RAFAELA ZANI COETI

Sperm storage in Neotropical snakes:
a spermatozoon physiological and ultrastructural approach

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RESUMO

COETI, R. Z. **Estocagem de esperma em serpentes Neotropicais:** uma abordagem fisiológica e ultra estrutural do espermatozoide. 2020. 86 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brasil, e Universidad de La Frontera, Temuco, Chile, 2020.

A estocagem de espermatozoides pelas fêmeas em vertebrados é bem descrita e ocorre por períodos de tempo distintos, em lugares diferentes ao longo do trato reprodutivo. Dentre todos os vertebrados, as fêmeas de répteis são conhecidas pelos mais longos períodos de estocagem de espermatozoides, sendo que em serpentes há relatos de até 5 anos, podendo ocorrer na região anterior da vagina, no útero posterior ou no infundíbulo, seja em criptas ou em tubos de estocagem de esperma especializados (SST). Além disso, os machos de serpentes também podem estocar espermatozoides durante pelo menos uma estação, dentro dos ductos deferentes. Entretanto, desde a primeira descrição de estocagem em serpentes, até trabalhos que relatam a quantidade estimada de tempo de estocagem e a variabilidade paterna da prole, a mesma pergunta permanece sem resposta, como é possível que os espermatozoides permaneçam vivos por tanto tempo? Há hipóteses que indicam que o oviduto feminino se encarregaria de manter esses espermatozoides, outros apontam que a manutenção dessas células se deve a secreções do segmento sexual renal masculino, mas poucos estudos focam em como a estrutura do espermatozoide e sua fisiologia poderiam ser a chave de sua longa sobrevivência. Assim, esse trabalho teve como objetivo analisar a célula germinativa masculina de serpentes Neotropicais, do ponto de vista ultra estrutural e fisiológico, com a finalidade de avaliar e descrever os espermatozoides maduros antes da cópula. Para tanto, esse estudo foi dividido nas seguintes partes: (1) um protocolo minimamente invasivo para obtenção de sêmen em serpentes; (2) parâmetros de avaliação seminal de motilidade, vigor e vitalidade das amostras; (3) a ultraestrutura dos espermatozoides e por fim (4) a integridade do material genético dos espermatozoides.

Palavras-chave: Estocagem de esperma. Avaliação espermática. Espermatozoides de serpente. Ultraestrutura. Citometria de fluxo.

RESUMEN

COETI, R. Z. **Almacenamiento de semen en serpientes Neotropicales:** un enfoque en la fisiología y ultraestructura de espermatozoides. 2020. 86 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brasil, Universidad de La Frontera, Temuco, Chile, 2020.

El almacenamiento de esperma por las hembras en los vertebrados está bien descrito y ocurre durante diferentes períodos de tiempo, en diferentes lugares a lo largo del tracto reproductivo. Entre todos los vertebrados, las hembras de reptiles son conocidas por los períodos más largos de almacenamiento de esperma, y en las serpientes hay informes de hasta 5 años, que pueden ocurrir en la región anterior de la vagina, en el útero posterior o en el infundíbulo, ya sea en criptas o en tubos especializados de almacenamiento de esperma (SST). Además, las serpientes machos pueden almacenar esperma durante al menos una temporada, dentro del conducto deferente. Sin embargo, desde la primera descripción del almacenamiento en serpientes, hasta estudios que informan la cantidad estimada de tiempo de almacenamiento y la variabilidad paterna de la descendencia, la misma pregunta permanece sin respuesta, ¿Cómo es posible que los espermatozoides permanezcan vivos durante tanto tiempo? Existen hipótesis que indican que el oviducto femenino estaría a cargo de mantener estos espermatozoides, otros señalan que el mantenimiento de estas células se debe a secreciones del segmento sexual renal masculino, pero pocos estudios se centran en cómo la estructura del esperma y su fisiología podrían ser la clave para su larga supervivencia. Por ésta razón, este trabajo tuvo como objetivo analizar la célula germinal masculina de las serpientes neotropicales, desde un punto de vista ultraestructural y fisiológico, con el propósito de evaluar y describir el esperma maduro antes de la copulación. Por lo tanto, este estudio se dividió en las siguientes partes: (1) un protocolo mínimamente invasivo para obtener semen de serpientes; (2) parámetros para la evaluación seminal de la motilidad, el vigor y la vitalidad de las muestras; (3) la ultraestructura del esperma y finalmente (4) la integridad del material genético del esperma.

Palabras clave: Almacenamiento de semen. Evaluación de esperma. Esperma de serpiente. Ultraestructura. Citometría de flujo.

ABSTRACT

COETI, R. Z. **Sperm storage in Neotropical snakes:** a spermatozoon physiological and ultrastructural approach. 2020. 86 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brasil, e Universidad de La Frontera, Temuco, Chile, 2020.

The sperm storage in vertebrate females is a well described process and occurs for distinct time periods, taking place in different portions of female reproductive tract. Among all vertebrates, the reptile females are known for the longer sperm storage and in snakes there are reports of up to 5 years, occurring inside the anterior vagina, posterior uterine tube and infundibulum, whether in crypts or in SST. In addition, male snakes can also store sperm for at least one season, inside the ductus deferens. However, since the first sperm storage description in snakes, until works reporting the estimated storage time and within-clutch multiple paternity, the same question stills without answer: How is possible that the spermatozoa be alive for so long? There are hypothesis indicating that female oviduct is in charged for sperm maintenance, others suggest that sperm cells maintenance are due to sexual segment of kidney (SSK) secretions, but few studies focus on how the spermatozoon structure and physiology could be the key for its long survival. Thus, this work aimed to analyse the Neotropical snakes' spermatozoa with a physiological and ultrastructural approach, for the purpose of evaluating and describing mature sperm before copulation. Therefore, we divided our study on four topics: (1) establishing a minimally invasive snake semen collection protocol; (2) evaluation of the snake semen parameters motility, vigor and vitality; (3) description the spermatozoa ultrastructure and (4) recognize if the genetic material of mature snake sperm is viable.

Keywords: Sperm storage. Sperm evaluation. Snake spermatozoa. Ultrastructure. Flow cytometry.

SUMMARY

1 INTRODUCTION

The sperm storage in vertebrate females is a well described process (HOLT; LLOYD, 2010) and occurs for distinct time periods, short as 36 hours in mammals or long as until 5 months in amphibians (HOLT; FAZELI, 2016). This process can take place in different portions of female reproductive tract, since inside of the terminal zone of the oviductal gland in sharks (HAMLETT et al., 2002) until in uterus and utero-tubal junction regions in bats (RACEY et al., 1987) and specialized sperm storage tubules (SST) in birds (BAKST; WISHART; BRILLARD, 1994). Among all vertebrates, the reptile females are known for the longer sperm storage, it is the case of turtles with until four years of storage (EWING, 1947) and snakes that recorded until five years (HAINES, 1940; BOOTH; SCHUETT, 2011). In reptile females the sperm storage registered sites are the anterior vagina, posterior uterine tube and infundibulum, whether in crypts or in SST (GIRLING, 2002) and in Squamata males the sperm storage is also registered, occurring in ductus deferens (TRAUTH; SEVER, 2011; ALMEIDA-SANTOS et al., 2006).

However, since the first sperm storage description in snakes (SAINT GIRONS, 1962), until works reporting the estimated storage time (HOLT; FAZELI, 2016) and within-clutch multiple paternity (ULLER; OLSSON, 2008; JELLEN; ALDRIDGE, 2011), the same question stills without answer: How is it possible that the spermatozoa be alive for so long?

There are hypothesis indicating that female oviduct is in charged for sperm maintenance (GIRLING, 2002), others suggest that sperm cells maintenance are due to sexual segment of kidney (SSK) secretions (BISHOP, 1959), but few studies focus on how the spermatozoon structure and physiology could be the key for its long survival, since female sperm storage is also used for sperm selection (ULLER; OLSSON, 2008; JELLEN; ALDRIDGE, 2011).

Thus, this work aimed to analyse the Neotropical snakes' spermatozoa with a physiological and ultrastructural approach, for the purpose of evaluating and describing mature sperm before copulation.

Therefore, we divided our study on four topics: (1) establishing a minimally invasive snake semen collection protocol; (2) evaluation of the snake semen parameters motility, vigor and vitality; (3) description the spermatozoa ultrastructure and (4) recognize if the genetic material of mature snake sperm is viable.

1.1 SEMEN COLLECTION PROTOCOL

The snake sperm collection is usually done by two techniques: (1) after the animal euthanasia, by direct compression of ductus deferens (ALMEIDA-SANTOS et al., 2004; TOURMENT et al., 2008; GRIBBINS et al., 2016) or (2) through ventral massages in the anterior region of the cloaca (FITCH, 1960; MENGDEN, 1980) that can includes local anaesthesia of this region (ZACARIOTTI et al., 2007; SILVA et al., 2015). Thus, the aim of this chapter was the improvement of a minimally invasive semen collection technique, for the purpose of maintaining the animal's welfare while obtaining a qualified seminal sample for physiological and morphological evaluations.

1.2 SEMINAL EVALUATION

Commonly the sperm evaluation is based in motility, concentration and morphology (DURRANT, 2009), however the plasmatic membrane integrity investigation can aggregate new information to the semen evaluation (JEYENDRAN et al., 1984; KERMANI-ALGHORAISHI et. al., 2010), mainly to snake sperm evaluation, where this type of analyses was poor studied (SILVA et al., 2017). The second chapter of this work had the objective to doing a snake sperm evaluation for the samples obtained in the chapter one and testing and include semen evaluation protocols of World Health Organization (WHO, 1999) for snake sperm, thus establishing a protocol for vitality.

1.3 SNAKES SPERM ULTRASTRUCTURE

The snake spermatozoon ultrastructure is related with its reproductive characteristics (TOURMENT et al., 2006, 2011). Spermatozoon that be storage in the female oviduct can presents a mid-piece with great development of multilaminar membranes (TOURMENT et al., 2006) just as, in species that perform reproductive strategies of sexual aggregation, sperm can be comparatively larger, because they undergo greater sperm competition (TOURMENT et al., 2009). The chapter 3 aim was characterizing the external and internal structures belonging to the snake spermatozoon in its final development and identify which of these morphological characteristics are related with male and female sperm storage.

1.4 SPERM INTEGRITY

The sperm storage strategy in males and females' snakes allows a distinct fertilization and copulation seasons and sperm selection (ULLER; OLSSON, 2008). The sperm selection inside of female reproductive tract choose the ones with better integrity of genetic material (HOLT; FAZELI, 2016). In this context, the use of a technique that allows the evaluation of the state of the genetic material of the sperm is essential. The flow cytometry is a technique used to measure multiples sperm attributes, including cellular viability, DNA content and structure, acrosomal integrity and mitochondrial function (CORDELLI et al., 2005). Thus, the chapter 4 tested a flow cytometry protocol to snake sperm that is effective for assessing its viability and the integrity of its genetic material.

2 SEMEN COLLECTION AND EVALUATION IN CAPTIVE CORAL SNAKE MICRURUS CORALLINUS

Running title: Semen collection and evaluation in coral snake

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ABSTRACT

The coral snake *Micrurus corallinus* is one of the Brazilian species kept in captivity to obtain venom for antivenom production. The difficulties in establishing a sizeable breeding colony and the small amount of venom produced per individual make it necessary to find alternatives that increase the reproductive efficiency of captive individuals. Here, we tested a semen collection protocol and characterized the seminal parameters of captive *M. corallinus*. Semen collection was performed during the mating season of the species (spring-summer). The spermatozoa of *M. corallinus* are elongated, filiform, and the tip of their heads are slightly curved. The midpiece is the longest part of the sperm. The successful collection of semen in the first attempt shows that this technique has great potential for use in future studies on semen transport, preservation, and artificial insemination. Our results will allow further studies to improve husbandry, reproductive rates, and conservation of captive *M. corallinus*.

KEYWORDS. Reproductive biotechniques. Reproduction. Reptile. Sperm parameters

2.1 INTRODUCTION

Reproductive biotechniques have been extremely useful in implementing conservation projects for endangered species (Silva et al., 2015). These techniques can also be used to increase the reproductive rates of animals that rarely breed in captivity. One example is the coral snake *Micrurus corallinus* (Elapidae), a medium-sized venomous species found in Atlantic forest areas from northeastern to southern Brazil (Marques et al., 2004; Campbell & Lamar, 2004). This species has a venom with a potentially lethal neurotoxic effect (Gutierrez et al., 2016). Therefore, individual *M. corallinus* have long been kept in captivity at the Butantan Institute to obtain venom for antivenom production (Mendes et al., 2018). However, the low encounter and capture rates of individual *M. corallinus* in the wild (Roze, 1996) make it difficult to establish a breeding colony with a considerable number of animals. Moreover, coral snakes produce little venom, and therefore, multiple extractions from several specimens are often required to obtain the necessary quantity of venom (Chacón et al., 2012; Mendes et al., 2018). Thus, finding strategies that increase the reproductive efficiency of captive snakes is crucial to establish a sizeable colony for venom extraction.

In this study, we tested a semen collection protocol and characterized the seminal parameters of captive *M. corallinus*.

2.2 MATERIAL AND METHODS

Animals and captive maintenance

This study was conducted under the approval of the Ethics Committee for Animal Research of the Butantan Institute (approval number 1447271016). We collected semen from seven clinically healthy adult males (snout-vent length > 390 mm).

Three individuals were housed in the vivarium of the Vital Brazil Institute (Rio de Janeiro) where the snakes were kept individually in enclosures formed by two boxes (with a transparent polycarbonate base, 200 × 230 × 300 mm) interconnected with PVC piping. The individuals were fed a diet prepared with animal protein (bovine liver and chicken eggs) and mineral-vitamin supplement (Hemolitan®, Vetnil, Brazil). This

diet (~20% of the snake body mass) was administered fortnightly by passing a thin catheter down the esophagus until reaching the stomach.

