Uttar Pradesh Journal of Zoology

Volume 45, Issue 13, Page 301-311, 2024; Article no.UPJOZ.3658
ISSN: 0256-971X (P)

Environmental Influences on Frozen Epididymal Sperm Cell Viability in White-Tailed Deer

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NCMH, MBM, IIAR and MBV designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors IIAR and MALV managed the analyses of the study. Authors CKLM, AV and MBV managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/upjoz/2024/v45i134157

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.mbimph.com/review-history/3658

Original Research Article

Received: 09/04/2024
Accepted: 13/06/2024
Published: 15/06/2024

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Cite as: *Muñoz-Hernandez, Nadia Carolina, Claudia Karina Lagunes-Moreno, Manuel Barrientos-Villeda, Miguel Angel Lammoglia-Villagomez, Ivan I. Avalos-Rosario, Anthony Valverde, and Manuel Barrientos-Morales. 2024. "Environmental Influences on Frozen Epididymal Sperm Cell Viability in White-Tailed Deer". UTTAR PRADESH JOURNAL OF ZOOLOGY 45 (13):301-11. https://doi.org/10.56557/upjoz/2024/v45i134157.*

ABSTRACT

The objective of this study was to evaluate the association between Temperature and Humidity Index (THI), age, and post-collection time with post-thawing sperm cell parameters such as progressive motility, viability, morphology, and cytoplasmic droplets. Samples were collected from 4 male white-tailed deer after regulated hunting. THI was calculated on collection days. Two THI categories were determined, ≥70 and <70. Thawed semen was placed on a slide and observed under a phase-contrast microscope to evaluate the progressively motile sperm cells. Morphology, viability, and cytoplasmic droplets were assessed on a smear stained with eosin-nigrosine and classified as normal or abnormal, viable or not viable, and present or absent, respectively. When THI was ≥70, the progressive motility (51.0 vs 30.67%), sperm viability (51.0 vs 36.33%), and the proportion of sperm cells with normal morphology (65.0 vs 37.67%) were higher than to days with a THI <70, without differences in the presence of cytoplasmic droplets. The 4-years bucks had a higher proportion of sperm cells with normal morphology (81.0 vs 35.5 and 26.0%) and cytoplasmic droplets (27.0 vs. 12.0 and 7.5%) compared to 2- and 3-year-old animals. Samples processed after 4 hours (51.0%) had a higher progressive motility than samples processed after 6 and 8 hours (32.0 and 25.0%, respectively). Spermatozoa processed after 8 hours (30.0%) had a lower viability than spermatozoa processed after 4 hours (51.0%). Compared to samples processed after 6 and 8 hours, those processed after 2 and 4 hours had a higher proportion of sperm cells with normal morphology (26.0 and 6.0 vs 81.0 and 65.0%, respectively). Samples processed after 2 hours had a higher (27.0 vs. 12.0%) proportion of sperm cells with cytoplasmic droplets than to those processed after 6 hours. High THI favors progressive motility and sperm viability. The older bucks had a greater proportion of sperm cells with normal morphology and cytoplasmic droplets than to the young ones. The greater progressive motility, viability, morphology, and presence of cytoplasmic droplets were found when the samples were processed within the first 4 hours.

Keywords: White-tailed deer; cryopreservation; spermatozoa; viability; morphology; motility.

1. INTRODUCTION

The white-tailed deer (*Odocoileus virginianus*) is one of Mexico's economically important wildlife species due to its importance in certain activities such as regulated hunting [1]. However, uncontrolled hunting practices without appropriate planning may have detrimental impacts, including reduced specimen number and genetic variation in low-density populations. This makes it necessary to find the means to improve the reproductive performance of white-tailed deer populations across the diverse geographical regions of Mexico.

Sperm cryopreservation continues to be a key tool in the breeding management of domestic and wildlife populations [2]. Utilizing sperm extracted from the epididymis from specimens recently hunted represents a significant avenue for recovering value germplasm [3]. Additionally, it has been shown that sperm obtained can be cryopreserved for long-term storage and subsequently utilized for different reproductive biotechnologies such as artificial insemination [4] protocols of superovulation and embryo transfer Waldhalm et al., [5], and *in vitro* embryo production Rubio-Santillanes et al., [6] in diverse subspecies white-tailed deer.

