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Characterization of ultramicroscopic parameters of rusa deer (Rusa Timorensis) spermatozoa

Caracterização de parâmetros ultramicroscópicos de espermatozoides de cervos rusa (Rusa timorensis)

Renata de Souza Gomes[1]; Maiana Silva Chaves[2]; Natanael Aguiar Braga Negreiros[3]; Leandro Rodrigues Ribeiro[4]; Vicente José de Figueiredo Freitas[5]; Andreia Maria da Silva[6]; Moacir Franco de Oliveira[7]; Alexandre Rodrigues Silva[8]; Dárcio Ítalo Alves Teixeira[9]*

Highlights

Rusa deer sperm is morphologically similar to other cervids but differs in size
The study identified head, midpiece, and tail length, along with head width.
These findings aid in rusa deer conservation and assisted reproduction.

Abstract

The rusa deer (Rusa timorensis) is a tropical species native to Asia, now found in Brazil, Malaysia, Thailand, New Caledonia, Australia, and Mauritius. Reproductive studies, including ultramicroscopic analyses, are essential for understanding species-specific sperm morphology and addressing potential reproductive issues that may impact conservation efforts. In this study, two adult male rusa deer were sedated using a dart containing ketamine (2 mg/kg) and xylazine (1 mg/kg). Semen was collected by electroejaculation, and a 60 µL aliquot of each sample was fixed in modified Karnovsky solution. The samples were sent for evaluation by scanning electron microscopy and transmission electron microscopy to observe

[1] Student of the Master's Course of the Postgraduate Program in Veterinary Sciences, Universidade Estadual do Ceará, UECE, Fortaleza, CE, Brazil. E-mail: resouzavet@gmail.com
[2] Researcher, National Council for Scientific and Technological Development/Cearense Foundation to Support Scientific and Technological Development (CNPq/ FUNCAP). Fortaleza, CE, Brazil. E-mail: maiana.chaves@uece.br
[3] Student of the Master's Course of the Postgraduate Program in Veterinary Sciences, UECE, Fortaleza, CE, Brazil. E-mail: natanael.negreiros@aluno.uece.br
[4] Veterinarian at Criadouro Fazenda Haras Claro, Caucaia, CE, Brazil. E-mail: leandrovet@gmail.com
[5] Prof. Dr., Physiology and Reproduction Control Laboratory, UECE, Fortaleza, CE, Brazil. E-mail: vicente.freitas@uece.br
[6] Post-Doc Researcher, Animal Germplasm Conservation Laboratory, Universidade Federal Rural do Semi-Árido, UFERSA, Mossoró, RN, Brazil. E-mail: andreia.m.silva@hotmail.com
[7] Prof. Dr., Animal Morphology Laboratory, Universidade Federal Rural do Semi-Árido, UFERSA, Mossoró, RN, Brazil. E-mail: moacir@ufersa.edu.br
[8] Prof. Dr., Animal Germplasm Conservation Laboratory, UFERSA, Mossoró, RN, Brazil. E-mail: alexrs@ufersa.edu.br
[9] Prof. Dr., Veterinary Imaging Diagnostic Laboratory, UECE, Fortaleza, CE, Brazil. E-mail: darcio.teixeira@uece.br
* Author for correspondence

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the cell surface and internal structures. Head length and width, midpiece length, and tail length were measured at 8.68, 5.55, 13.89, and 36.69 µm, respectively. The internal structures observed included the mitochondrial sheath, outer dense fibers, axoneme, nucleus, acrosome, and plasma membrane. These findings demonstrate that the spermatozoa of the rusa deer share structural similarities with those of other mammals, such as pigs, humans, and guinea pigs, while also exhibiting specific morphometric characteristics that may contribute to the development of effective conservation and assisted reproduction strategies. These results not only underscore the importance of preserving this species but also provide a foundation for future research aimed at optimizing reproductive techniques and maintaining genetic diversity in cervids.
Key words: Wildlife. Conservation. Cervids. Sperm Morphology.