Two animals were maintained in the vivarium of the Biological Museum of the Butantan Institute (São Paulo). Snakes were kept individually in glass containers (700 × 500 × 400 mm) with ventilated lids. Animals was fed (20–25% of their body mass) snakes or amphisbaenids provided by the Animal Reception Section or the Biological Museum weekly. In both conditions, the containers were held at room temperature (22–27°C) under a natural photoperiod. All animals were given water ad libitum.

We also collected semen from two newly captured individuals that were temporarily housed in the Animal Reception Section of the Butantan Institute. Individuals were kept in a makeshift container (like plastic bottles in which they were brought to the institute by the collectors). Snakes were not fed, handled, and prophylactically treated until semen collection, which occurred within 24 h after the animal arrived at the institute.

Semen collection and evaluation

Semen collections were conducted between 2016 and 2018, always between November and March (austral spring and summer), which corresponds to the mating season of the species (Marques, 1996; Almeida-Santos et al., 2017) in where the males copulated with sperm that are storage in their ductus deferentia (Almeida-Santos et al., 2006; Marques et al., 2013). Snakes were restrained in a transparent plastic tube. Semen was collected directly from the urogenital papilla using microhematocrit capillary tubes after massaging the ventral portion of the snake towards the cloaca (Mengden, 1980).

We measured semen volume and evaluated semen appearance and color. Next, semen samples were divided into two groups. The first group was diluted in Ham's F10 medium (dilution 1:100 µL) to evaluate sperm motility and progressive motility, and the second group was fixed in 10% saline formaldehyde solution to evaluate sperm concentration and morphology. Sperm motility and progressive motility were assessed using an optical microscope (Eclipse E100; Nikon Corporation, Tokyo, Japan) at ×100 magnification. For the motility analysis, we estimated the percentage of motile sperm cells. For the progressive motility analysis, we estimated the percentage of sperm cells showing forward progressive movement. Sperm

concentration analysis was performed in a Neubauer hematimetric chamber using an optical microscope (Leica DM 400B; Leica Microsystems, Wetzlar, Germany) at ×400 magnification. To assess sperm morphology, we first imaged the cells of each semen sample using a phase-contrast microscope (at ×1000 magnification). We then measured sperm head length, midpiece length, tail length, and total length of 50 sperm using ImageJ, version 1.52a (Schneider, Rasband and Eliceiri, 2012).

Statistical analysis

Data were checked for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). We tested whether male morphological traits (body mass and snout-vent length) were correlated with semen parameters (semen volume and sperm concentration). Correlations were considered significant at $p < 0.05$. Statistical analysis was performed using Statistica, version 7 (StatSoft, 2004).

2.3 RESULTS

In all sampled individuals, semen was successfully collected in the first attempt. We observed no contamination of semen samples with feces or urate (Figure 1). The semen of *M. corallinus* was white and thick (Figure 1B). Male morphological traits and seminal parameters are shown in Table 1. We found no significant correlations between male morphological traits and seminal parameters (Table 2).

The spermatozoa of *M. corallinus* were elongated, filiform, and the tip of their heads were slightly curved (Figure 2). Sperm total length averaged 103.73 ± 1.35 µm. The midpiece was the longest part of the sperm (64.18 ± 1.16 µm), followed by the tail (29.05 ± 0.68 µm) and the head (10.50 ± 0.08 µm).

2.4 DISCUSSION

Semen collection techniques in snakes have improved in efficiency over the last six decades. In this work, we used Mengden's (1980) technique to collect semen from *M. corallinus*, by a digital ventral massage. This technique was effective and minimally invasive, preserving the behavior and health of the animals studied. Thus, the reproductive status of male *M. corallinus* was assessed directly for the first time.

The spermatozoa of *M. corallinus* have three regions: head, midpiece, and tail. This morphology is similar to that described in other reptiles (Oliver, Jamieson and Schelting 1996). The sperm morphology of elapid snakes has been poorly studied (Oliver et al., 1996; Gribbins; Freeborn; Sever, 2016). In *Micrurus*, sperm morphology has only been described in *M. fulvius* (Austin, 1965). Therefore, this is the first paper describing the sperm morphology of *M. corallinus*.

The volume of semen obtained depends on factors such as collection technique, species, and reproductive timing. For example, Almeida-Santos et al. (2004) obtained approximately 1 mL of semen from individual rattlesnakes (*Crotalus durissus*), and an adult male of this species weighs 30 times more than an adult male *M. corallinus*. Moreover, the technique used by Almeida-Santos et al. (2004) was very destructive and laborious, involving euthanasia and then excision, stretching, and washing of the ductus deferens in a Petri dish. The semen volume obtained for *M. corallinus* (4.15 ± 2.13 µL) was similar to that obtained for other snake species using similar techniques (e.g., 2-5 µL for *Pantherophis guttatus*: Mattson et al. 2007; 3.21 ± 1.38 µL for *B. insularis*: Silva et al., 2015).

The high motility and progressive motility (≥ 70%) confirm that the semen stored in the ductus deferentia during the mating season has viable. The adoption of effective techniques to collect and characterize semen parameters are fundamental for the planning and applicability of other reproductive biotechniques such as artificial insemination, cooling, and cryopreservation. Thus, our results will allow further studies to improve husbandry, reproductive rates, and conservation of captive *M. corallinus*.

We obtained semen in every first collection attempt, this shows that the technique works to coral snakes, besides that, we proved that the *Micrurus corallinus* semen have good parameters in the reproductive season. Thus, the protocol described in this work have great potential to be applied in future studies on semen evaluation, morphology and reproductive management in this snake genera.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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2.6 TABLES

Table 1. Body measurements and seminal parameters of captive Micrurus corallinus (n = 7).

Parameter	Mean	SD[1]	Range
SVL[2] (mm)	488.1	6.6	392–565
Body mass (g)	32.7	10.1	19.2–51.0
Semen volume (µL)	4.4	2.1	3.0–8.4
Sperm motility (%)	85	5	80–90
Sperm progressive motility (%)	68.5	9	60–80
Sperm concentration (×10^9sptz[3]/mL)	1.3	0.6	0.3–1.9

[1]standard deviation

[2]snout-vent length

[3]spermatozoa

Table 2. Results of the correlation between the biometric data and seminal parameters of captive *Micrurus corallinus*.

Correlation	*P*-value	*R*
SVL[1] × semen volume	0.327	0.436
SVL[1] × sperm concentration	0.612	-0.234
Semen volume × sperm concentration	0.132	0.626
Body mass × sperm concentration	0.571	-0.261
Body mass × semen volume	0.679	0.192

[1]snout-vent length

2.7 FIGURES

FIGURE LEGENDS

FIGURE 1 Semen collection from a captive male *Micrurus corallinus*. A) Massage of the ventral portion of the snake's abdomen; B) Semen collection using a microhematocrit capillary. White arrow = cloaca, black arrow = sperm.

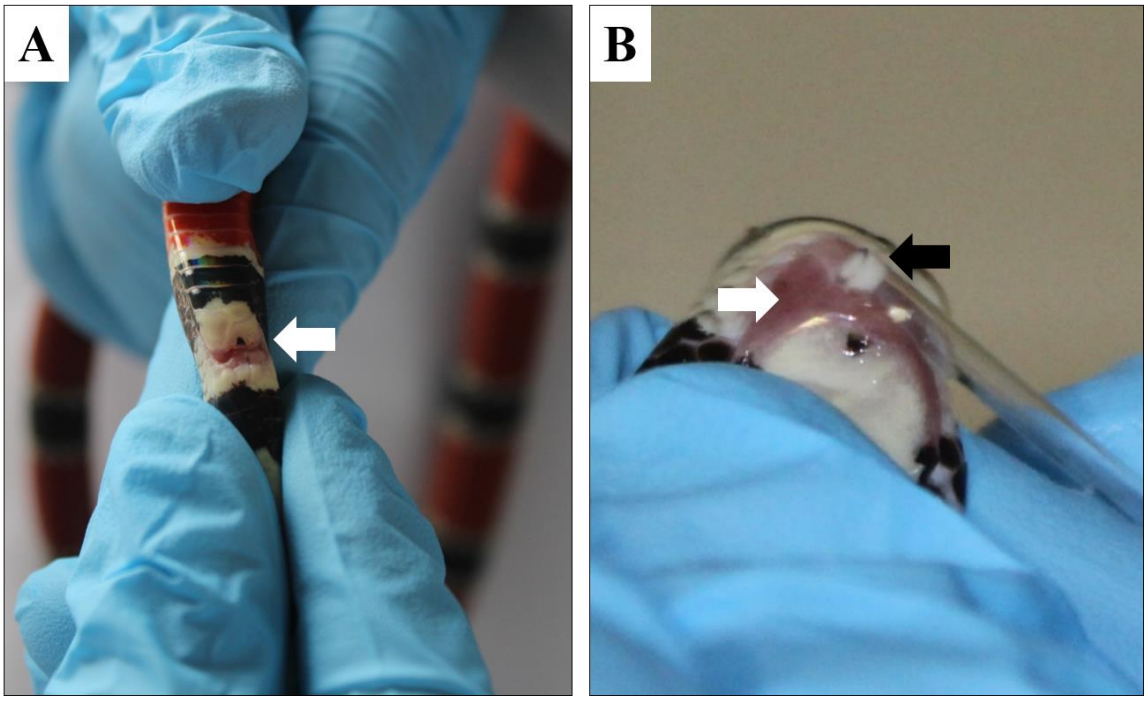
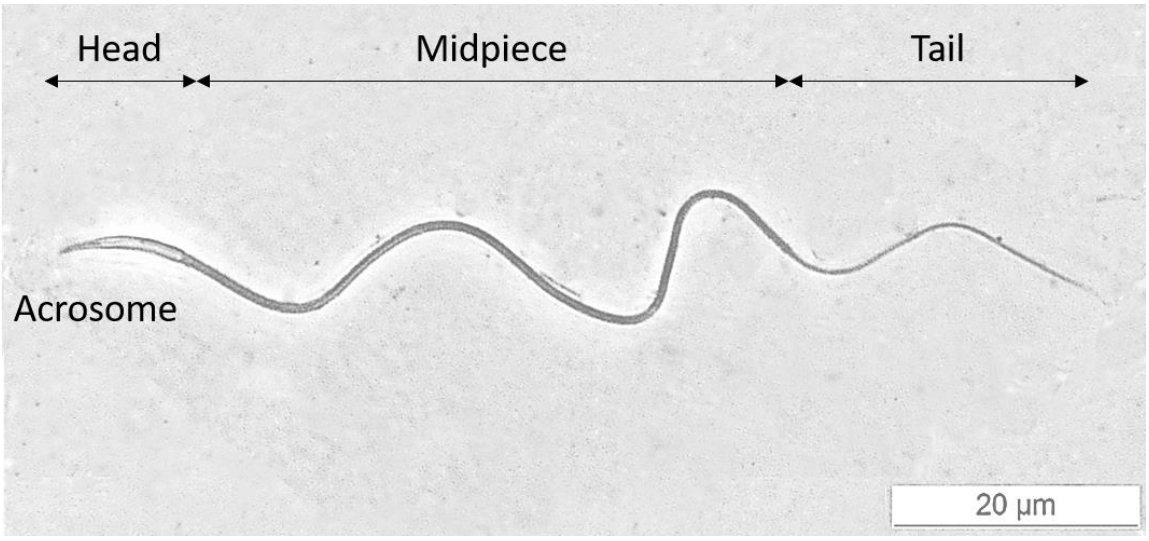


FIGURE 2 A spermatozoon of *Micrurus corallinus* observed in a phase-contrast microscope.



3 ARE THE ABNORMAL SNAKE SPERMATOZOA ALIVE? A FIRST VIEW OF WHO PROTOCOLS TO EVALUATED SNAKE SPERM AND THE CYTOPLASMIC DROPLETS

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ABSTRACT

Micrurus frontalis is a triadal coral snake maintained in captivity for antivenom serum production and its reproduction in this situation is a challenge. The development of biotechniques that enable breeding in captivity is essential and one of them are semen collection and evaluation, important procedures in assessment of male fertility. Membrane integrity investigation is a complementary test and could aggregate information to traditional semen analysis that was poorly studied in snakes semen, what makes it difficult to establish a valid protocol and elucidate the importance of some morphological aspects such as, the presence of cytoplasmic droplets in snake spermatozoon. Thus, the aim of this work was to test semen analysis protocols, adapted from the World Health Organization laboratory manual for the examination and processing of human semen in *Micrurus frontalis* semen, and evaluate its sperm quality and presence of cytoplasmic droplets. To assess the sperm vitality, a test was performed using a 0.5% eosin dissolved in 0.9% NaCl solution and, for morphological evaluation, we used the staining methods Hematoxylin-Eosin and Rapid staining procedure. Thus, we were able to adapt the protocols for analyses of vitality, motility and morphology of the snake sperm from WHO manuals. So that, the standardized protocol enabled us to characterize spermatozoa of *Micrurus frontalis,* that presented cytoplasmic droplets, as alive and viable.

KEYWORDS. Reproductive biotechniques. Membrane integrity. Snake sperm. Cytoplasmic droplets

3.1 INTRODUCTION

Micrurus frontalis is a triadal coral snake, midsize (considering a snout-vent length, SVL, ȳ = 928 mm) which is characterized by white scales ringing the black scales of the snout and which can be found in the central region of Brazil (Goiás, Mato Grosso, Mato Grosso do Sul, São Paulo, Espírito Santo) and in the north of Paraguay (Silva Jr., el al, 2016). Due to the risks of human envenomation and death (Norris et al., 2009), maintaining coral snakes in captivity for antivenom serum production is important, but not easy since its reproduction in captivity is still a challenge (Mendes et al., 2018). In Brazil, the venom of *Micrurus frontalis* is used specifically for producing the antivenom serum in biological research institutions, such as Fundação Ezequiel Dias (FUNED-MG) (Raw et al. 1991).