Several factors such as age and time lapse between the collection and processing of sperm may impact sperm cell parameters in white-tailed deer Texan subspecies (Ake-Lopez et al., 2010) and red deer [7]. However, there are other 14 subspecies distributed in Mexico [8], including the *veracrucis* subspecies. Therefore, this study aimed to evaluate the association of THI, age, and post-collection time with sperm cell parameters such as progressive motility, viability, morphology, and cytoplasmic droplets in the Veracruz subspecies of white-tailed deer.

2. MATERIALS AND METHODS

This study was performed in Comapa, Veracruz, Mexico, at "La Puerta del Morillo" Wildlife Management Unit (WMU), registered under SEMARNAT-UMA-EX-0338-VER/14 permit. The area has a warm sub-humid climate with moderate humidity (61.35%) and a temperature range of 24 °C to 30 °C (Fig. 1). Recovery of biological samples occurred from September to November 2023. Sample analysis and processing were conducted at the Reproductive

Biology Laboratory, in the Torreón del Molino ranch of the Faculty of Veterinary Medicine and Animal Sciences at the Universidad Veracruzana, located at kilometre 14.5 of the Veracruz-Xalapa highway.

2.1 Animals

The animals used in this study consisted of four male specimens of white-tailed deer (*Odocoileus virginianus veraecrucis)* in their natural habitat within the land of the WMU. The bucks ranged in age from 2 to 6 years old and with a body condition score ranging from 3 to 5. The biological samples from every animal were obtained through regulated hunting collection. The animals were extracted according to the general provisions of the General Wildlife Law in Mexico [9].

2.2 Recovery and Cryopreservation of Sperm Cells

The time lapse between the recovery and process of samples was quantified and used as an independent variable during the statistical analysis. The specimens were maintained in a cooler at 5 ºC until they arrived at the lab. The recovery of the sperm was according to the procedure established by Garde et al. [7]. Briefly, the testes were dissected, isolating the epididymis and the deferent ducts. These were thoroughly cleaned, and the superficial blood vessels of the tailed were punctured to remove most of the blood. Subsequently, spermatozoa were extracted from the epididymal tailed through incisions made with a scalpel, removing the white fluid from the cut tubules. Immediately after extraction, the sample was diluted 1:1 (v:v) with Optidyl® (Biovet, France) diluent [10]. The samples were placed in a water bath at 37 °C, followed by incubation in a cold chamber at 5 °C for 3 to 4 hours, as indicated by manufacturers. The samples were loaded into 0.5 ml semen straws and manually frozen after incubation, with the straws positioned 4 cm above the liquid nitrogen level for 15 minutes. Straws were then stored in the liquid nitrogen tank for further analysis.

2.3 Thawing and Evaluation of Sperm Parameters

The semen straws were thawed at 37 °C for 1 minute. Subsequently, 3,7 µL of diluted semen was placed on a slide previously warmed to 37 °C. The slide covered with a coverslip was observed under a phase-contrast microscope (Olympus CX4). The percentage of active, progressively motile sperm cells was estimated and observed at 40x. Sperm morphology and viability were evaluated on a smear stained with eosin-nigrosine at 100x. To assess morphology, 100 spermatozoa were observed in different fields and classified as normal or abnormal. To assess viability, 100 spermatozoa were observed and classified as viable or not viable (Fig. 2).

