cells) were analyzed and measured using the images from the transmission electron microscope. An average was calculated from the values obtained from all studied sperm cells.

Ultrastructural cytochemistry: detection of basic proteins

Phosphotungstic acid was used for detection of basic proteins. Small sections of male gonads were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, containing 3% w/v sucrose, pH 7.2. Samples were washed with the same buffer and dehydrated in alcohol, adding 1% phosphotungstic acid to the alcohol in the last washing step (Sousa *et al*., 1998). Subsequently, they were embedded in EPON resin. Ultrathin sections were examined with a Zeiss Leo 906 TEM.

Results

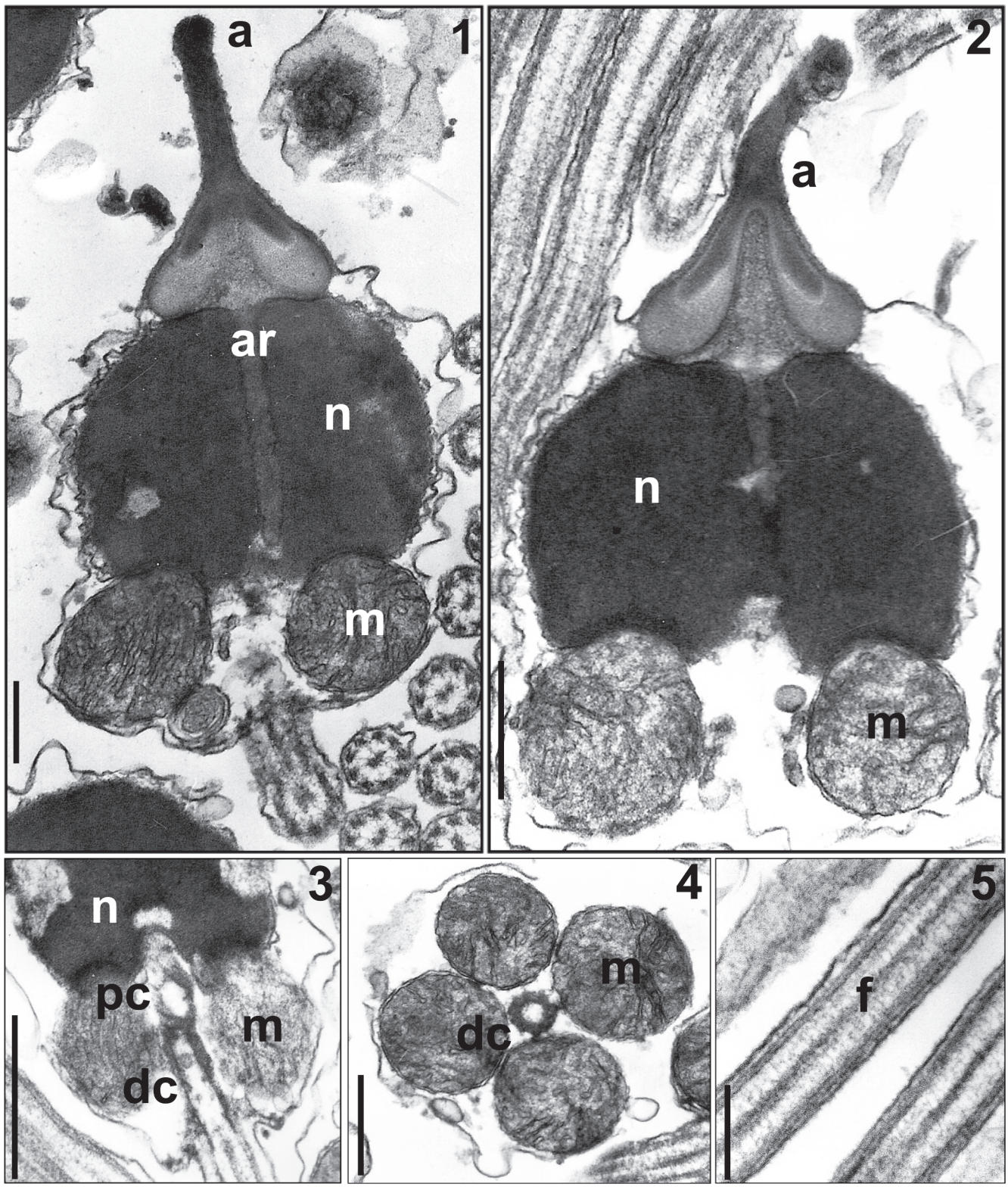
TEM analysis of *Mytella* spermatozoa revealed identical ultrastructural patterns in specimens from populations that live buried in muddy-sand sediment and those anchored to rocky substrates (Table 1). The present results showed a primitive sperm with a head and flagellum, adapted for external fertilization. The flagellum originated from the distal centriole, which, together with proximal centriole and mitochondria, formed the intermediate portion. The apical part of the nucleus was capped by a cone-like elongated acrosome and a subacrosomal area (Figs. 1-2 and 6-7). The apex of the vesicle contained electron-dense content, whereas the basal part of the cone was comparatively less electron-dense. There was a conspicuous indentation in its base, where it was possible to observe the presence of a subacrosomal area, consisting of heterogeneous material and an axial rod formed by a bundle of filaments. The nucleus was a slightly oblate spheroid and contained extremely condensed and homogeneous chromatin. There was a conspicuous anterior tubular-like nuclear depression where the axial rod projects from its basal location. The axial rod extended throughout the longitudinal axis of the nucleus (Figs. 1-2 and 6-7). There was a depression in the nuclear outline facing the midpiece: the posterior nuclear fossa (Figs. 3 and 8). The proximal centriole was anterior and perpendicular in relation to the distal centriole and was located inside the nuclear fossa. The distal centriole gave rise to the axoneme. The spermatozoon intermediate portion contained four to five spherical mitochondria encircling the distal and proximal centrioles (Figs. 3-4 and 8-9). There was a single flagellum (Figs. 5 and 10), whose microtubules were distributed in the basic 9+2 distribution pattern. The slight difference showed by the means of the