The maintenance and reproduction of captive exotic animals is an important and current issue (Durrant, 1990; Schulte-Hostedde et al., 2015). Hence, the development of biotechniques to make it feasible is quite required. Despite it be used as an ordinary tool for assessing male fertility, predict hormone levels and function of male reproductive system, the evaluation of sperm quality is still, an important first step procedure that makes the development of more advanced techniques, such as artificial insemination, possible (Durrant, 2009; Kermani-Alghoraishi et al., 2010).

Semen collection and evaluation in reptiles was successfully done in a large number of species, ranging from crocodilians (Larsen et al., 1992; Johnston et al., 2014) to turtles (Platz et al., 1980; Tanasanti et al., 2009) until lizards (Zimmerman and Mitchell, 2017; Juri et al., 2018) and snakes (Zacariotti et al., 2007; Silva et al., 2015). Differently from semen collection of some turtles and lizards that needs electroejaculation methodology (Zimmerman and Mitchell, 2017; Juri et al., 2018), in slender snakes, for instance *Micrurus frontalis*, just containment and ventral massages are enough (Coeti et al., in preparation).

Traditionally, the semen evaluation is performed through analysis of sperm motility, concentration, and morphology (Durrant, 2009). However, the investigation of cell membrane integrity is a complementary test that easily demonstrates if the spermatozoon is alive or dead (Jeyendran et al., 1984; Kermani-Alghoraishi et al., 2010). These analyses are rarely performed in snake's semen (Silva et al., 2017), which makes difficult the establishment of a valid protocol. Nonetheless, laboratory

protocols designed for humans have been adjusted and used, with reasonable success rates, for evaluating semen characteristics of different animal species (Durrant, 2009; Vieira et al., in preparation).

Furthermore, the investigation of the integrity of cellular membrane of snake spermatozoon can elucidate some issues regarding sperm viability. As the presence of cytoplasmic droplets at the midpiece of snake spermatozoon is generally considered an abnormality (Fahrig et al., 2007; Silva et al., 2015), unlike for other species already investigated that, under some very specific conditions, it may not be taken as an anomaly (Krutzsch, Crichton and Nagle 1982; Gist, Hess and Thurston, 1992; Cervantes et al., 2008; Zhang et al., 2015; Gatimel et al., 2017).

In this regard, in the present study we have established a protocol, based on World Health Organization laboratory manual for the examination and processing of human semen (WHO, 2010), for evaluating sperm quality as well as the presence of cytoplasmic droplets in semen of *Micrurus frontalis* collected through a non-invasive approach.

3.2 MATERIALS AND METHODS

Animals

This study was approved by the Scientific Ethics Committee of Animal Research from Fundação Ezequiel Dias (FUNED), registered under the protocol number 126/2017. Five clinically healthy adult males (SVL > 700 mm) of *Micrurus frontalis*, kept in captivity at FUNED, were used in this work. These animals were kept in individual plastic box (30.5 x 46.5 x 33.0 cm) lined with paperboard, had a balanced diet composed of euthanized snakes offered at least once a month, free access to water and monitored temperature (22-28°C).

Seminal collection

Semen was collected on 25th January 2019, as previously described by Coeti et al., (in preparation). Briefly, five snakes were restrained in a transparent plastic tube and then, finger massage was performed in the ventral part of the snake body towards

the tail. The semen was collected directly from the urogenital papilla, using microhematocrit capillary tubes, and the semen volume were evaluated. Only two animals provided samples. Semen samples were diluted in culture medium HAM's F10 (dilution 1:100 µL) for analysis.

Sperm parameters

Sperm motility analysis was performed using 10 µm of semen diluted in HAM's F10 solution (SHS) in a wet preparation on a slide covered with a 22mm x 22mm coverslip. At least 200 spermatozoa were counted under an optical microscope at 400x magnification, and graded as A (progressive motility), B (non-progressive motility) or C (immotile). To access the correlation between the sperm motility and the presence of cytoplasmatic droplets, we also counted 200 spermatozoa for each individual and classified it as normal spermatozoon (Sptz n) or with cytoplasmatic droplets (Sptz cd).

If agglutination was present, it was evaluated as isolated (<10 spermatozoa per agglutinate), moderate (10-50 spermatozoa per agglutinate), large (>50 spermatozoa per agglutinate) or gross (all spermatozoa agglutinated).

To evaluate the sperm vitality, cytoplasmic membrane integrity and viability of the spermatozoon with cytoplasmic droplets, the test was performed using a 0.5% eosin dissolved in 0.9% NaCL solution. We mixed 5µl of SHS with 5µl of eosin in a microscope slide covered with a 22mm x 22mm coverslip. We counted at least 200 sperm cells under an optical microscope at 400x magnification until 2 minutes after the addition of the eosin. Spermatozoa are considered alive if unstained and dead if stained in a dark pinkish colour.

To assess the sperm morphology, the smear was prepared spreading out 10µL of the sample on a glass slide surface and fixed with 95% ethanol for 15 minutes. The sperm smears were stained using two methods. The first staining method used was Hematoxylin-Eosin, in which the slide was immerged in histological hematoxylin for 5 minutes, quickly washed in tap water and followed by 5 minutes in histological eosin, washed and allowed to air dry. Rapid staining procedure (Renylab) was also performed. Briefly, after to be treated with the fixative solution, the slides were dipped for 30 seconds in each stain solutions 1 and 2, washed in water and let to air drying. In both staining methods we randomly photographed 10 spermatozoa using an optical

microscope (Olympus BX60) with 1000x magnification. The ImageJ software version 1.52a (Schneider, Rasband and Eliceiri, 2012) was used to measure the length of the head, midpiece and tail as well as the total length of spermatozoon.

Semen concentration was evaluated through the sperm counting in a Neubauer chamber (4x4; Lo-Laboroptik), with 10 µl of the sample in the central grid, in which at least 200 spermatozoa were counted. The concentration was calculated using the formula C = (N / n) x 1/25 x dilution factor, where N is the number of spermatozoa counted and n is the number of columns examined.

3.3 RESULTS

The semen collection was well succeeding only in two out of five *Micrurus frontalis* specimens. The physical analyses of the samples returned a whitish coloration as well as a milky and thick consistency of the semen (Figure 1A). The biometric, semen volume and cell composition data are shown in table 1.

The vitality test using saline eosin solution, was effective in distinguishing the dead cells from the alive ones (Figure 1B), however the time lack to proceed with the analysis was limited, once after 2 minutes of exposition to the eosin solution we observed that the head-acrosome complex was burst. Nonetheless, spermatozoa alive corresponded to approximately 90% of the total evaluated (table 2) whereas the average of spermatozoa presenting cytoplasmic droplets was around 20% (table 1).

M. frontalis spermatozoon is filiform and the head have a slender form. Both Hematoxylin-Eosin and Rapid staining method presented a good performance, enabling a satisfactory morphology examination. However, Hematoxylin-Eosin showed a good intensity of staining in the acrosome, head, mid-piece and tail (figure 1C) whereas Rapid staining method has resulted in weak staining of sperm tail and, it may have caused the sperm head to burst, as observed in some of these cells (figure 1D). Spermatozoon morphological measures were similar in both staining methods (table 3).

3.4 DISCUSSION

The non-invasive semen collection in snakes is recently well succeeding (Zacariotti et al., 2007; Fahrig et al., 2007; Silva et al., 2015), however the individual variation must be taken into account, what can explain the low percentual of successful semen collection in *Micrurus frontalis*. Despite this, the colour and texture of semen was similar to other snakes (Silva et al., 2015) showed no alterations between samples, as well as a high vitality percentage.

Semen volume, SVL and body mass in snakes seem not be related (Schulte-Hostedde and Montgomerie, 2006; Silva et al., 2015); thus, the difference herein observed between specimens of *M. frontalis* regarding the volume of semen may be associated with an individual feature, as also indicated through the percentage of spermatozoon with cytoplasmic droplets (sptz cd).

In the present study, we were able to evaluate and quantify the vitality and motility of the *M. frontalis* spermatozoa. These analyses are important to demonstrate the sperm performance, predicting the fertility rates and testis function (Barth & Oko, 1989; Zinaman et al., 2000). The sperm vitality test is performed through the evaluation of the integrity of cell membrane, since a damaged membrane is permeable to stains (WHO, 2010). However, the eosin prepared with NaCl solution, as used in this study, resulted in an important change in the morphology of the sperm heads, which has led them to swollen only few minutes after the staining procedure has finished. In this regard, we tried another preparation using PBS instead of NaCl for eosin dilution. In this attempt, we observed that even 30 minutes later, the spermatozoa were still presenting the usual morphology of the head. These results reflect that the osmolarity of the stain solution may affect the sperm vitality, and the evaluation needs to be performed with no delay.

Spermatozoa with cytoplasmic droplets were find in testis samples of herpetofauna animals like salamanders (George et al., 2005), turtles (Zhang et al., 2015) and snakes (Esponda and Bedford, 1987), however in *M. frontalis* the sperm samples were collected from the final portion of the ductus deferens, non-invasively, which make them quite similar to the spermatozoa present in the naturally ejaculated semen. For the first time it has been shown that the spermatozoa with cytoplasmic droplet are alive and viable in this coral snake species. Thus, males of *M. frontalis*

copulate and are able to produce offspring, despite the presence of a high percentage of cytoplasmic droplet spermatozoa, since they are alive and viable. Therefore, still remains to be further investigated the evolutive meaning of keeping the viability of sperms even though the presence of these potentially hazardous features. It has been suggested that cytoplasmic droplet might be playing a role for sperm survival during long-term storage inside female genital tract (Zhang et al., 2015). This could be an explanation for case of *Micrurus frontalis*, once the females do sperm storage (Bassi et al., 2019).

The morphological characteristics of spermatozoon of the coral snake, such as the filiform head covered by a conical acrosomal, is similar to the observed for spermatozoa in other snakes species (Silva et al., 2015; Silva et al., 2017). However, the total motility found for *M. frontalis* spermatozoa was around 94%, in contrast to the observed for *Bothrops insularis* (64%; Silva et al., 2015) and *Erythrolamprus poecilogyrus* (80%; Silva et al., 2017). The total cell length was also different among species. Hence, for the coral snake, we found a range between 60.6 – 61.4 µm for the spermatozoa length although it has been larger in *B. insularis* (96.34 - 100.54 µm; Silva et al., 2015) and *Nerodia sipedon* (110.2 – 113.9 µm; Schulte-Hostedde and Montgomerie, 2005).

Many staining methods are used to assess sperm morphology. In our study two methods were tested: Hematoxylin-Eosin (HE), normally used for the histological analysis of several tissues, and also has shown satisfactory results for evaluation of sperm in different animal species; and the Rapid Staining procedure, commonly used for human sperm analyses. HE satisfactorily evidenced the head, middle piece and tails of spermatozoa, without damaging the cell. These data corroborate with other studies that highlight HE as one of the best staining methods for evaluation of sperm from different species (Aksoy et al., 2012; Vieira et al., in preparation). In contrast, the Rapid staining method caused a head burst in some spermatozoa, impairing the detailed visualization of the morphology of these cells. This result is in agreement with our hypothesis that the osmolarity of the stain solution could be compromising the performance of the coloration method tested.

In conclusion, we were able to adapt the protocols of vitality, motility and morphology from WHO (2010), showing that it is suitable for snake sperm analyses, generating and enabling further information regarding sperm quality in these animals.

In addition, we showed that in *Micrurus frontalis* spermatozoa containing cytoplasmic droplets are alive and viable, which is in agreement with the hypothesis that these structures may have an important meaning within the reproductive cycle of this Neotropical snake species.

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3.6 TABLES

Table 1. *Micrurus frontalis* body measures and semen volume and composition.

ID	**SVL (mm)**	**W (g)**	**SV (µl)**	**Sptz cd (%)**	**Sptz n (%)**
M. frontalis 1	880	66.8	4.7	35	65
M. frontalis 2	710	53.1	2.1	12	88

SVL = snout–vent length; W = Weight; SV = semen collected volume; Sptz cd = spermatozoa with cytoplasmic droplet; Sptz n = normal spermatozoa.

Table 2*. Micrurus frontalis* sperm parameters*.*

Sperm parameters	***M. frontalis 1***	***M. frontalis 2***	**Mean (SSD)**
Concentration (10^6/ml)	511	554	532.5 (30.4)
Vitality (%)	91.2	92.6	91.9 (0.70)
Motility A (%)	15.3	15.4	15.3 (0.07)
Motility B (%)	81.5	75.7	78.6 (4.12)
Motility C (%)	3.1	8.8	6.0 (4.05)
Agglutination	absent	isolated	

SSD = sample standart deviation; sptz = spermatozoa; Motility A = mobile with progressive movement; Motility B = mobile without progressive movement; Motility C = immobile.

Table 3. *Micrurus frontalis* morphological measures of spermatozoon using different staining methods.

Morphological measures (mm)	**HE**	**RS**
Head mean (SSD)	5.3 (0.5)	6.0 (0.1)
Mid-piece mean (SSD)	35.2 (1.4)	32.8 (1.4)
Tail mean (SSD)	20.0 (1.3)	22.5 (1.6)
Total cell length (SSD)	60.6 (1.5)	61.4 (1.9)

SSD = sample standard deviation; HE = Hematoxylin-Eosin; RS = Rapid staining.