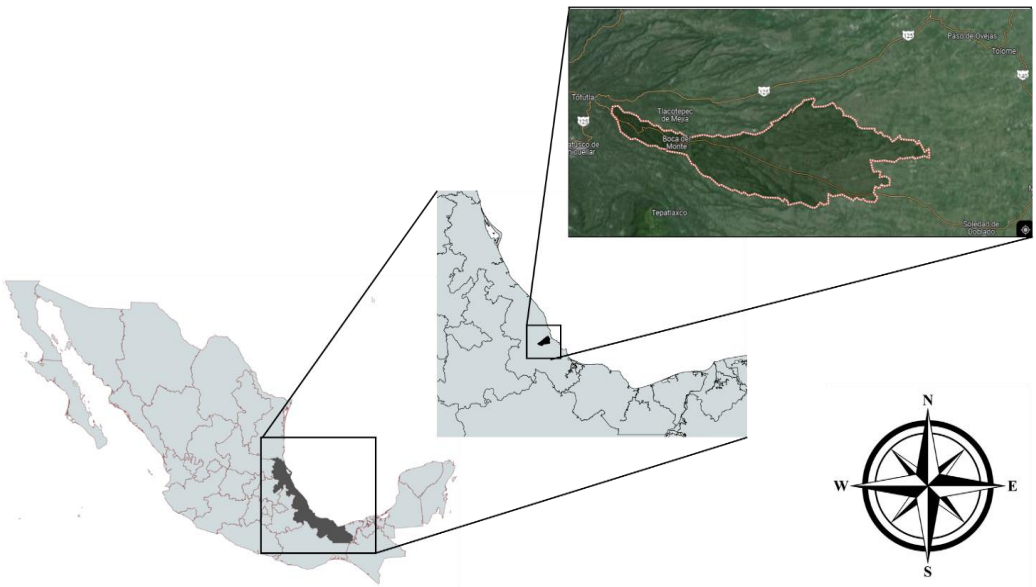

Fig. 1. Localization of the Wildlife Management Unit "La Puerta del Morillo" in Comapa, Veracruz, Mexico

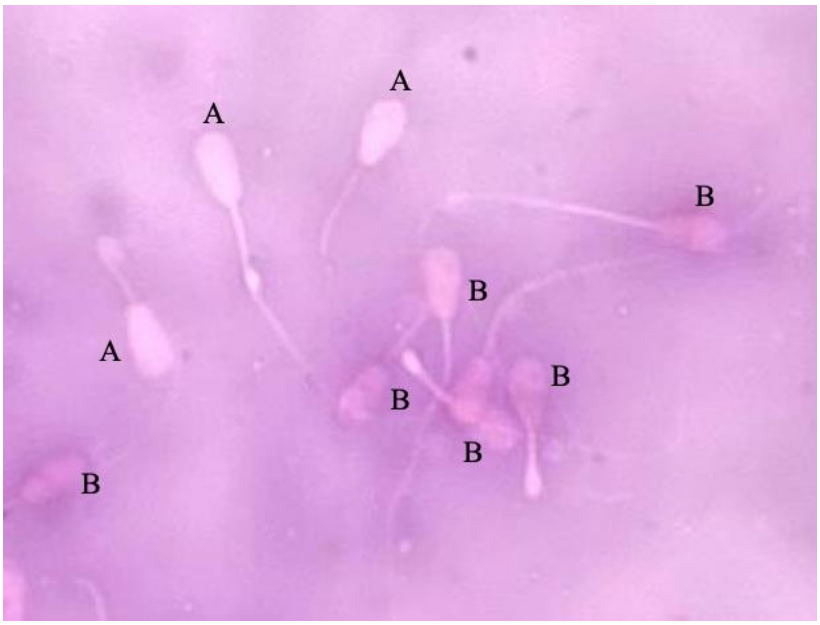

Fig. 2. Evaluation of the viability of sperm cells of white-tailed deer (*Odocoileus virginianus veracrucis*) with the Eosin-Nigrosin staining. Normal live sperm appear in white (A), and dead sperm appear in purple (B)

2.4 Calculation and Evaluation of THI

Agroclimatology information was retrieved from the database of the National Aeronautics and Space Administration Prediction of Worldwide Energy Resources NASA POWER; Rockett et al., [11]. Temperature (T) and humidity (H) data were retrieved from the geographical position (± 2 m) of the site (19.2034, -96.6242) during the study duration. Briefly, the THI was calculated as previously described (Avalos-Rosario, 2023) for every sampling date using the following equation:

$$\mathrm{THI} = (0.8\ x\ T) + \frac{H}{100} x\ (T - 14.3) + 46.5$$

A cut-off of THI 70 was implemented to determine two categories, ≥70 and <70, and used as independent variables in the statistical analysis.

2.5 Statistical Analysis

The data analysis was performed with the software SAS (SAS® OnDemand, SAS Institute Inc., Cary, NC, USA) using the GLIMMIX procedure. Because the results were presented as binary data, a statement was included to specify the binary distribution and logit link function, therefore the results are presented as proportions. The statistical model for every dependent variable (progressive motility, viability, morphology, and cytoplasmic droplet) included the effect of the THI category, buck's age, and post-collection time. The statistical significance level was characterized by $p<0.05$.