TABLE 1.

Morphometric and numerical data of sperm structures (n= 5 individuals from each population)

Population from	Nucleus length	Acrosome length	Nucleus width	Number of mitochondria
Muddy-sand sediment	1.8	1.68	2.2	4
Rocky shore	1.8	1.72	2.2	4 (92%) or 5 (8%)

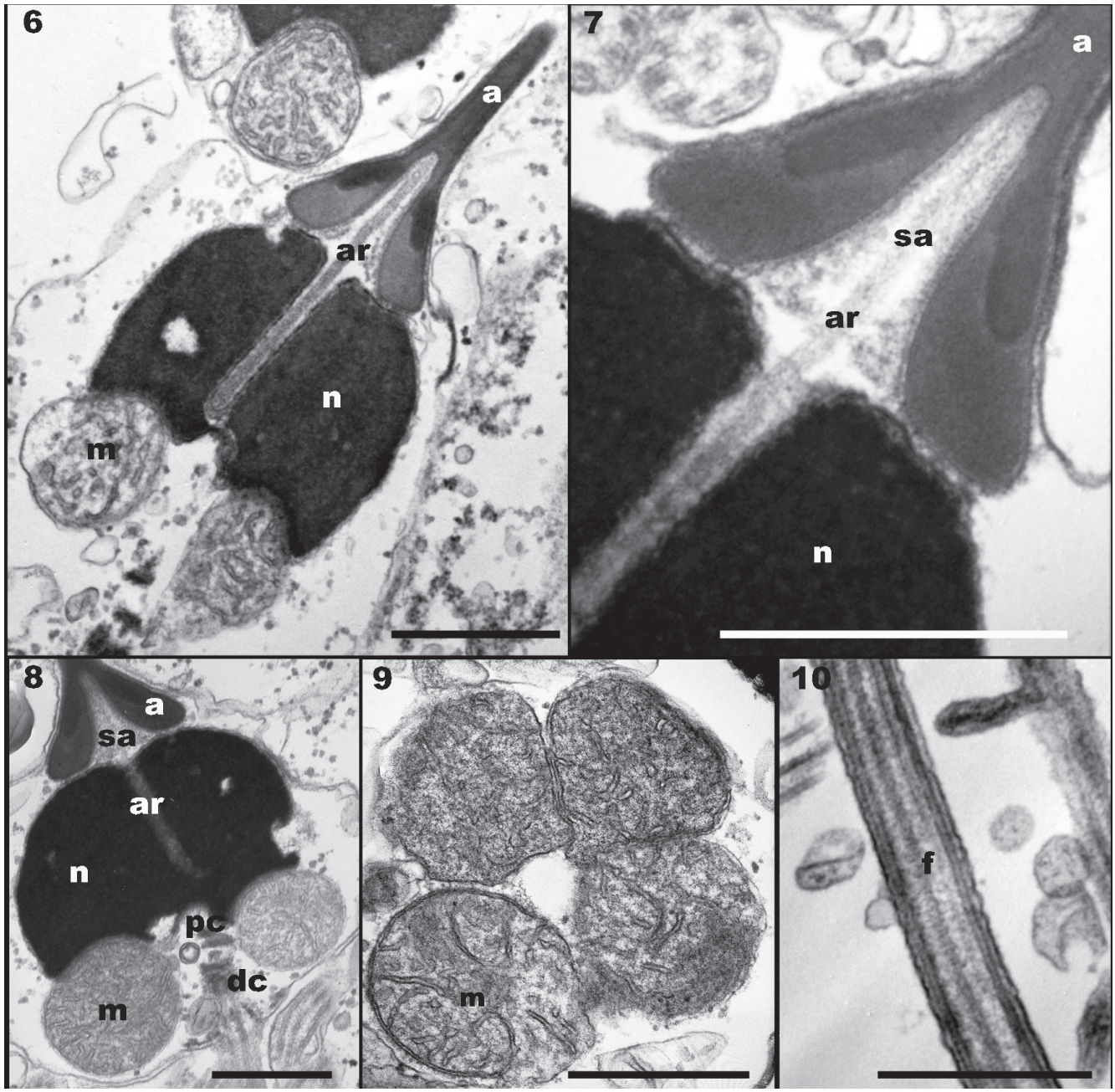
The values above correspond to an average based on all sperm cells analyzed. All morphometric measurements are in mm, except for the number of mitochondria.