3.7 FIGURES

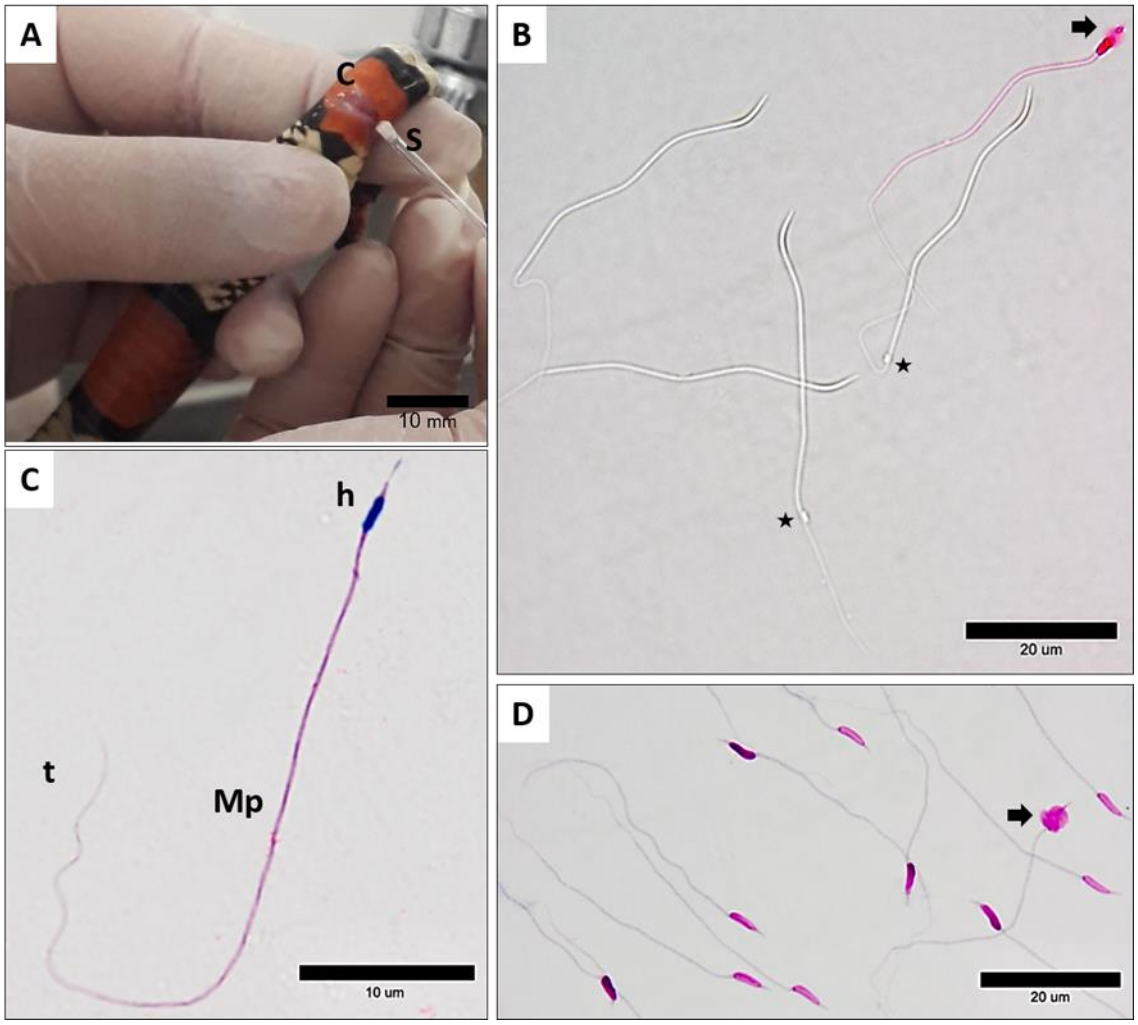

Figure 1. A) *Micrurus frontalis* semen collection, C= cloaca, S = semen; B) spermatozoa in saline solution eosin preparation, pink spermatozoon is dead, Arrow = acrosome burst, Star = cytoplasmic droplet; C) spermatozoon in Hematoxylin-Eosin staining, h = head, Mp = mid-piece and t= tail; D) spermatozoa in Rapid staining procedure staining, Arrow = head burst.

4 SPERM STORAGE IN CORAL SNAKES: AN ULTRASTRUCTURAL APPROACH

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ABSTRACT

Reproduction in snakes employs a wide range of mechanisms such as long-term sperm storage, mating aggregations and male-male combat. These varied mechanisms can be better understood when combined with study of the ultrastructural morphological variability of spermatozoa. Within the coral snakes, genus *Micrurus*, there are differences in the reproductive strategies adopted between monads (BRM) and triads (BRT); the aim of this work was to relate these two strategies to morphological differences among spermatozoa within different species. The semen of six *Micrurus* was collected using a non-invasive technique, fixed and processed for scanning and transmission electron microscopy. *Micrurus* spermatozoa have the same ultrastructure as those of other snake species, comprising the head, divided into acrosome, nucleus and neck; the midpiece; and the two parts of the tail, the principal piece and the end piece. *Micrurus corallinus* and *Micrurus surinamensis* presented evident multilaminar membranes occurring along all the pieces, while *Micrurus frontalis* and *Micrurus altirostris* showed cytoplasmic droplets occurring mainly in the midpiece. The differences found in spermatozoa morphology may be related with sperm storage in these four coral snakes, since the development of multilaminar membranes in the midpiece and the maintenance of cytoplasmic droplets in the mature sperm are both features related to extra energy provision for the spermatozoa while stored in the female's oviduct.

Key-words: Snake spermatozoon. Sperm ultrastructure. Multilaminar membranes. Cytoplasmic droplets.

4.1 INTRODUCTION

Reproduction in snakes employs a wide range of mechanisms such as polyandry (Clark et al., 2014), long-term sperm storage (Almeida-Santos and Salomão, 1997), sperm competition (Tourmente et al., 2006), mating aggregations (Duvall, Arnold and Schuett, 1992; Shine 2003) and male-male combat (Almeida-Santos et al., 1999; Missassi et al., 2017). These varied strategies have stimulated new approaches to snake reproductive studies.

One of these approaches is the ultrastructural study of the morphological variability of spermatozoa (Oliver et al., 1996; Tavares-Bastos et al. 2002; Tourmente et al., 2006; Rheubert et al., 2010; Gribbins et al., 2016), which can be applied to a reproductive perspective of sperm competition and storage (Vieira et al., 2004; Cunha, Tavares-Bastos and Báo, 2008). The size of the spermatozoa could indicate levels of sperm competition (Tourmente et al., 2009). Additionally, sperm competition can act as a sexual selection force to modify sperm ultrastructure in order to maximize success in female fertilization (Tourmente et al., 2009; Blengini et al. 2014; Blengini et al., 2017). Other ultrastructural characteristics like the development of multilaminar membranes in the midpiece could be related to provision of an extra energy source for the spermatozoon while it is stored in the female oviduct (Tourmente et al., 2006). Understanding of the possible connections between snake sperm dynamics, life history, and mating system features is still limited, so an increase in the number of species studied by this perspective is needed (Tourmente et al., 2011).

Snake ultrastructural spermatozoa studies have produced data on the families Colubridae, Pythonidae, (Jamieson and Koehler 1994; Oliver, Jamieson and Scheltinga, 1996), Viperidae (Al-Dokhi 2004), Typhlopidae (Harding et al. 1995), and Boidae (Tavares-Bastos, Colli and Báo, 2008). However, a few studies were dedicated to describe the spermatozoa of some Elapidae (Oliver, Jamieson and Scheltinga, 1996; Gribbins et al., 2016) and the neotropical Elapidae remains completely unknown.

The neotropical Elapidae is a large group of dangerous venomous snakes, with coral color pattern, represented by three genera: *Micrurus*, *Leptomicrurus* and *Micruroides* (Slowinski, 1995). The most studied of these is *Micrurus* (Jackson and Franz, 1981; Roze, 1996; Silva Jr. and Sites, 2001; Goldberg, 2004; Almeida-Santos, Pizzatto and Marques, 2006; Pires et al., 2014; Marques et al., 2017) and the number

of works on reproduction of *Micrurus* species has increased recently (Marques, Pizzatto and Almeida-Santos 2013; Missassi et al., 2017; Bassi, Coeti and Almeida-Santos, 2019).

Micrurus species can use different mating strategies to reproduce according to their lineage (Marques, Pizzatto and Almeida-Santos, 2013), thus species with black rings arranged in monads (BRM) have mating aggregations (Almeida-Santos, Coeti and Bassi, in press) while species where the black rings are arranged in triads (BRT) (Slowinski, 1995) present male-male combat (Almeida-Santos, Schidmit and Balestrin 1998; Missassi et al., 2017); in both lineages the female is able to perform sperm storage (Bassi, Coeti and Almeida-Santos 2019).

In agreement with studies that relate spermatozoa ultrastructure with reproductive characteristics (Tourmente et al., 2006; Tourmente, Giojalas and Chiaraviglio, 2011), the reproductive differences in the genus *Micrurus* can be evidenced through ultrastructure studies of the spermatozoon. Thus, the aim of this work was to describe the morphology of the spermatozoa in coral snakes of the genus *Micrurus* and to relate the morphological differences among spermatozoa with different reproductive strategies in the species.

4.2 MATERIAL AND METHODS

Specimens

Six adult male *Micrurus* snakes were obtained from 2017 to 2019, under the approval of the Ethics Committee for Animal Research from Instituto Butantan reception, Biological Museum of Instituto Butantan (approval number 1447271016), Instituto Vital Brazil (approval number of Faculdade de Medicina Veterinária e Zootecnia da USP 8531090816) and Fundação Ezequiel Dias (FUNED) (approval number 126/2017). We must emphasize the difficulty of gaining access to these captive animals, since most are kept exclusively for the production of antivenom and have a low life expectancy (Mendes et al., 2018).

The individuals came from different lineages, according to the recent phylogeny of Zaher et al. (2016). The BRM clade was represented by *Micrurus corallinus* (n= 2)

specimens and the BRT clade by *Micrurus frontalis* (n=2)*, Micrurus altirostris* (n=1) and *Micrurus surinamensis* (n=1).

Semen collection

Mature spermatozoa were obtained according to the protocol of Coeti et al. (in preparation, Chapter 1). Initially, the individuals are physically restrained inside plastic tubes to empty the cloaca of feces or urine by ventral massages. The animal's cloaca is then washed with saline solution. Subsequently, a new ventral massage is done by applying slight pressure on the sides where the vas deferens is located until the animal ejaculates. The semen is collected from the cloacal urogenital papilla using microhematocrit capillary tubes. We measured the semen volume with the help of a ruler, calculating the conversion from millimeters to milliliters.

Spermatozoon microscopic analysis

A small fraction of the collected semen was prepared in the form of a smear for immediate confirmation of the presence of spermatozoa under a light microscope. The remaining semen was fixed in Karnovsky solution (Morris, 1965).

After fixation, the sample was washed with 0.1 M sodium cacodylate buffer, pH 7.2. At this moment the sample was divided into two parts for scanning and transmission electronic microscopy (SEM and TEM respectively).

Spermatozoon SEM

For SEM processing, round coverslips were used as support for the samples. They were covered with a drop of 1% polylysine, kept inside a well culture plate, initially for 10 min at room temperature, and then at 37 °C for 5 min. The excess of polylysine was then removed and the semen sample, already in sodium cacodylate buffer, was dripped onto the coverslips and allowed to adhere on the surface for 15 min. They were then incubated in 1% osmium tetroxide in the 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h, followed by three 15-min washes in the buffer, and dehydrated in a crescent series of ethanol. The samples in 100% ethanol were finally dried by the critical point method, using CO_2 as the transition fluid, assembled on aluminium stubs using copper

conductive tape, gold-plated using a sputtering system, and examined under a FEI Quanta 250 scanning electron microscope, operating at 10 kV.

Spermatozoon TEM

The sample was initially centrifuged for pellet formation. After three washes (15 min each) in 0.1 M sodium cacodylate buffer, pH 7.2, the sample was post fixed in buffered 1% osmium tetroxide for 1 h; washed again three times (15 min each) in the same buffer, dehydrated in crescent ethanolic series, and embedded in epoxy resin, using propylene oxide as intermediate agent. Centrifugation was repeated in each solution exchange whenever was necessary.

Ultrathin sections (approximately 60 nm) were mounted in sequence, on 400 mesh copper grids for observation under a transmission electron microscope LEO 906E, operating at 80 kV. The ultrastructural morphometric and morphological analysis was conducted on the TEM micrographs.

Statistical analyses

The snout-vent length (SVL) and weight of each animal and the volume of semen collected were recorded for statistical analysis, applying the Spearman nonparametric correlation.

The length of the head, midpiece and tail were measured by the ImageJ software (Schneider, Rasband and Eliceiri, 2012) in photographs taken under FEI Quanta 250 scanning electronic microscope (10 kV). Comparisons between species were investigated with a non-parametric analysis of variance (Kruskal-Wallis).

All tests were performed with GraphPad Prism version 5 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com); the significance level was set at $P < 0.05$.

4.3 RESULTS

Sample collection and sperm characteristics

The non-invasive method was successfully performed in all the animals (Figure 1A). The semen samples were thick and white, and no contamination with faeces or urate was visible (Figure 1B). The volume of the samples varied from 1.05 to 6.3 µl (Table 1) and presented no correlation with the SVL (r = -0.151; *P* = 0.802) or weight (r = -0.394; *P* = 0.419) of the animals.

Morphometrical data showed that *M. corallinus* have the smallest spermatozoa and *M. frontalis* the largest one (table 2), but the differences in size were not significant among all four species (H = 0.683; *P* = 0.877).

Basic spermatozoa ultrastructure of the four species

All species presented an elongated spermatozoa (Figures 2A, 2B, 2C and 2D) divided into head, midpiece and tail. The sickle shaped heads are clearly divided into the acrosome, the nuclear region and the neck (Figure 3A). The midpiece is the biggest part of the cells (table 2), and the tails are divided into a principal and an end piece (Figure 4E).