3. RESULTS AND DISCUSSION

On collection days when the THI was ≥70, the progressive motility ($p<0.01$), sperm viability ($p=0.01$), and the proportion of sperm cells with normal morphology ($p<0.01$) were higher compared to collection days with a THI <70, however, no differences were identified by evaluating the presence of cytoplasmic droplets (Fig. 3). This study showed that post-mortem cryopreservation of sperm cells of the Veracruz subspecies of white-tailed deer provided optimal parameters after thawing. On average, the viability (40.00%), progressive motility (35.75%), presence of cytoplasmic droplets (13.50%), and normal morphology (44.50%) are aligned with the consulted literature [7,12,13].

Fig. 3. Association between Temperature and Humidity Index (THI) on the collection day and epididymal sperm cell parameters of white-tailed deer (*Odocoileus virginianus* veracrucis) in the Veracruz tropics, Mexico. Columns with different superscripts (a,b) indicate differences between THI levels by $p<0.05$.

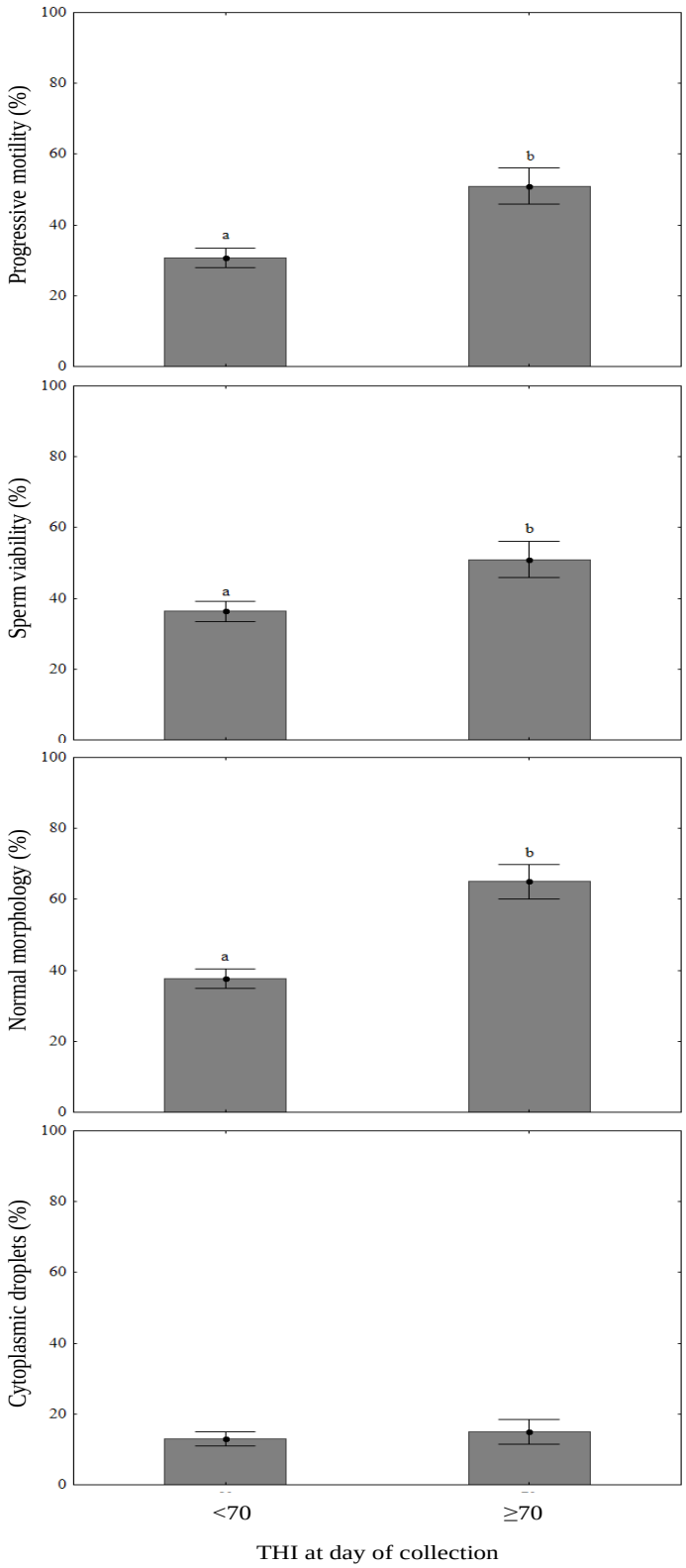

Fig. 3. Association between Temperature and Humidity Index (THI) on the collection day and epididymal sperm cell parameters of white-tailed deer (*Odocoileus virginianus veracrucis*) in the Veracruz tropics, Mexico