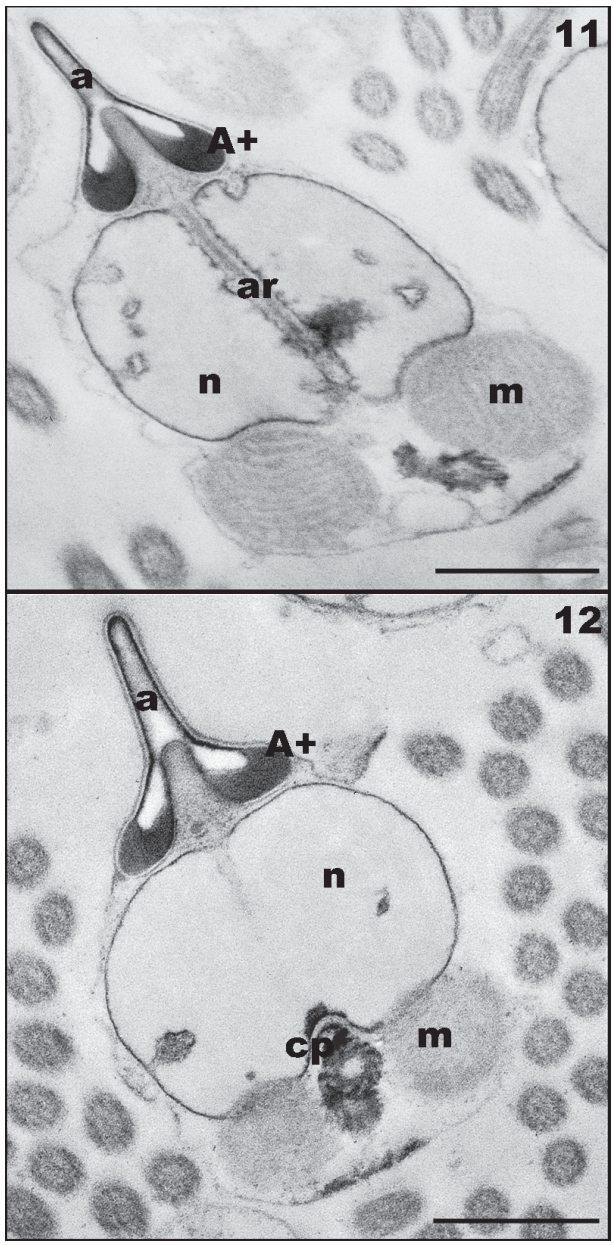
FIGURES 1-5. Spermatozoa of *Mytella* samples from mud-sandy sediment. 1-2: Longitudinal sections of the sperm cell head showing nucleus, subacrosomal region, axial rod and the acrosome that occasionally shows an inclined position. 3: Longitudinal section of the intermediate portion. 4: Transversal section of the intermediate portion. 5: Flagellum. Bars = 0.5 µm. a= acrosome; ar = axial rod; dc= distal centriole; pc= proximal centriole; f= flagellum; m= mitochondrion; n= nucleus.