The first part of sperm, the acrosome complex, is generally elongated (Figures 3A and 3B) and covers the apical portion of the nucleus (Figure 3B). The acrosome has two layers, the external acrosomal vesicle and the internal subacrosomal cone, separated by the acrosomal ridge (Figure 3D). Inside the acrosomal vesicle there is a perforatorium (Figure 3C) that was identified only when observed in cross sections. The nuclear rostrum, surrounded by the subacrosomal cone cover, is the most apical part of the nucleus (Figure 3B). The nucleus is generally elongated, curved and shows a massive electrondense chromatin (Figure 3E and 3F) occupying the majority of the sperm head. It terminally finishes in a concave depression that holds the proximal centriole (Figure 3B).

The neck region connects the head with the midpiece of the sperm and comprises the proximal and distal centriole, wrapped in pericentriolar material (Figure 4A). The proximal centriole is perpendicular to the longitudinal distal centriole, which is

surrounded by a dense collar of cytoplasm mass and whose microtubules make up the basal body of the axoneme that runs along all the midpiece and tail (Figure 4B).

The medullar part of the midpiece is composed by the axoneme, a circular combination of 9 plus 2 microtubules, which is involved by a fibrous sheath, externally followed by a layer of dense bodies mixed with mitochondria (Figure 4C). This arrangement longitudinally extends until the annulus, which is the final part of the midpiece (Figure 4D).

Following in terminal direction, after the annulus region, where there are no more dense bodies and mitochondria, the tail begins, divided into the principal piece and the end piece. The principal piece is covered by the axoneme and the fibrous sheath (Figure 4F) which becomes thinner towards the end of the cell until it disappears, marking the beginning of the end-piece and the end of the sperm (Figure 4G).

Spermatozoa ultrastructural differences observed among species

The ultrastructural examination of the spermatozoa pointed to clear differences for each species. In the midpiece of *M. frontalis* (Figure 5A) and in the head of *M. altirostris* (Figure 5C) cytoplasmic droplets, composed mainly of lipids, mitochondria and vacuoles were observed (Figure 5B). In *M. corallinus* (Figure 5G) and *M. surinamensis* (Figure 5F) the spermatozoa were surrounded by multilaminar membranes together with the plasma membrane. In addition, in some of the spermatozoa of *M. surinamensis* the neck region presents more pericentriolar material and mitochondria (Figures 5D and 5E).

4.4 DISCUSSION

This work describes and compares for the first time the sperm ultrastructure of four species of *Micrurus* coral snakes. The non-invasive collection method used here and in other snake studies (Oliver, Jamieson and Scheltinga, 1996; Tourmente et al., 2006) allows access to mature spermatozoa for morphological ultrastructure analysis without harming the animals. However, the semen volumes obtained in this work by this method presented individual variation that was not correlated with the SVL or weight of the animals. This fact can be explained because this method does not cause a true

ejaculation, and probably only a portion of the total semen maintained in the ductus deferens was obtained (Silva et al., 2015). In addition, individual variation in the volume of semen is probably more related with seasonal spermatogenesis and storage in each male (Marques, Pizzatto and Almeida-Santos, 2013).

Morphometry shows that the midpiece is the biggest part of *Micrurus* spermatozoon, which is indicative of sperm competition or reproductive period variation (Tourmente et al., 2009; Blengini et al., 2016). The absence of significant differences among the four coral snakes, was already expected since other studies demonstrate that spermatozoa ultrastructure does not vary in closely related species (Rheubert et al., 2017; Blengini et al., 2017). In comparison with other species of snake, like *Bothrops alternatus*, *Bothrops diporus* (Tourmente, Giojalas and Chiaraviglio, 2008) and *Boa constrictor occidentalis* (Tourmente, Giojalas and Chiaraviglio, 2011), *Micrurus* species have bigger spermatozoa with a more elongated midpiece, which according to Tourmente et al., (2009) means that it undergoes a greater sperm competition when compared to other genera.

Coral snake spermatozoa have conserved the same characteristics as those of Serpentes (Oliver, Jamieson and Scheltinga, 1996; Tourmente et al., 2006; Tavares-Bastos et al., 2007; Cunha, Tavares-Bastos and Báo, 2008; Tourmente, Giojalas and Chiaraviglio, 2008, Tourmente et el., 2009; Rheubert et al., 2010; Rheubert et al., 2017; Hao and Zhang, 2018) being divided into the head (composed of the acrosomal and nucleus regions), neck, midpiece and tail (composed of the principal and end piece) although with some variations. In *Micrurus* the acrosomal complex has an acrosomal ridge separating the external acrosomal vesicle from the internal subacrosomal cone, but not so lucent as that present in *Agkistrodon contortrix* (Rheubert et al., 2017); a perforatorial base plate was not seen, or has been lost as in other snakes (Oliver, Jamieson and Scheltinga, 1996).

Although the spermatozoa in *Micrurus* species have a similar morphology, we found some different structures like the presence of multilaminar membranes investing the whole cell in *Micrurus corallinus* and *Micrurus surinamensis*. This structure was described for sperm collected from epididymal ducts and testes in *Lampropeltis getulus* (Hamilton and Fawcett, 1968); testicular biopsy or ejaculation in *Boiga irregularis*, *Stegonotus cucullatus*, *Oxyuranus mioolepidotus*, *Oxyguranus microlepidotus* and *Aspidites melanocephalus* (Oliver, Jamieson and Scheltinga, 1996); ejaculation in *Boa*

constrictor occidentalis (Tourmente et al., 2006); epididymal biopsy in *Leptotyphlops koppesi*, *Typhlops reticulatus* and *Liotyphlops beui* (Tavares-Bastos et al., 2007); epididymal biopsy in *Crotallus durissus* (Cunha, Tavares-Bastos and Báo, 2008); epididymal biopsy in *Seminatrix pygaea* (Rheubert et al., 2010); vas deferens biopsy in *Agkistrodon contortrix* (Rheubert et al., 2017) and epididymal biopsy in *Myrrophis chinensis* (Hao and Zhang, 2018). All these data, collected from different male reproductive tract segments, confirm that the multilaminar membranes are present in immature (from testes and epididymis) and mature spermatozoa (from ejaculate); however, they are not present in the sperm of all snakes, being absent from the sperm of *Bothrops alternatus* and *Bothrops diporus* (Tourmente, Giojalas and Chiaraviglio, 2008), as well *Micrurus frontalis* and *Micrurus altirostris* here studied.

The presence of multilaminar membranes in snake spermatozoa is related with ATP accumulation as an extra energy source (Hamilton and Fawcett, 1968; Tourmente et al., 2006) and with the maintenance of sperm viability over long periods, as when the sperm is stored in females (Cunha, Tavares-Bastos and Báo, 2008). *Micrurus corallinus* females have a period of sperm storage (Bassi, Coeti and Almeida-Santos, 2019) and besides that, the males copulate with sperm stored in the ductus deferens (Almeida-Santos, Pizzatto and Marques, 2006; Coeti et al., in preparation). Thus, the presence of multilaminar membranes in these spermatozoa, which pass through at least two storage periods, sustains the idea that this structure is important for the viability and maintenance of the cell in this period. This suggests that other species of *Micrurus* that possess the same structures, like *Micrurus surinamensis*, probably pass through a comparable process.

Nonetheless, some species with recognized female sperm storage, like *Bothrops alternatus* (Almeida-Santos and Salomão, 2002) and *Micrurus frontalis* (Bassi, Coeti and Almeida-Santos, 2019), do not have multilaminar membranes (Tourmente, Giojalas and Chiaraviglio, 2008; present work). Long-term sperm storage is described for snakes (Booth and Schuett, 2011), and although we do not know for how long *Micrurus* females do this, the process of sperm storage in *Micrurus corallinus* and *Micrurus frontalis* occur (Bassi, Coeti and Almeida-Santos, 2019) in different manners.

Assuming that, spermatozoon multilaminar membranes help *Micrurus corallinus* and, probably, *Micrurus surinamensi*s in the process of storage, perhaps other different structures can assume a similar role in the case of *Micrurus frontalis* and, presumably,

Micrurus altirostris spermatozoa. The cytoplasmic droplets found in the last two species are similar to those described for other animals that store sperm, like bats (Krutzsch, Crichton and Nagle 1982; Cervantes et al., 2008) and turtles (Gist, Hess and Thurston, 1992; Zhang et al., 2015). These droplets are associated with the provision of extra energy to spermatozoon still in maturation inside the epididymis (Yuan et al., 2013), especially when this process involves long-term storage (Zhang et al., 2015). Although cytoplasmic droplets have previously been reported in snake sperm (Esponda and Bedford, 1987), this is the first time that they have been found in mature spermatozoa from the ductus deferens rather than spermatozoa from the epididymis. The presence of such structures in *M. frontalis* and *M. altirostris* spermatozoa might be linked with male sperm storage, since these species present sperm in the ductus deferens all year round (Marques, Pizzato and Almeida-Santos, 2013). However, in the case of these two coral snakes, the fact that these sperm are mobile and viable (Coeti et al., in preparation), and are ejaculated with cytoplasmic droplets, suggests the hypothesis that the spermatozoa carry “backpacks” containing extra energy that will give them the possibility of penetrate the female, ready to spend a “vacation time” stored in her reproductive tract.

The last morphological difference we have detected in the coral snakes’ spermatozoa may also be related with reproduction. Our observations show that the mitochondria are generally present in different arrangements in the midpiece of the sperm of *Micrurus* species. However, in *M. surinamensis* mitochondria also appear in the neck region where they are larger than those in the midpiece. This differentiation in mitochondria location and size is similar to other reptiles like the Chinese soft-shelled turtle (Zhang et al., 2015) and lizards (Scheltinga et al., 2001; Tavares-Bastos et al., 2002). The elongation of the midpiece in snake spermatozoon increases the number of mitochondria and consequently the amount of energy produced by the cell (Tourmente et al., 2006; Tourmente et al., 2009). Furthermore, in the turtle spermatozoon neck, mitochondria lipoprotein membranes are digested during the period of storage within the female reproductive tract (Zhang et al., 2015). Thus, even without information on the reproductive cycle of *Micrurus surinamensis*, our finding showing the presence of mitochondria in the spermatozoa neck in this species is an indicative of preparation for sperm storage periods.

The variation in sperm ultrastructure indicates a male strategy to produce variable spermatozoa aiming at maximizing fertilization success in different reproductive situations (Zhang et al., 2015) and seasons (Blengini et al., 2016). Thus, the morphological differences found individually and interspecies in *Micrurus* spermatozoa highlight the long path that spermatozoa travel along the whole reproductive cycle and how this trajectory selects the cell that finally will fertilize the oocyte.

In summary, the presence of multilaminar membranes, cytoplasmic droplets and mitochondria in the neck of the spermatozoa of *Micrurus* species may be related with the reproductive strategy of sperm storage in males and in females belonging to this genus. It was not possible yet to direct connect such morphological evidences with different mating strategies or phylogenetic lineages, so more reproductive data are needed to clarify these relationships.

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4.6 TABLES

Table 1. *Micrurus* information and collection data.

Species	Date	Institution	SVL (mm)	Weight (g)	Volume (µl)
M. corallinus	August, 21, 2017	IVB	422	19.2	3.15
M. corallinus	November, 9, 2018	IB	545	38.1	6.3
M. frontalis	February, 21, 2017	IVB	597	142.4	3.15
M. frontalis	January, 25, 2019	FUNED	880	66.8	4.7
M. altirostris	June, 28, 2017	IB	930	149	3.15
M. surinamensis	December, 10, 2018	IB	671	98.7	1.05

SVL = snout-vent length

IB = Butantan Institute

IVB = Vital Brazil Institute

FUNED = Ezequiel Dias Foundation

Table 2. *Micrurus* sperm measurements

Species	Mean (SD) µm			
	Head	Mid-piece	Tail	Total
Micrurus corallinus	9.28 (0.76)	57.07 (4.87)	30.08 (3.86)	96.44 (4.04)
Micrurus frontalis	10.90 (1.22)	60.84 (4.33)	32.64 (3.13)	104.39 (3.80)
Micrurus altirostris	10.09 (0.73)	57.53 (2.04)	34.98 (2.27)	102.60 (3.29)
Micrurus surinamensis	9.83 (0.64)	57.33 (1.44)	33.24 (1.85)	100.40 (2.13)

SD = Standard Deviation

4.7 FIGURES

Figure 1.