Resumo

O cervo rusa (Rusa timorensis) é uma espécie tropical originária da Ásia, atualmente distribuída no Brasil, Malásia, Tailândia, Nova Caledônia, Austrália e Ilhas Maurício. Estudos sobre reprodução, incluindo análises ultramicroscópicas, são essenciais para a compreensão da morfologia espermática específica da espécie e para abordar possíveis problemas reprodutivos que afetam os esforços de conservação. Dois cervos rusa machos adultos foram sedados com um dardo contendo cetamina (2 mg/kg) e xilazina (1 mg/kg). A coleta de sêmen foi realizada por eletroejaculação e uma alíquota de 60 µL de cada amostra foi fixada em solução de Karnovsky modificada. As amostras foram enviadas para avaliação em microscópio eletrônico de varredura e microscópio eletrônico de transmissão, onde foi possível observar a superfície celular e suas partes, estruturas internas, e o comprimento e largura da cabeça, peça intermediária e cauda, medindo 8,68, 5,55, 13,89 e 36,69 µm, respectivamente. As estruturas internas observadas incluíam a bainha mitocondrial, fibras densas externas, axonema, núcleo, acrossomo e membrana plasmática. Tais achados evidenciam que os espermatozoides do cervo rusa compartilham características estruturais com os de outros mamíferos, como suínos, humanos e porquinhos-da-índia, além de exibirem particularidades morfométricas que podem ser úteis para aprimorar estratégias de conservação e reprodução assistida. Esses dados não apenas destacam a relevância de preservar a espécie, mas também oferecem bases para pesquisas futuras, visando à otimização de técnicas reprodutivas e à manutenção da diversidade genética em cervídeos.
Palavras-chave: Vida Selvagem; Conservação; Cervídeos; Morfologia Espermática.

Introduction

With the increasing impact of anthropogenic activities, wildlife populations are increasingly unable to maintain their biological cycles, putting their survival at risk. Therefore, studies on species population diversity are essential to prevent exploitation and support conservation strategies. The loss of diversity reduces an organism's ability to respond to environmental changes, eliminating potentially valuable biological information for humans, such as undiscovered biochemical compounds (Cruz et al., 2020).

Due to the difficulty of obtaining genetic material from wild animals, studies often focus on analyzing closely related, non-threatened species as models for studying physiological parameters or technical

adaptations (Comizzoli, 2015). The rusa deer (Rusa timorensis) is a tropical species native to Asia that is currently distributed across several countries, including Brazil, Malaysia, Thailand, New Caledonia, Australia, and Mauritius (Samsudewa & Capitan, 2011). Reproductive studies are crucial for understanding intraspecific variability in spermatozoa and the influence of geographical differences, both of which are essential for developing effective conservation strategies (Comizzoli, 2015). Sperm morphology varies among individuals and populations, serving as a tool for species classification within a phylogenetic context (Fitzpatrick et al., 2022). Additionally, morphometric analysis of spermatozoa helps identify attributes that routine tests cannot detect, such as subtle variations in size, shape, and membrane integrity.

For a more detailed characterization of spermatozoa, tools such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) allow for the description of both surface characteristics and internal morphology, including the nucleus, acrosome, mitochondrial sheath, and axoneme (Silva et al., 2015). TEM studies have described morphological aspects (Bartoov et al., 1980), the effects of different extenders (Badr et al., 2015) and diets (Younan et al., 2017) on spermatozoa, as well as disturbances in spermatogenesis, spermiogenesis, and sperm capacitation (Linn et al., 2021). On the other hand, SEM has been used to characterize the spermatozoa of deer species such as Dama dama, Axis axis, Panolia eldi, Cervus nippon taivunus, Odocoileus virginianus, and Rusa timorensis, focusing on their cell surface (Mahre et al., 2014; Cunha et al., 2021).

Given the importance of understanding sperm morphological diversity as a taxonomic tool and the need for detailed morphometric data to improve species identification and classification, this study aims to analyze the microscopic aspects of rusa deer spermatozoa raised in environments with edaphoclimatic conditions different from their native habitat. Their potential as animal models will enhance our understanding of sperm attributes. These findings are crucial for developing semen cryopreservation strategies and assisted reproductive techniques. Advancing knowledge of rusa deer reproductive biology will contribute to effective conservation strategies not only for this species but also for a wide range of threatened mammals.

Materials and Methods

Bioethics

This study was approved by the Ethics Committee on Animal Use (CEUA) of the State University of Ceará (approval number 09045821/2022). All procedures were conducted in accordance with animal care guidelines (Mellor et al., 2009).

Location and experimental animals

Two adult male rusa deer, kept under semi-confined conditions, were used in the experiment. They were fed Brachiaria grass (Brachiaria sp.) and maintenance feed for horses (Equimix, Integral Mix, Fortaleza, Brazil). The animals belonged to Haras Claro, a private farm located in Caucaia, CE, northeastern Brazil.