acrosome length was not significant, since morphometrical variations were found in both populations. Measurements of the acrosomal vesicle extension of male gametes from both populations ranged from 1.65 to 1.75 μm, possibly reflecting a cellular pliability (Table 1).

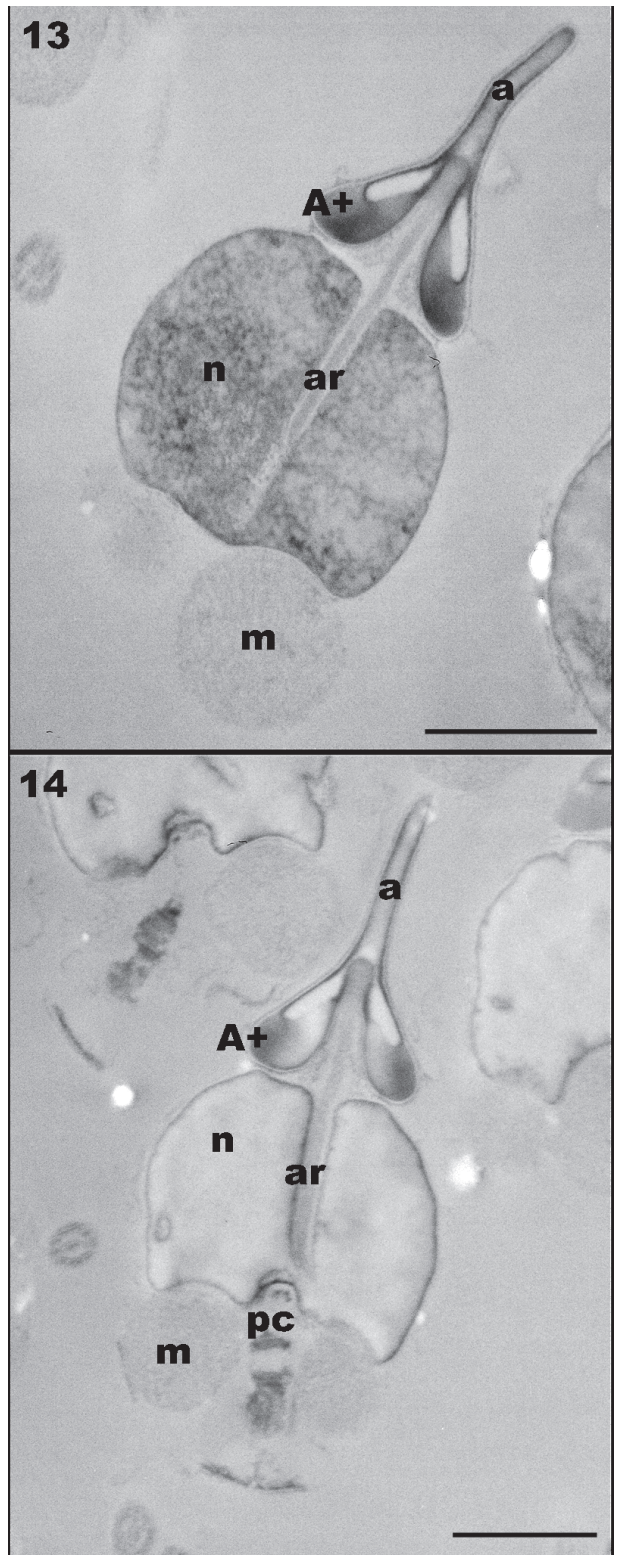
With respect to the cytochemical investigation (Figs. 11-15), the base of the acrosomal vesicle reacted positively to the phosphotungstic acid technique, indicating potentially high amounts of basic proteins. When images obtained from conventional transmission electron microscopy were compared with those from samples treated with phosphotungstic acid, an inversion in electron density patterns of the acrosomal vesicle was observed: the clear areas became electron dense and vice-versa (Fig. 15).