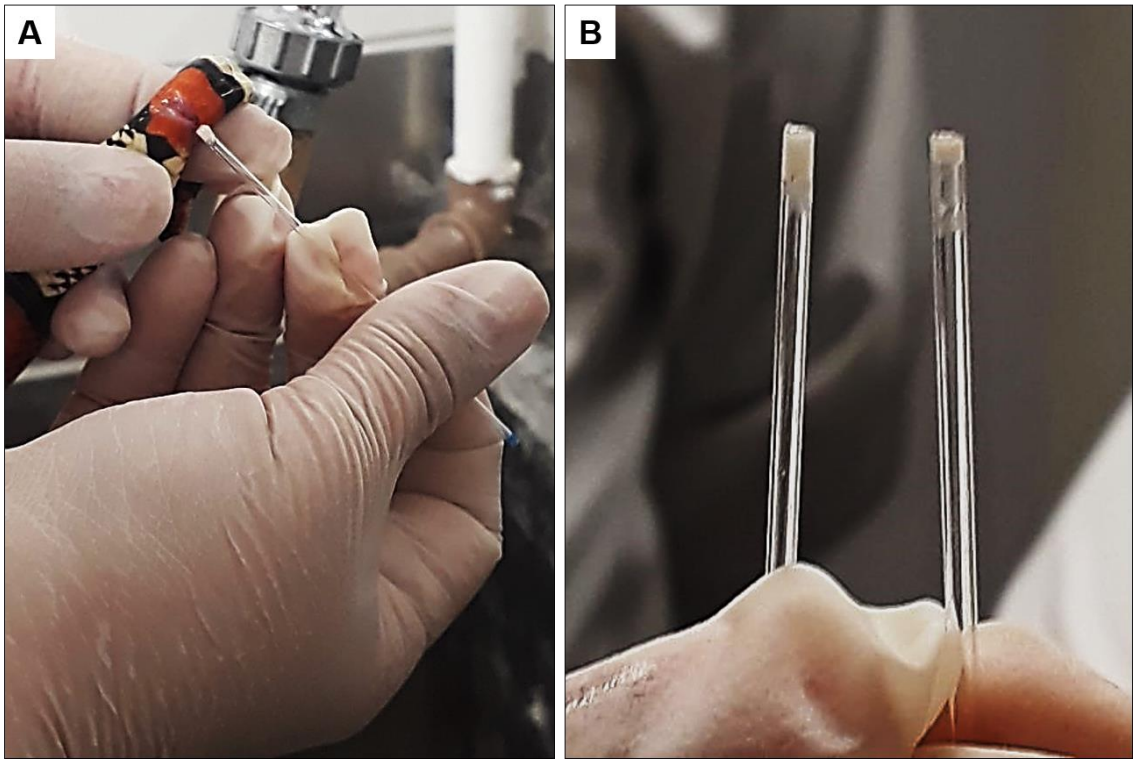

Figure 1. Semen collection in *Micrurus frontalis*. **A)** Semen collection from cloacal gap with microhematocrit capillary tube; **B)** Microhematocrit capillary tubes with semen showing white coloration.

Figure 2.

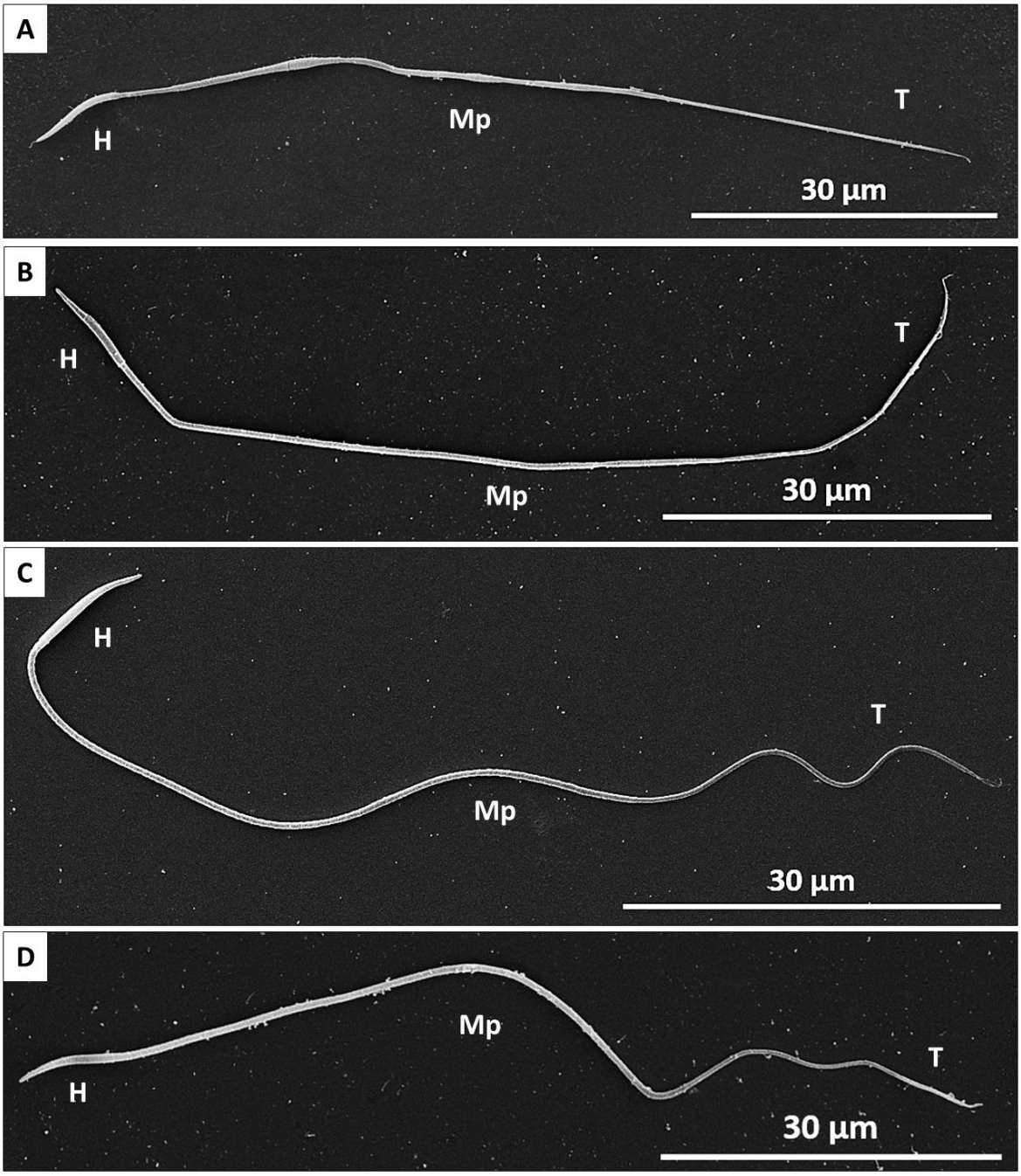

Figure 2. SEM of *Micrurus* of spermatozoa. **A)** *Micrurus frontalis*; **B)** *Micrurus altirostris*; **C)** *Micrurus surinamensis*; **D)** *Micrurus corallinus*. Head (H), mid-piece (Mp) and tail (T).

Figure 3.

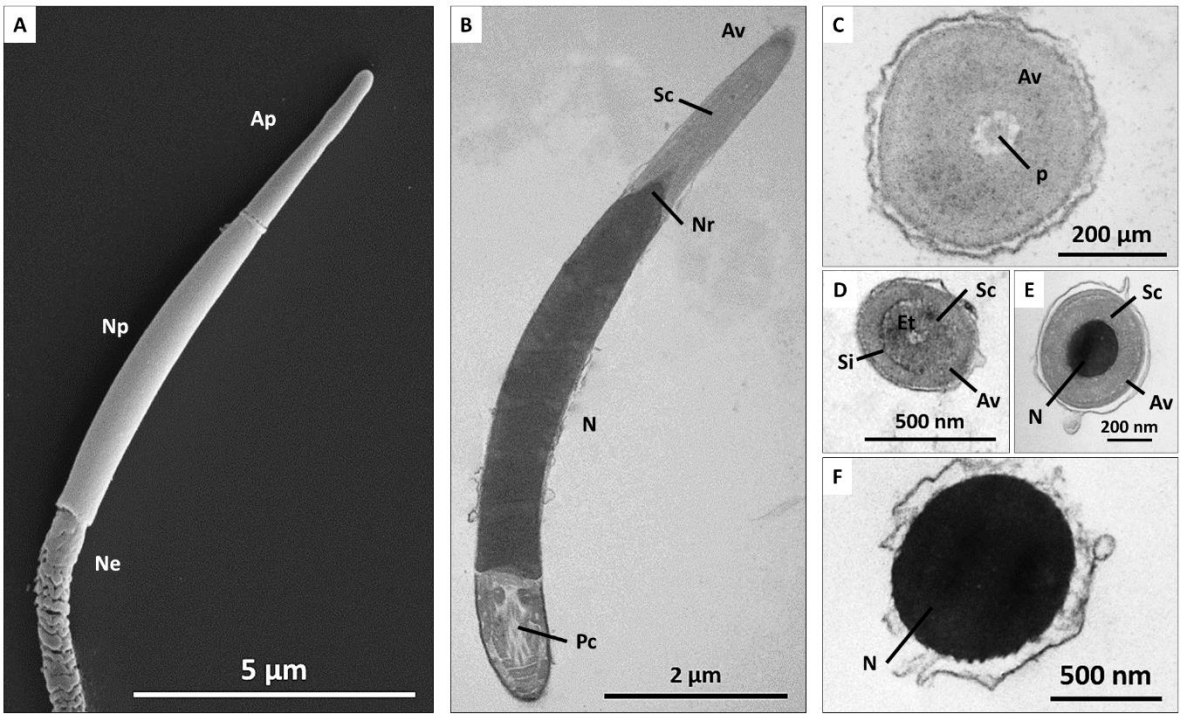

Figure 3. *Micrurus* spermatozoon head. **A)** External view showing division in parts: acrosome (Ap), nucleus (Np) and neck (Ne); **B)** Longitudinal section, showing the head and the neck. Acrosomal vesicle (Av), sub acrosomal cone (Sc), nuclear rostrum (Nr), nucleus (N) and proximal centriole (Pc); **C)** Transversal section of the acrosome with acrosomal vesicle (Av) and perforatorium (p); **D)** Transversal section of the acrosome with acrosomal vesicle (Av), acrosomal lucent ridge (Si), sub acrosomal cone (Sc) and epinuclear lucent zone (Et); **E)** Transversal section of the transition from the acrosomal to the nucleus part, with acrosomal vesicle (Av), sub acrosomal cone (Sc) and nucleus (N); **F)** Transversal section of the nucleus part showing containing the nucleus (N). **(A)** SEM; **(B)** to **(F)** TEM.

Figure 4.

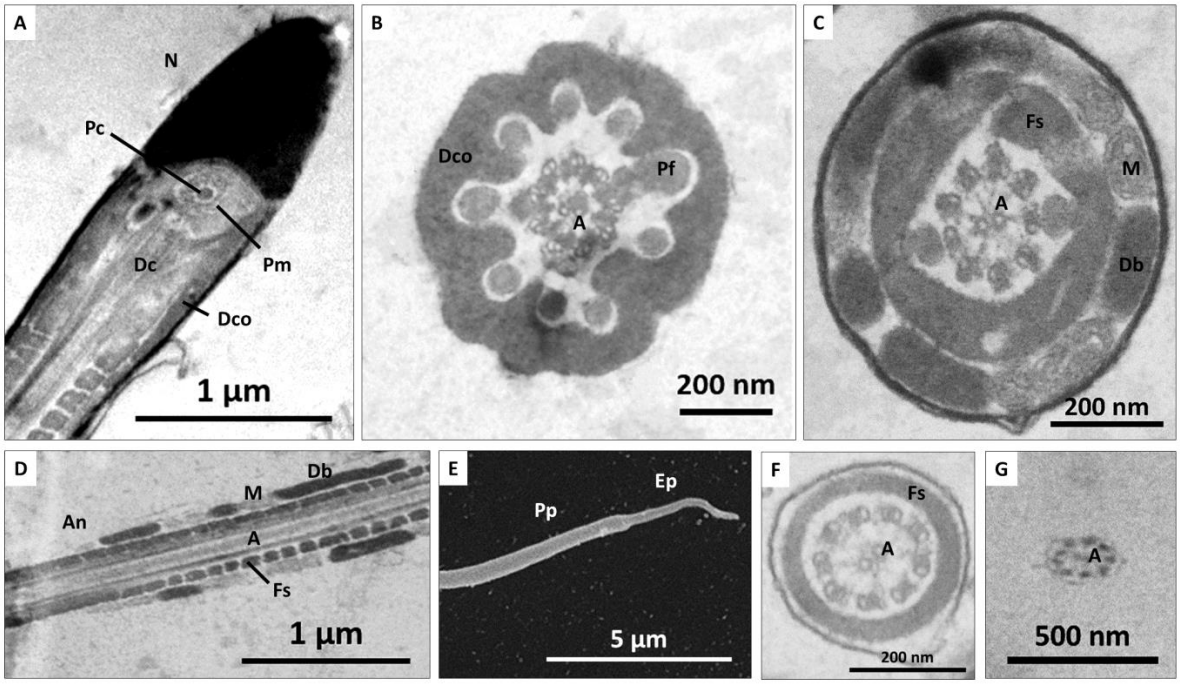

Figure 4. *Micrurus* spermatozoon neck, midpiece and tail. **A)** Longitudinal section of the neck with nucleus (N), proximal centriole (Pc) involved in pericentriolar material (Pm), distal centriole (Dc) and dense colar (Dco); **B)** Transverse section of neck with dense colar (Dco), peripheral fibers (Pf) and axoneme (A); **C)** Transverse section of the mid piece with axoneme (A), fibrous shield (Fs), dense body (Db) and mitochondria (M); **D)** Longitudinal section of the mid piece showing the same parts as described in **(C),** followed by the terminal part, the annulus (An); **E)** Tail with visible divisions into principal piece (Pp) and end piece (Ep); **F)** Transverse section of the principal piece with axoneme (A) and fibrous shield (Fs); **G)** Transverse section of the end piece with axoneme (A). **(A)**, **(B)**, **(C)**, **(D)**, **(F)** and **(G)**, TEM; **(E)**, SEM.

Figure 5.