Caucaia is part of the Metropolitan Region of Fortaleza, in the state of Ceará, and has a semi-arid climate (Superintendência do Desenvolvimento do Nordeste [SUDENE], 2021). The predominant biome is the Caatinga, which features diverse shrub and tree vegetation with remnants of dry forests (Galdino et al., 2011).

Semen collection

For semen collection, the animals were sedated using a dart containing ketamine (2 mg/kg) and xylazine (1 mg/kg). Once sedated and positioned in lateral recumbency, the prepuce was cleaned with sterile saline solution. An electroejaculator probe (Neovet Autoejac v2) was then inserted into the rectum, and electrical stimulation was applied in three sequential series, following the protocol described by Fitri et al. (2017).

In the first series, electrical stimuli were applied with increasing voltage from 1 to 6 V in 1-V increments. Each voltage level was applied three times, with each stimulus lasting three seconds, followed by a one-second pause. In the second series, after a 1-min rest interval, stimuli were applied from 7 to 12 V, following the same pattern. In the third and final series, after another one-minute interval, voltages from 13 to 18 V were applied, maintaining the same protocol.

The ejaculate was collected in a Falcon tube and protected from light. A 60-µL aliquot of each sample was fixed in modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4) and stored under refrigeration for ultramicroscopic analysis.

Ultramicroscopic evaluation

Scanning and transmission electron microscopy analyses were performed in collaboration with the Animal Germplasm Conservation Laboratory of the Federal Rural University of the Semi-Arid Region.

Scanning electron microscopy (SEM)

For ultrastructural analysis, the methodology of Bezerra (2018) was adapted. Seminal samples fixed in Karnovsky solution were centrifuged at 800 × g for 10 min. The resulting pellet was washed three times for 5 min with 0.2 M phosphate buffer, post-fixed in 1% osmium tetroxide diluted in distilled water, and dehydrated through increasing concentrations of ethanol (50%, 70%, 90%, and 100%) for 10 min each. The pellets were broken apart, and a drop of the suspension was placed on a glass slide and air-dried. The slides were mounted on stubs using carbon tape. For metal coating, the stubs were placed in a metallizer and coated with a 20 nm gold layer for subsequent observation under a scanning electron microscope (Tescan Vega3; Tescan Analytics, Fuveau, France).

Transmission electron microscopy (TEM)

The fixed samples were washed with phosphate buffer, post-fixed with 1% osmium tetroxide, dehydrated in propylene oxide, and embedded in Epon resin (Embed 812; Electron Microscopy Sciences, Hatfield, USA). Ultrathin sections (60-70 nm) were manually stained with uranyl acetate, and contrast was detected with lead citrate. The samples were evaluated according to the parameters established by Silva et al. (2019).

Statistical analysis

Approximately 28 spermatozoa were observed, photographed, and measured using electron microscopy. Statistical analyses were performed using mean ± standard error of the mean, with significance set at $p < 0.05$. For sperm morphometric evaluation (µm), ImageJ software was used to measure head length and width, as well as tail length. SPSS for Windows 23 (IBM Corporation, 2014) was used to calculate the mean and standard deviation.

Results and Discussion

Two semen samples were collected from each animal following the previously described electroejaculation protocol. Fresh semen was evaluated immediately after collection, and a 60-µL fraction of each ejaculate was separated. In the laboratory, the material was pooled for analysis by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Animal 1 exhibited a higher ejaculate volume in both collections, with an average of 1.5 mL. The semen had a watery appearance, translucent coloration, pH of 8.5, total motility of 70%, progressive motility of 75%, vigor score of 4, viability of 97%, and sperm concentration of 45×10^6. In contrast, Animal 2 produced a lower ejaculate volume, averaging 0.7 mL. The semen from Animal 2 showed altered coloration and consistency, with significantly lower total and progressive motility and vigor compared to Animal 1. Its ejaculate had a brownish color, a milky appearance, total and progressive motility of 20%, vigor score of 1, sperm concentration of 510×10^6, and a pH of 8.