FIGURES 6-10. Spermatozoa of *Mytella* samples from rocky shores. 6: Longitudinal section of the sperm cell head showing nucleus, subacrosomal region, axial rod and the acrosome. 7: Longitudinal section of the subacrosomal area. 8: Longitudinal section of the intermediate portion. 9: Transversal section of the intermediate portion. 10: Flagellum. Bars = 1 μm (Figs. 6-9), 0.5 μm (Fig. 10). a= acrosome; ar = axial rod; dc= distal centriole; pc= proximal centriole; f= flagellum; m= mitochondrion; n= nucleus, sa= subacrosomal area.



FIGURES 11-12. Spermatozoa of *Mytella* samples from mud-sandy sediment treated with E-PTA, at low pH, for detection of basic proteins. The acrosomal portion lays on the nucleus as a cap and the base of the acrosomal vesicle reacts positively to the E-PTA technique, indicating relatively high amounts of basic proteins. Note an inversion in electron density patterns of the acrosomal vesicle, comparing images obtained from conventional transmission electron microscopy and from samples treated with E-PTA; in this case, the clear areas become electron dense and vice-versa. Bars = 1 μm. a= acrosome; A+= E-PTA positive reaction, which reveals basic proteins; ar = axial rod; cp= proximal centriole; m= mitochondrion; n= nucleus.



FIGURES 13-14. Spermatozoa of *Mytella* samples from rocky shore treated with E-PTA, at low pH, for detection of basic proteins. The acrosomal portion lays on the nucleus as a cap and the base of the acrosomal vesicle reacts positively to the E-PTA technique, indicating relatively high amounts of basic proteins. Note an inversion in electron density patterns of the acrosomal vesicle, comparing images obtained from conventional transmission electron microscopy and from samples treated with E-PTA; in this case, the clear areas become electron dense and vice-versa. Bars = 1 μm. a= acrosome; A+= phosphotungstic acid positive reaction, which reveals basic proteins; ar = axial rod; pc= proximal centriole; m= mitochondrion; n= nucleus.

Discussion

Sperm ultrastructure characteristics have been used as tools in studies of the taxonomic and phylogenetic relationships of Bivalvia, including the family Mytilidae. To date, spermatozoa of Mytilidae have been described as a primitive type, which is typical of invertebrates that reproduce by external fertilization. In these species, the sperm acrosome is conical and can be either long- or short-shaped, the nucleus is relatively small, the ultrastructural organization of the intermediate piece is similar to the one described herein, in which spherical mitochondria are grouped as a ring encircling the distal and proximal centrioles, and the sperm has a single flagellum in the posterior region (Kafanov and Drozdov, 1998; Reunov *et al.,* 1999; Introíni *et al*., 2004).

According to Kafanov and Drozdov (1998), the genera within the superfamily Mytiloidea (*Adula*, *Arcuatula*, *Aulacomya*, *Brachidontes*, *Bathymodiolus*, *Choromytilus*, *Crenomytilus*, *Modiolus*, *Musculista*, *Musculus*, *Mytilus*, *Perna*, *Perumytilus*, *Semimytilus*, *Septifer* and *Trichomya*) can be classified in numerous groups according to the size of the acrosomal vesicle, chromatin condensation pattern, number of mitochondria and presence of an axial rod extending from the subacrosomal region to the acrosome (Reunov and Hodgson, 1994; Garrido and Gallardo, 1996; Healy *et al*., 2000). The available data on sperm ultrastructure and conchology of Mytiloidea species constitute a set of traits that have suggested taxonomic changes in these taxa. Consequently, Mytilidae is the only family formally remaining in the recent bivalve superfamily Mytiloidea. Currently, the family Mytilidae is subdivided into two subfamilies according to the presence or absence of an acrosomal rod. Species without the axial rod are grouped within the family Modiolinae, while species that contain the axial rod are considered members of the subfamily Mytilinae. There is strong evidence that all the sperm types of Mytilidae species are derived from the genus *Modiolus* (Kafanov and Drozdov, 1998).