Columns with different superscripts (^{a,b}) indicate differences between THI levels by $p<0.05$

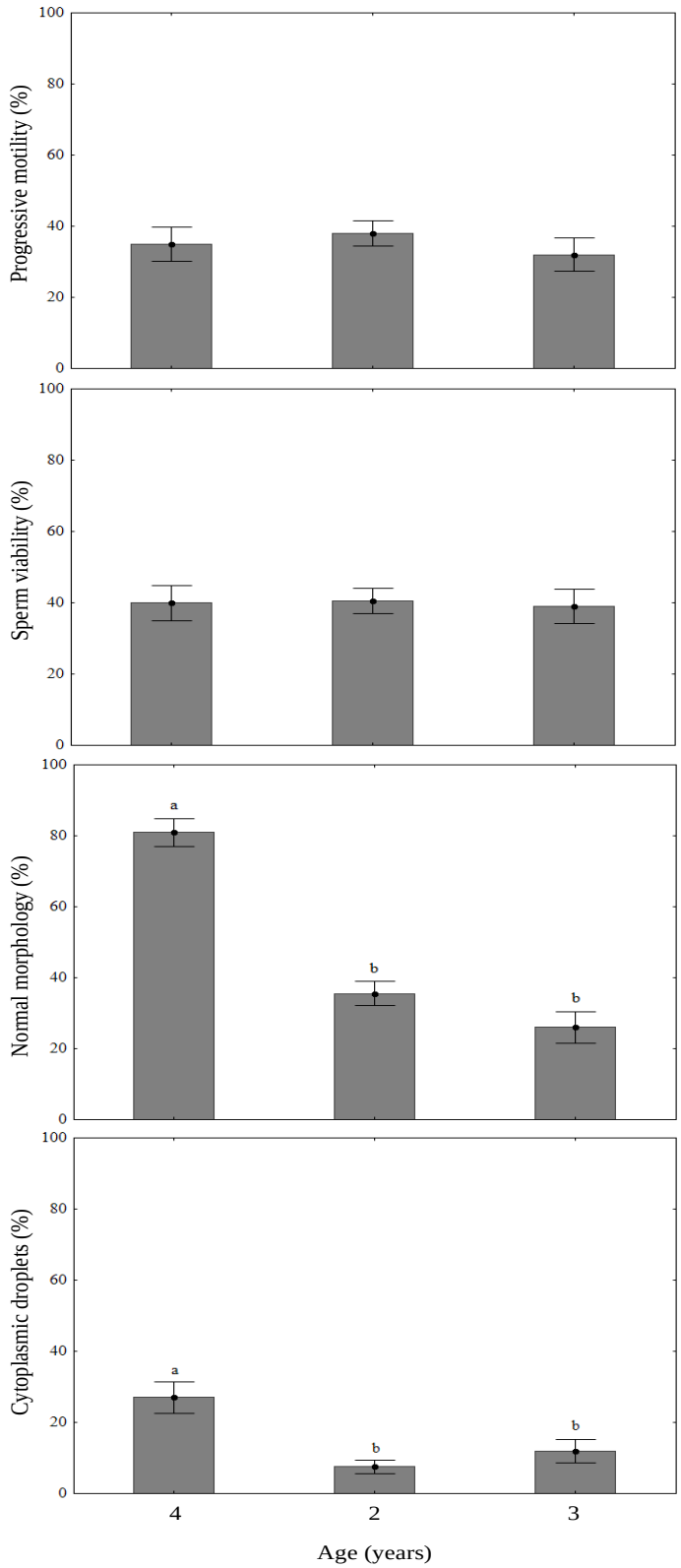

Fig. 4. Association between age of bucks and epididymal sperm cell parameters of white-tailed deer (*Odocoileus virginianus veracrucis*) in the Veracruz tropics, Mexico