SEM analysis revealed that the sperm surface morphology (Figure 1A and 1B) showed a flat, broad head measuring 8.67 ± 0.34 µm in length and 5.11 ± 0.28 µm in width. The tail length was 51.79 ± 7.01 µm. TEM images allowed for the observation of internal structures, such as the mitochondrial sheath, nine outer dense fibers, and the axoneme composed of nine outer and two inner microtubules (Figure 2A). A longitudinal section revealed that the sperm head contains a nucleus surrounded by the nuclear membrane and an acrosome, with the nucleus occupying most of the head (Figure 2B). An intact plasma membrane, along with a well-preserved acrosome and nucleus, was also evident.

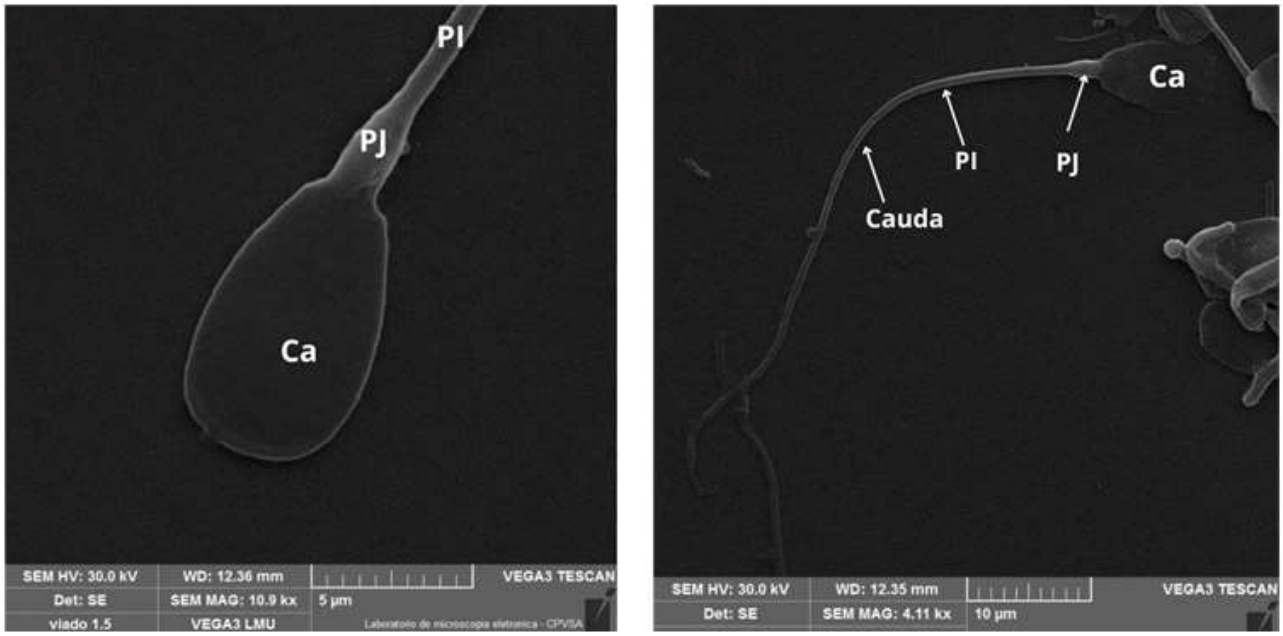

Figure 1. A and B. Representative images of rusa deer sperm viewed by scanning electron microscopy. Legend: **Ca** = Head, **PJ** = Junction Piece and **PI** = Intermediate Piece.