The spermatozoon morphology of the *Mytella* specimens studied in the present work is in agreement with the known sperm characteristics of the family Mytilidae. The presence of the long axial rod, extend-

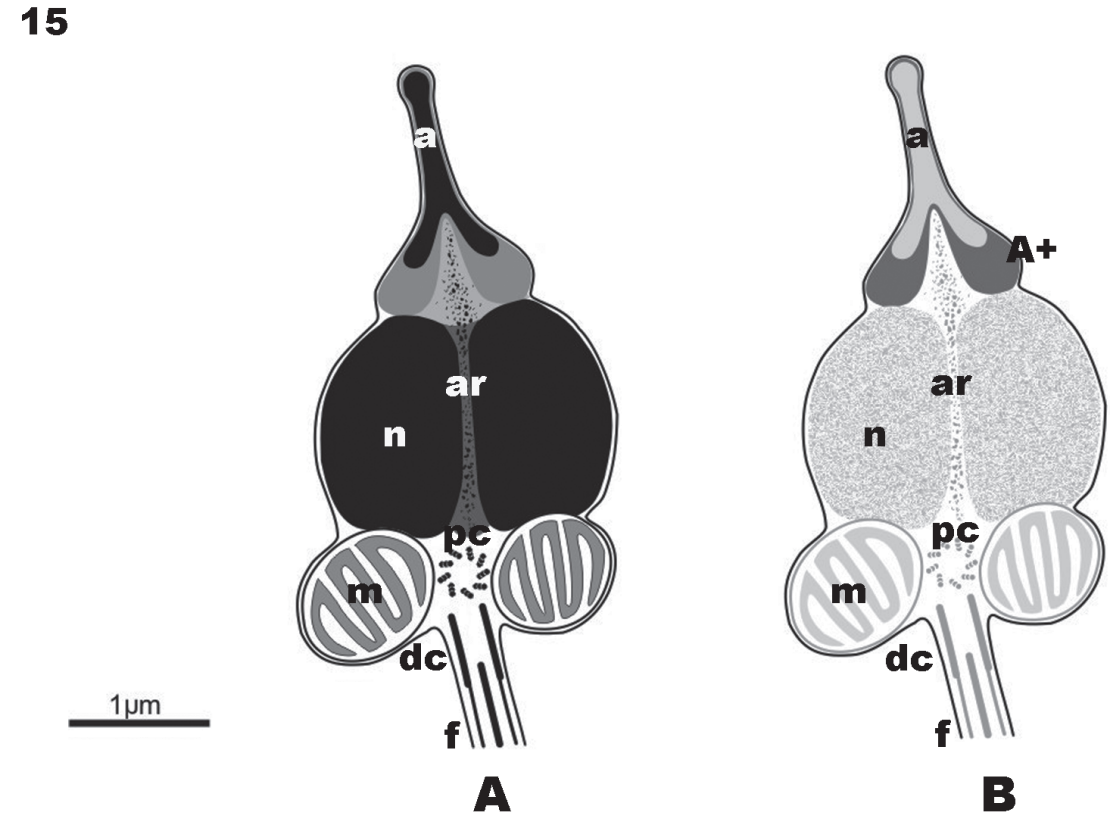


FIGURE 15. Schematic illustrations of *Mytella charruana* sperm cells. A: Sperm cell fixed with glutaraldehyde and osmium. **B:** Sperm cell treated with E-PTA. Bar = 1µm.
a= acrosome; A+= phosphotungstic acid positive reaction, which reveals basic proteins; ar = axial rod; dc= distal centriole; pc= proximal centriole; f= flagellum; m= mitochondrion; n= nucleus.

ing from the sperm nucleus to the acrosomal vesicle, justifies inclusion of the genera *Perna* and *Mytilus* in the subfamily Mytilinae, together with the two *Mytella* populations studied in this work. However, various other genera, such as *Modiolus* and *Brachidontes*, do not have axial rods and are considered members of the subfamily Modiolinae (Introíni *et al*., 2004).