Columns with different superscripts (^{a,b}) differ by p<0.05

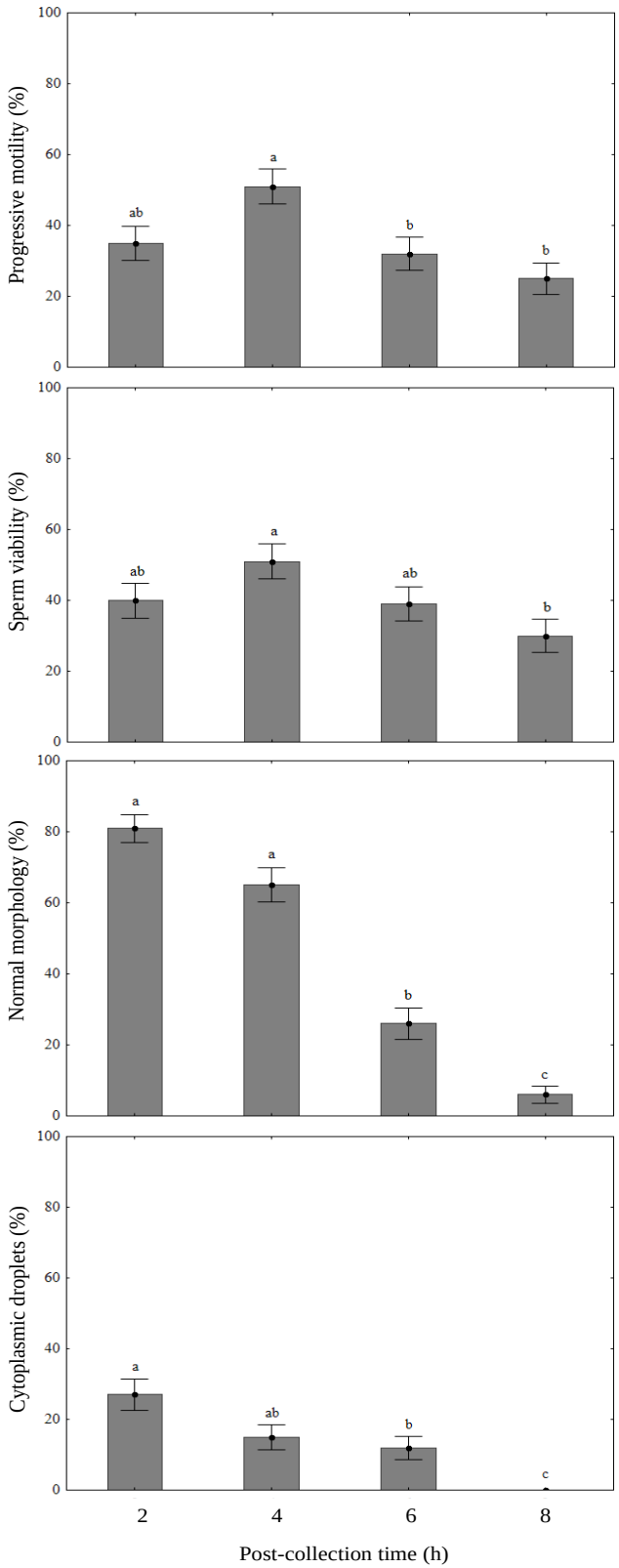

Fig. 5. Association between post-collection time and epididymal sperm cell parameters of white-tailed deer (*Odocoileus virginianus veracrucis*) in the Veracruz tropics, Mexico

Columns with different superscripts (^{a,b,c}) differ by p<0.05

When evaluating the effect of age on the sperm parameters, old bucks (around 4 years) had a higher proportion of sperm cells with normal morphology ($p<0.01$) and cytoplasmic droplets ($p<0.01$) compared with younger animals (2 and 3 years old). Nevertheless, age was not associated with progressive motility or sperm viability (Fig. 4).