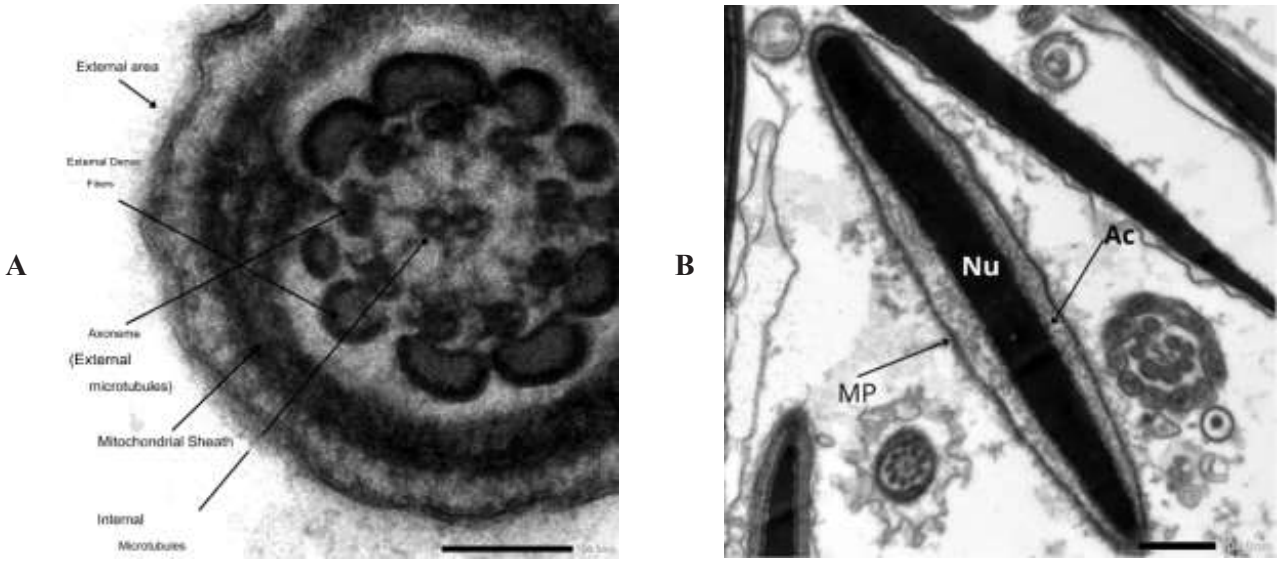

Figure 2. Ultrastructural image of Rusa deer sperm visualized by transmission electron microscopy. **A.** General structure of the sperm. **B.** Longitudinal section of the sperm head, highlighting the following structures: **Nu** = Nucleus, **MP** = Plasma membrane, **Ac** = Acrosome.

The results of this study indicate that spermatozoa from rusa deer raised in northeastern Brazil exhibit specific characteristics that can serve as a baseline for identifying alterations induced by management techniques and practices. The surface structure of rusa deer spermatozoa follows the pattern observed by Maher et al. (2014), with the head and tail divided into three parts: midpiece, principal, and terminal. However, the morphometric values observed differ from those reported by Maher et al. (2014), who described a sperm head length of 7.8 ± 0.28 µm and a width of 4.2 ± 0.15 µm.

In other deer species, such as the gray brocket deer (*Mazama gouazoubira*), the sperm head measures 8.5 ± 0.5 µm in length and 4.4 ± 1.0 µm in width (Cunha et al., 2021). These variations may be related to geographic distribution and species phylogeny, as discussed by Fitzpatrick et al. (2022). Additionally, these characteristics can be used to assess fertility and potential chromatin structure alterations in the male reproductive system (B. L. Sailer, 1996a).

Regarding internal morphology, rusa deer spermatozoa exhibit fundamental characteristics similar to those described in other mammalian species. Gu et al. (2019) examined sperm ultrastructure in ten mammalian species and found that all contained a set of nine outer dense fibers in the connecting piece, indicating an ultrastructural conservation pattern in these cells. The observed characteristics of the sperm head, with the nucleus occupying most of the space and a small cytoplasmic area, highlight the vulnerability of these cells to metabolic adaptations and stress, requiring seminal plasma factors and proteins for reproductive biotechnologies. Studies have shown that antioxidant proteins in seminal plasma play a crucial role in protecting spermatozoa from oxidative damage, contributing to cell integrity and male fertility (E. R. Sailer, 1996b).

This study provides detailed information on the morphology and ultrastructure of rusa deer spermatozoa, highlighting similarities and differences with other mammalian species. These findings are essential for developing effective conservation and assisted reproduction strategies, as well as supporting future research on the reproductive biology of this species.

Conclusion

The findings of this study indicate that rusa deer spermatozoa maintain the morphological pattern observed in other mammalian species, such as pigs, humans, and guinea pigs. However, they exhibit slightly larger morphometric measurements compared to other deer species evaluated in previous studies.

This difference may indicate modifications resulting from the evolutionary process, while the structural similarity suggests that rusa deer could serve as a model species for assisted reproduction protocols aimed at other endangered deer species.

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Author Contributions

Dárcio Ítalo Alves Teixeira was the advisor; Dárcio Ítalo Alves Teixeira and RSG designed the methodology; RSG conducted the experiment; LRR and GDPS performed handling and anesthesia; MSC, NABN, ARS, MFO, and AMS assisted in morphometric and electron microscopy analysis; Dárcio Ítalo Alves Teixeira and VJFF provided equipment and reagents; RSG wrote the manuscript; Dárcio Ítalo Alves Teixeira and MSC critically reviewed the manuscript.

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