Studying *Mytilus edulis*, Wada *et al*. (1956) observed that the sperm cell set the egg-membrane lysin free during the acrosomal reaction. The substance responsible for this process has since been identified as the component that corresponds to an annular deposit in the "basal ring" of the intact acrosome. This material is entirely solubilized in a very short period of time after the apex of the acrosome bursts at the beginning of the reaction.

Based on the available literature, the data on phosphotungstic acid acrosomal staining of species of the Mytilidae family are barely sufficient for a suitable discussion; however, they are enough to outline a brief comparison (Endo, 1976; Sousa and Oliveira, 1994; Sousa *et al*., 1998; Introíni *et al*., 2004, 2009). There are only two publications about phosphotungstic acid staining of mussels. Endo (1976) described the phosphotungstic acid staining of the acrosome of *Mytilus edulis*, which was subdivided into various regions (diagrammatically schematized) based on electron density patterns and structural differences. Phosphotungstic acid stained a relatively slender strand in the anterior portion of the acrosome, a peripheral proximal section and, more conspicuously, "the basal ring". According to this author, basic amino acids may well be responsible for the pattern of phosphotungstic acid staining found in the *Mytillus edulis* acrosomal vesicle. When comparing the species *Brachidontes darwinianus* and *B. solisianus,* Introíni *et al*. (2004) reported that the acrosome of both species showed two distinct regions: the vesicle base was stained by phosphotungstic acid, whereas the acrosomal apex was not stained. Hence, the results found in the present work corroborate those of Endo (1976) and Introíni *et al*. (2004). Although all spermatozoa share similar cytochemical patterns, it should be emphasized that Endo (1976) observed two additional acrosomal sites that reacted positively to the phosphotungstic acid technique.

According to Wada *et al*. (1956) and Endo (1976), the vesicle base (termed the "basal ring" by these authors) of *Mytilus edulis* is rich in a substance that can cause lysis of the egg membrane. Based on these results and observations, we can infer that the region of the acrosome that was similarly stained by phosphotungstic acid in *Mytella* and *Brachidontes* may also be rich in lysin. However, further studies that examine phosphotungstic acid staining after pronase digestion, or describe the acrosomal reaction of species of other Mytilidae genera, are required to definitively confirm whether the "basal ring" of the acrosome of mussels is rich in lysin.

Sperm morphology and cytochemical features were identical between the populations from the muddy-sand sediment or rocky habitats. Hence, the data suggest that these populations belong to the same species, even though they live in distinct habitats. All of the specimens studied were further examined for conchological characteristics and identified as *Mytella charruana* (Dr. Osmar Domaneschi and Dr. Eliane Arruda, personal communication).

According to Oliveira *et al*. (2005), the fact that *M. charruana* populations form huge aggregates, living at high population densities, could partially explain the high genetic variability in this species. For instance, in Lepanto beach, Costa Rica, *M. charruana* individuals were recorded at the density of 5,400 individuals per m^2, with an impressive number of 61 million individuals in a single population (Sibaja, 1985 *apud* Oliveira *et al.,* 2005). *M. charruana* preferentially lives in muddy-sandy bottoms rather than on rocky substrates (Nishida *et al*., 2006 *apud* Carranza *et al*., 2009). According to Gorbushin (1996), competition is an interaction among individuals that is triggered by similar demands on limited supplies, resulting in lower survival and decreased growth and reproductive rates of the competitors. *M. charruana* populations frequently reach high densities in muddy-sand banks, leading to a significant intraspecific competition. Most likely, *M. charruana* individuals are occasionally recruited away from this habitat and consequently establish new populations on neighboring rocky substrates. Considering the sperm morphology of *Mytella charruana*, this species possibly produces planktotrophic larvae (Franzén, 1983). The planktonic larval stage allows large dispersion of species before recruitment.

The spermatozoon ultrastructural traits described in this work are consistent with sperm characteristics of diverse species in the family Mytilidae. The presence of a sperm axial rod upholds the classification of *M. charruana* in the subfamily Mytilinae. Moreover, the data reinforce the conchological *Mytella charruana* identification of all the examined specimens from the two distinct habitats. Strong intraspecific competition could be associated with the requirements of *M. charruana* populations for additional space and available supplies, expanding their colonization to diverse habitats.

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