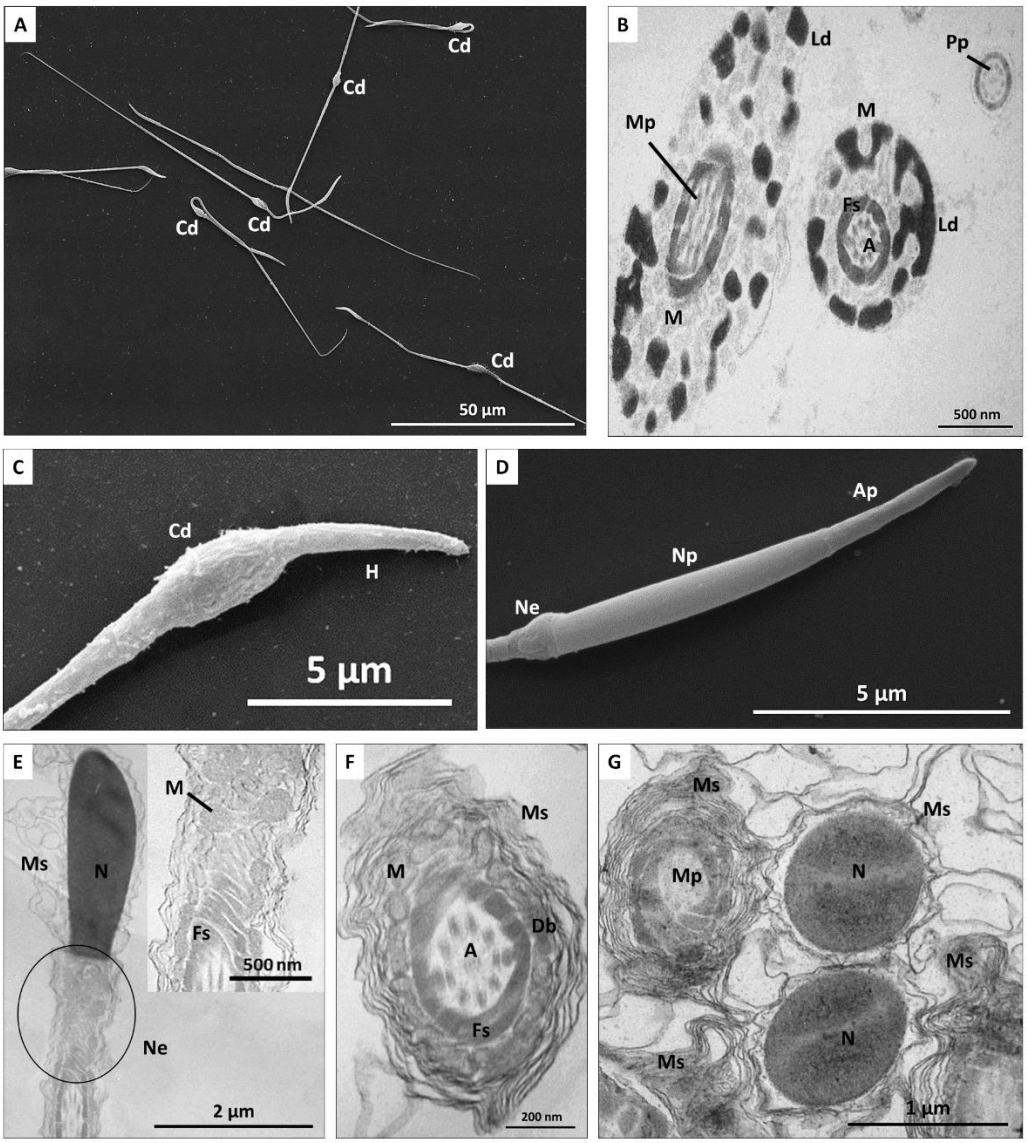

Figure 5. Spermatozoon morphological differences among *Micrurus* species. **A)** *Micrurus frontalis* spermatozoa sample with cytoplasmatic droplets (Cd); **B)** Transverse sections of *Micrurus frontalis* mid piece (Mp) with axoneme (A) and fibrous shield (Fs) involved for cytoplasmatic droplets with mitochondria (M) and lipids (Ld) and principal piece (Pp); **C)** External morphology of *Micrurus altirostris* sperm head (H) with a cytoplasmatic droplet (Cd); **D)** External morphology of *Micrurus surinamensis* sperm head with visible divisions, acrosomal part (Ap), nucleus part (Np) and bulky neck (Ne); **E)** Longitudinal section of *Micrurus surinamensis* sperm head and neck with multilaminar membranes (Ms) and nucleus (N). The insert shows a higher magnification of the neck (Ne) with fibrous shield (Fs) highlighting the different mitochondria (M); **F)** Transverse section of *Micrurus surinamensis* mid piece with

axoneme (A), fibrous shield (Fs), mitochondria (M), dense body (Db) and multilaminar membranes (Ms); G) Transverse section of spermatozoa of *Micrurus corallinus* with mid piece (Mp) and nucleus (N) wrapped by multilaminar membranes (Ms). **A)**, (**C)** and **(D)**, SEM; **(B), (E)**, **(F)** and **(G)**, TEM.

5 A PROTOCOL FOR EVALUATING SNAKE SPERM BY FLOW CYTOMETRY

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ABSTRACT

The methodologies for evaluating the semen of humans and other animals has evolved to determine the fertilizing potential of a sample using more sophisticated and rapid technologies, such as flow cytometry. Sperm parameters commonly determined by flow cytometry include viability, reactive oxygen species (ROS), and mitochondrial membrane potential (MMP). However, although the protocols for semen evaluation by flow cytometry are well established for many animal groups, no protocol is available to examine snake semen. Here, we established and tested, for the first time, a flow cytometry protocol to assess the viability, ROS production and MMP of snake semen. For this, we collected semen from captive individuals of *Erythrolamprus miliaris* and tested three fluorochromes to verify different sperm parameters: propidium iodide to assess sperm viability, dihydroethidium to assess ROS production, and tetramethylrhodamine methyl ester perchlorate to assess MMP. Although further experiments are needed, we successfully established protocols and flow cytometry settings for assessing snake sperm by flow cytometry and took the first step towards a more objective and replicable evaluation of snake semen parameters.

Key-words: Semen evaluation. Flow cytometry. Snake sperm. Viability.

5.1 INTRODUCTION

The search for the "viable sperm" goes beyond determining sperm motility; it involves mainly finding motile cells with intact plasma membranes (Graham, 2001; Hossain et al., 2011). In this context, the methodology for evaluating the semen of humans and other animals has evolved to determine the fertilizing potential of a sample using more sophisticated and rapid technologies, such as flow cytometry (Graham, 2001). Flow cytometry can reveal sperm defects, such as DNA damage, lack of acrosomal integrity, and mitochondrial dysfunction (Cordelli et al., 2005). Accordingly, flow cytometry has been successfully used to evaluate a wide range of seminal parameters in several animal groups as fish (Yang, Daly and Tiersch, 2016), bovines (Malama et al., 2017), and humans (Uribe et al., 2014; 2017; Cabrillana et al., 2016).

Several sperm parameters are commonly determined by flow cytometry. Sperm viability can be assessed by staining the sample with propidium iodide (PI), a fluorochrome that stains the nuclei of cells with damaged plasma membranes (Graham, 2001). Reactive oxygen species (ROS) can be assessed by staining the sample with dihydroethidium (DHE), which penetrates the sperm nucleus through membrane (Burnaugh, Sabeur and Ball, 2007). The mitochondrial membrane potential, a parameter that evaluates flagellar movement (Paoli et al., 2011), can be determined by using tetramethylrhodamine methyl ester perchlorate (TMRM). This fluorochrome penetrates spermatozoa and accumulates in the mitochondria being the mean fluorescence intensity proportional to the state of MMP (Uribe et al., 2017). When evaluating sperm by flow cytometry, it is important to combine these fluorochromes with other solutions (positive or negative controls) to validate the results obtained. For example, Triton X-100 is a detergent that disrupts the plasma membrane (Vishwanath, Swan and White 1986) and thus kills the sperm cells, and ionomycin induces the mitochondrial permeability transition (Uribe et al., 2017).

The protocols for semen evaluation by flow cytometry are well established for many animal species such as fish (Yang, Daly and Tiersch, 2016), birds (Partyka, Niżański and Łukaszewicz, 2010), and mammals (Cheuquemán et al., 2013). However, there are no protocols available for performing this analysis on snake semen. The military ground snake, *Erythrolamprus miliaris*, an abundant semi-aquatic species

widely distributed in South America (Marques et al., 2004; Nogueira et al., 2019). The reproductive biology of the species is well known. Females produce multiple clutches throughout the year and exhibit obligatory sperm storage (Pizzatto and Marques, 2006; Rojas, Barros and Almeida-Santos, 2017). In captivity, males produce high semen volume (Tatiane Lima, pers. comm.). Thus, the species is an excellent candidate to be used as a model to stablish and set new protocols in snake.

According to this background, the objective of this work was to established and test a flow cytometry protocol to assess the viability, ROS production and MMP of snake semen.

5.2 MATERIAL AND METHODS

Semen collection and preparation

This study was conducted under the approval of the Ethics Committee for Animal Research of the Butantan Institute (approval number 8381030519). We collected semen from three clinically healthy adult *E. miliaris* kept at the Laboratory of Ecology and Evolution of the Instituto Butantan (São Paulo) for at least one year. Semen collections were conducted in January 2020 using the same collection protocol described for coral snakes (Chapter 1; Coeti et al., in review). Snakes were restrained in a transparent plastic tube. Semen was collected directly from the urogenital papilla using microhematocrit capillary tubes after massaging the ventral portion of the snake towards the cloaca. Because of its high viscosity, the semen was diluted with 200 µl phosphate-buffered saline (PBS) solution. The medium was immediately centrifuged, and the supernatant was discarded. The resulting pellet was used to assess sperm concentration in a Neubauer hemocytometer chamber.

Experimental design and flow cytometry analysis

Semen suspension was diluted in PBS to a final concentration of 2×10^6 sperm/mL in a final volume of 500 µl. The suspension was aliquoted equally into seven tubes. We then tested three fluorochromes to verify different sperm functions. Tube 1

was used as a negative control to check for autofluorescence. Samples from tubes 2 and 3 were treated with propidium iodide (PI; Sigma-Aldrich Inc., St Louis, MO, USA) to assess sperm viability. Samples from tubes 4 and 5 were treated with dihydroethidium (DHE; Molecular Probes, Life Technologies, Carlsbad, USA) to assess reactive oxygen species (ROS) production. Lastly, samples from tubes 6 and 7 were treated with tetramethylrhodamine methyl ester perchlorate (TMRM; Sigma-Aldrich Inc., St Louis, MO, USA) to show the mitochondrial membrane potential. All tests were performed in duplicate because we used a control group for each one. Thus, tube 3 was pre-treated with Triton X-100 before PI, and tubes 5 and 7 were pre-treated with ionomycin before DHE and TMRM, respectively. The experimental design, concentration of each solution, and duration of each part of the experiment are shown in Figure 1. Next, all tubes were washed by centrifugation at 6,400 rpm for 2 min in a minicentrifuge (Kasvi, K14-0602, São José do Pinhais, PR, Brazil), and each sperm pellet was resuspended in 300 µl PBS medium. Fluorescence analysis was performed on a FACS Canto II flow cytometer (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA) using a 585/42 nm band pass filter and data from at least 10,000 sperm events were recorded. Samples were acquired and analyzed with the software FACSDiva^{TM} version 8.0.2 (Becton, Dickinson and Company).

5.3 RESULTS

The autofluorescence of the sperm of *E. miliaris* was similar in all flow cytometer filters used (figure 1), which means that the reading of the dyes in the cells by the flow cytometer is assured. Flow cytometry showed that the dead sperm changed their morphology. This finding was supported by the PI control with Triton X-100 (Figure 3D-F) since the events stained with PI were found in a different quadrant (Figure 3A-C). Moreover, the percentage of events stained with PI control was higher than that stained with PI only (Table 1). Therefore, PI stains only non-viable sperm. DHE stained more events in the control group, and ionomycin seemed to improve ROS production in the sperm cells, except in individual 3 (Table 1). TMRM stained fewer events in the control group, which means that ionomycin decreases the mitochondrial membrane potential of the cells (Table 1).

5.4 DISCUSSION

Autofluorescence is an intrinsic and natural fluorescence of cells with multispectral detection ranging from blue to red (Chorvat and Chorvatova, 2008). The variation in cell autofluorescence can be a problem in cytometry because it makes it difficult to recognize between stained and unstained cells (Alberti, Parks and Herzenberg, 1987). Thus, checking for autofluorescence is necessary before applying a flow cytometry protocol to a new cell type. Our results show that autofluorescence is not a problem to assess snake sperm.

We tested various concentrations of PI, DHE, and TMRM, including the control groups with Triton X-100 and ionomycin, based on successful protocols for the same parameters using human sperm (Uribe et al., 2014, 2017, 2018). However, we found that snake sperm is less resistant to Triton X-100 and ionomycin than human sperm, probably because the head of snake sperm is slender (Hamilton and Fawcett, 1968), which may indicate a less complex nucleus. Thus, a short exposure period in a small amount of Triton X-100 caused cell death and morphological changes in the sperm of *E. miliaris*, presumably because of damage to the plasma membranes (Vishwanath, Swan and White 1986), as successfully detected by PI staining in flow cytometry. The duration of exposure and the concentration of ionomycin were monitored and gradually adjusted until they caused only the membrane permeabilization and did not kill the cells, making it possible to evaluate the DHE and TMRM stains for snake sperm.

DHE stained events according to ROS production in individuals 1 and 2, but not in individual 3. The percentage of events increased in the control group with ionomycin, showing that DHE is a suitable indicator for snake sperm, as observed in equines (Burnaugh, Sabeur and Ball, 2007). The DHE staining results for individual 3 were probably wrong due to some methodological mistake and are therefore uninformative.

TMRM detected the mitochondrial membrane potential, and the control group with ionomycin showed fewer events in snake sperm, as observed in human sperm (Uribe et al., 2017). However, the percentage of events measured in snake sperm was

very low compared to human sperm. The snake sperm has more mitochondria than human sperm, probably because of its longer midpiece (Tourment et al., 2009). Thus, we expected more events of mitochondrial membrane potential. A possible explanation for this result is that the time and/or concentration of exposure to TMRM is below the necessary to detect the correct number of events.

We established protocols for the evaluation of snake sperm by flow cytometry for the first time. Although further experiments are needed, this is the first step towards a more objective and replicable evaluation of the seminal parameters of snakes. Once these protocols are established, our future goals are to test better dilutions and diluents for the semen and seasonal variation in the sperm viability of captive snakes.