In our understanding, this is the first study reporting an association between an environmental factor, such as THI and sperm cell parameters in white-tailed deer. Interestingly, a THI greater than 70 enhanced the proportion of live, motile, and normal sperm cells. Our results differ from research on Holstein bulls which have demonstrated a negative association between high THI and sperm quality [14]. However, the cited study was performed in temperate climates in the Netherlands, therefore, another factor to consider is the adaptation of white-tailed deer to the tropic climate [1].

The time lapse between the collection and process of samples was the main factor affecting the sperm parameters. Sperm cells from samples processed after 4 hours had a higher ($p=0.01$) progressive motility than those processed after 6 and 8 hours, with no differences compared to those processed after 2 hours. When viability was evaluated, spermatozoa processed after 8 hours had a lower ($P=0.02$) vitality than spermatozoa processed after 4 hours, moreover, the viability of sperm cells processed after 2 and 6 hours showed no differences with the rest of the groups. Compared to samples processed after 6 and 8 hours, those processed after 2 and 4 hours had a higher ($P<0.01$) proportion of sperm cells with normal morphology. Besides, the proportion of sperm cells cryopreserved after 8 hours was lower ($P=0.02$) compared to those processed after 6 hours. Samples processed after 2 hours had a higher ($P=0.04$) proportion of sperm cells with cytoplasmic droplets compared to samples processed after 6 hours, with no differences in samples processed after 4 hours. Samples processed after 8 hours had no sperm cells with cytoplasmic cells (Fig. 5).

In our study, the highest survival percentage was found between 2 and 4 hours post-mortem with a rate of 50%, compared to 6 and 8 hours. Similar results were obtained by Ake-López and colleagues [12] who pointed out that the proportion of live sperm cells declined from 58% (3-5 hours) to 37% (5-7 hours post-mortem). These results are also aligned with the study by Garde et al [7] in which a lower proportion of sperm cells with normal morphology was found in samples processed after 12 hours. These results may be explained by the reduction in sperm motility from the epididymis before cryopreservation, caused by cellular hypoxia, due to the cessation of blood flow to testicular and epididymal tissues after death (Reyes et al., 2012).

It can be observed that as post-collection time increases, the number of sperm with normal morphology decreases. Surprisingly, opposite results were obtained by Ake-Lopez et al. [7] who reported a higher proportion of normal morphology sperms in samples processed after 7 hours compared to 0-5 hours.

Our sampling period (September to November) matches the rut period of the white-tailed bucks reported previously in the Veracruz tropic in which a higher testosterone concentration was found Ahuja-Aguirre et al., [15]. Studies in bovine species have shown a positive correlation between testosterone levels with sperm motility and morphology [16]. Therefore, more research is needed to investigate whether there is a variation in the sperm parameters over the different seasons of the year.

Bucks' age was a factor associated with morphology and the presence of cytoplasmic droplets. A higher proportion of normal-morphology sperm cells was found in older bucks than young ones. Similar results were reported in red deer where a higher proportion of normal morphology spermatozoa was found in 4-year-old deer compared to younger bucks [7]. However, a study in the Texan subspecies reported no differences in motility and morphology between bucks of different ages [12]. This age difference might be linked to sexual maturity and testosterone concentrations. It has been proved that old bucks (≥3.5 years old) were found with higher testosterone levels compared to young bucks 1.5–2.5 years old; [17]. High testosterone levels are usually associated with social hierarchy (Gomes et al., 2021), as well as sperm motility and morphology Dasrul et al., [16], which may explain why the oldest bucks had a lower incidence of abnormal spermatozoa. Additionally, the data show a positive relationship between age and the presence of distal cytoplasmic droplets. At four years old, 25% of the spermatozoa were visualized with this characteristic, while at 2 years old, the percentage decreased. In this context, some

authors assert that the droplets are indicative of sperm maturation by providing the necessary energy to be competent and develop progressive motility after ejaculation [18,19], especially in those sexually mature bucks [20-23].

4. CONCLUSION

The factors evaluated in this study showed influence over the sperm cell parameters of white-tailed deer. High THI favoured progressive motility and sperm viability. Older bucks had a greater proportion of sperm cells with normal morphology and cytoplasmic droplets than young ones. The post-collection time was the most important factor influencing all the evaluated parameters, with the best progressive motility, viability, morphology, and presence of cytoplasmic droplets when the samples were processed within the first 4 hours.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://prh.mbimph.com/review-history/3658