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5.6 TABLES

Table 1

L. miliaris	**Live cells**	**Pi**	**Pi + triton**	**DHE**	**DHE + Io**	**TMRM**	**TMRM + Io**
1	88.7	1.9	21.2	5.3	41.1	1.8	1.2
2	97.3	1.0	39.9	14.4	40.2	3.3	2.2
3	99.6	3.9	11.9	93.6	92.0	1.5	0.7

Table 4. Evaluation of the semen samples of three individuals of the military ground snake, *Erythrolamprus miliaris*. All data are shown as percentages of live cells. PI = propidium iodide; DHE = dihydroethidium; Io = ionomycin; TMRM = tetramethylrhodamine methyl ester perchlorate.

5.7 FIGURES

Figure 1

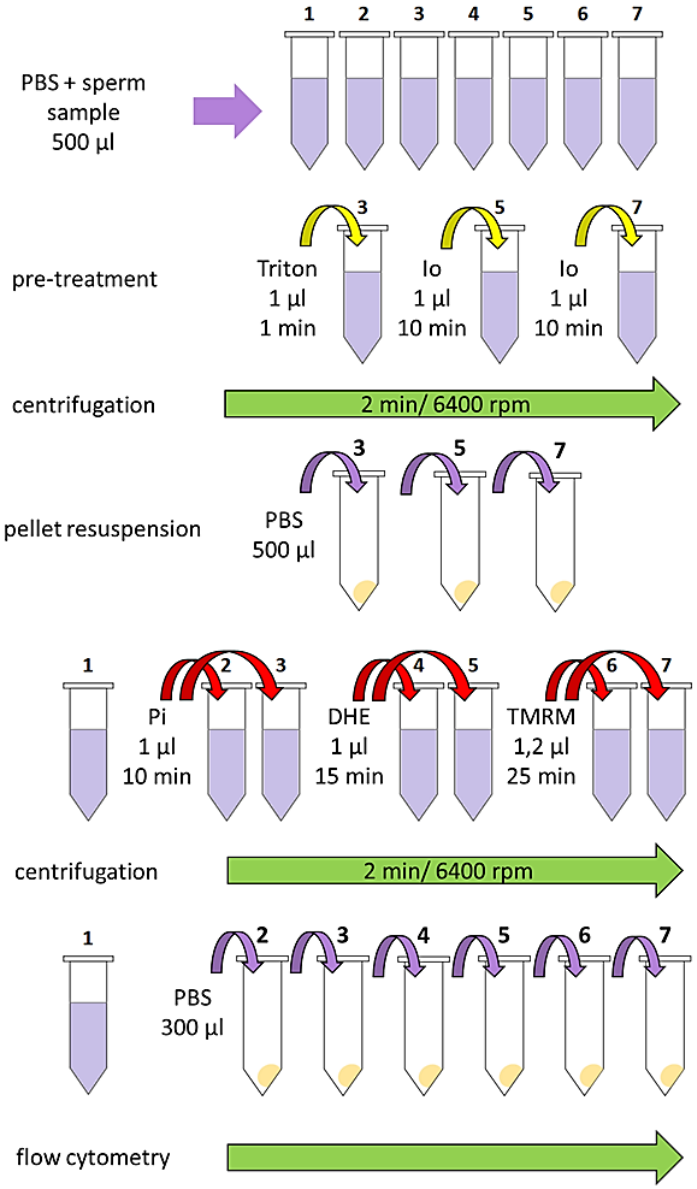

Figure 2. Experimental design for evaluating the semen of the military ground snake, *Erythrolamprus miliaris*. The semen suspension (volume = 500 µl) was aliquoted equally into seven tubes. Positive control groups (tubes 3, 5, and 7) were pre-treated with 1 µl Triton X-100 for 1 minute (tube 3) and 1 µl ionomycin (Io) for 10 minutes (tubes 5 and 7). The tubes were then centrifuged for 2 minutes at 6400 rpm, and the resulting pellet was resuspended in 500 µl PBS. Next, the fluorochromes were added in a time-ordered sequence as follows: tubes 6 and 7 were incubated with 1,2 µl of TMRM for

25 minutes; tubes 4 and 5 were incubated with 1 µl of DHE for 15 minutes; and tubes 2 and 3 were added 1 µl of PI for 10 minutes. All tubes were then incubated in the dark at 37 ºC. After incubation, tubes 2 to 7 were centrifuged for 2 minutes at 6400 rpm, and the resulting pellet was resuspended in 300 µl PBS solution before flow cytometry analysis.

Figure 2

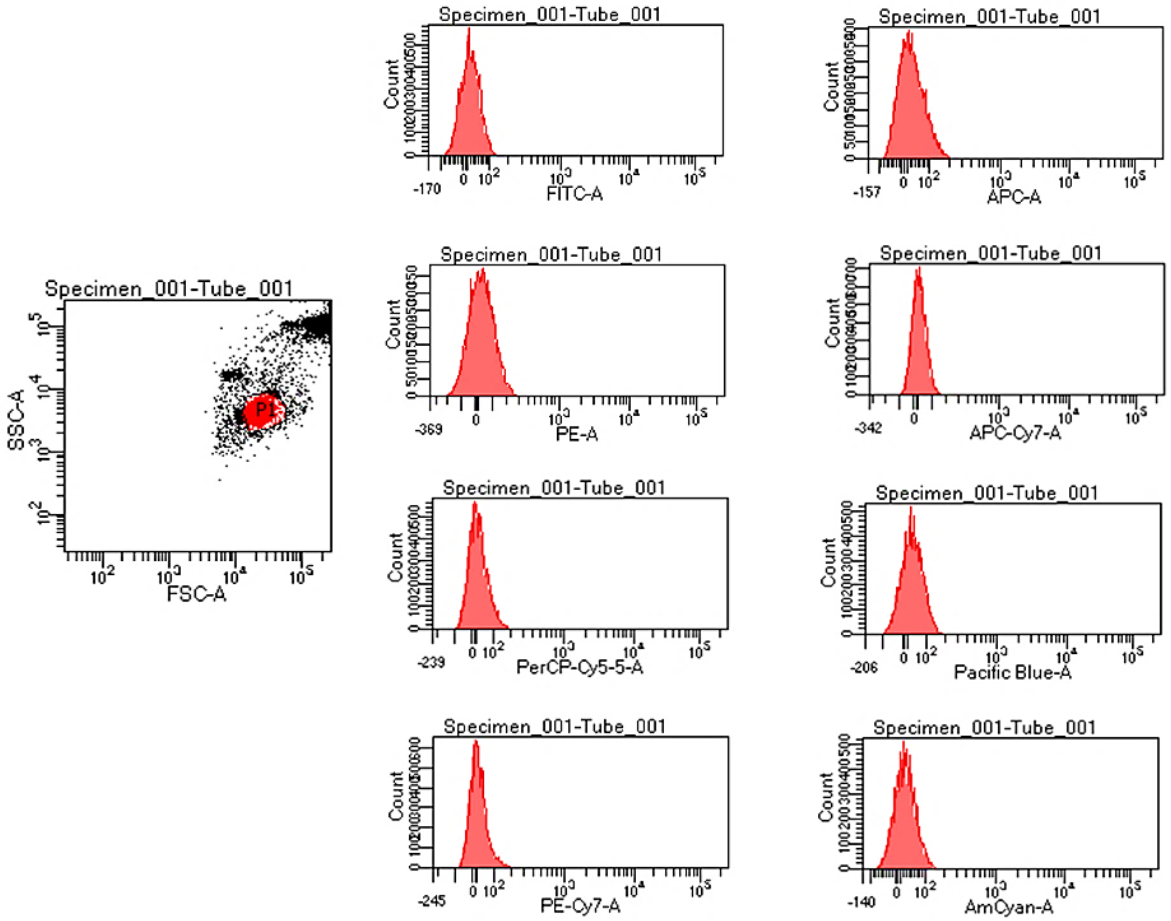

Figure 3. Autofluorescence of the sperm of the military ground snake, *Erythrolamprus miliaris*. All tested flow cytometer filters produced the same result, demonstrating that the autofluorescence in snake sperm is the same at different emissions. FSC = forward scatter, SSC = side scatter, PE = red-orange fluorophore, FITC = green fluorophore, PE-Cy5 = orange fluorophore, PE-Cy7 = red fluorophore, APC = far-red fluorophore, Pacific blue = violet fluorophore, AmCyan = green fluorophore.

Figure 3

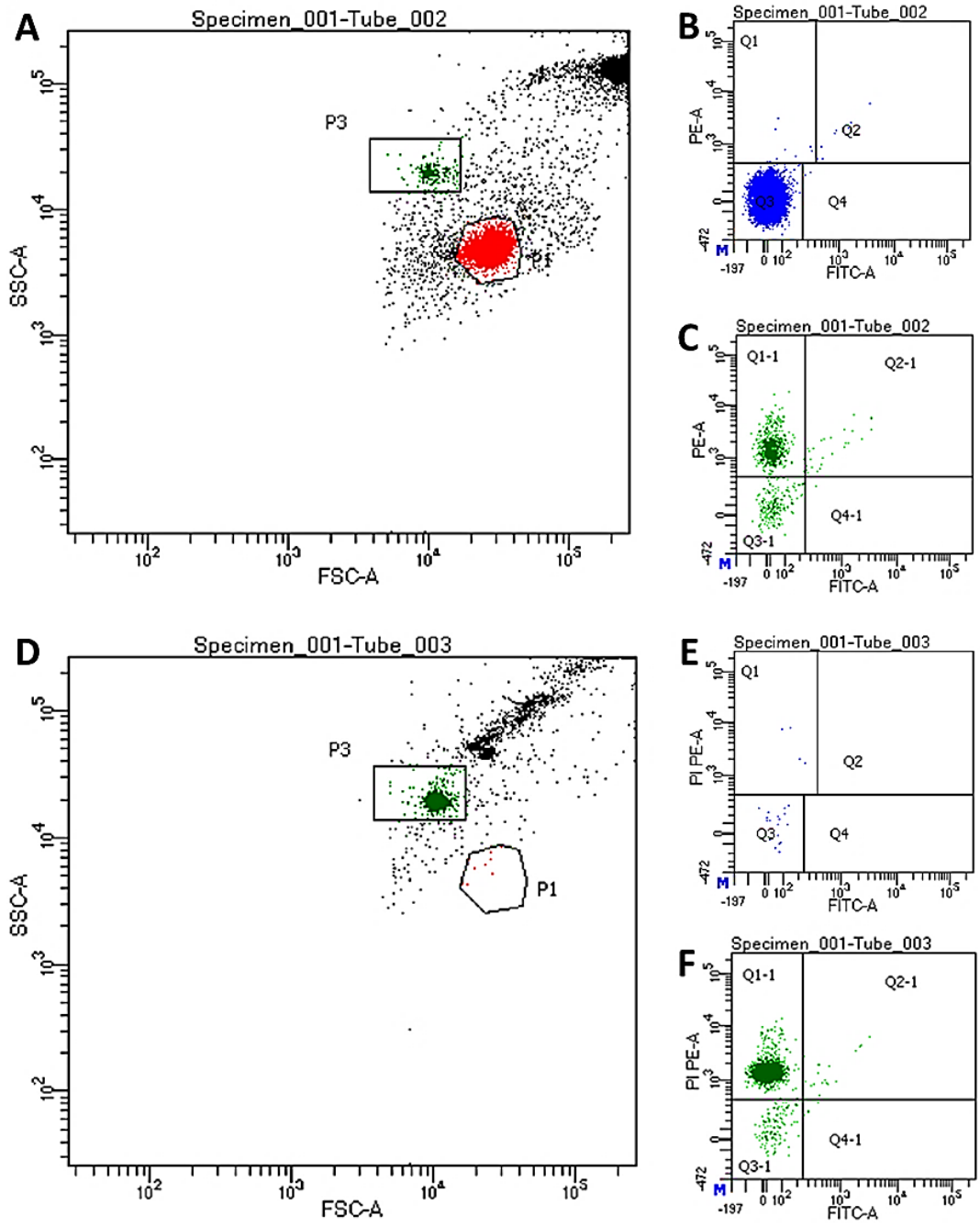

Figure 4. Dot plot of the events stained with PI and PI+Triton X-100 (control). A) Events of viable (red, P1) and non-viable (green, P3) sperm from tube 2 (without Triton X-100). B) Population of live cells (blue) in Q3 (without PI. C) Population of non-viable cells (green) in Q1-1 stained with PI. D) Events of viable (red, P1) and non-viable (green, P3) sperm from tube 3 showing the noticeable effects of Triton X-100 in

decreasing the red population. E) Decrease in the cell population considered alive in blue (Q3). F) Increase in the population of dead sperm (stained with PI) in green (Q1-1). FSC = forward scatter, SSC = side scatter, PE = Phycoerythrin, FITC = green fluorophore.

6 CONCLUSION

The semen collection protocol established in this work was effective both *Micrurus* and *Erythrolamprus miliaris* sperm quality evaluation, however in larger species like Micrurus frontalis the collection success rate decreases. Thus, it is a good technique when is needed small semen volumes and for small and slender snakes.

Snake sperm evaluation can be done with protocols for humans and mammals, but its necessary caution mainly with osmolarity and spermatozoa head permeability.

Morphological measurements of snake spermatozoon have differences when done at optical and electronic microscopy, so it is more reliable to measure cell size from scanning microscopy.

The snake spermatozoa go a long way within the reproductive cycle and they ultrastructural characteristics evolved to support storage periods inside male and female reproductive tract. The multilaminar membranes and cytoplasmic droplets described here, for the first time, to *Micrurus* spermatozoa are related with the sperm storage strategy presents in these genera.

We finally conclude that mature snake sperm are physiologically and morphologically prepared to storage and the particularities of these cell are largely what make this process possible.

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