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Different staining techniques evaluation for the study of sperm morphology and morphometry in bats (Mammalia: Chiroptera)

Evaluación de diferentes técnicas de tinción para el estudio de la morfología y la morfometría de los espermatozoides en murciélagos (Mammalia: Chiroptera)

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ABSTRACT

The study of sperm morphology involves the use of various staining techniques that allow visualization of different structures of the spermatozoon and their variability. Each technique varies depending on the dyes used and the necessary steps for execution. The aim of this work was to evaluate different staining techniques for sperm morphology and morphometry analysis in bats. Samples were collected from the epididymis of 57 adult specimens, which were macerated in Farmer's solution and used to prepare smears. The smears were stained with Toluidine Blue, Giemsa, May Grünwald-Giemsa, Gram stain, Hematoxylin-Eosin, DAPI (4',6-diamidino-2-phenylindole), Basic Fuchsin, and Janus Green. Criteria for comparing the techniques were complexity, time required, and associated costs., Microphotographs were taken using light and epifluorescence microscopy for morphometric analysis, and 50 spermatozoa were measured per individual. Spermatozoa were obtained from 16 specimens: *Artibeus planirostris* (n=5), *Sturnira erythromos* (n=3), *Molossus molos-*

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sus (n=4), *Molossops temminckii* (n=2), *Histiotus laephotis* (n=1), and *Myotis albescens* (n=1). Among the evaluated techniques, Toluidine Blue proved to be a quick, simple, and cost-effective method in order to determine the presence of spermatozoa and describe their general morphology. Dicromatic stains like Hematoxylin-Eosin and May Grünwald-Giemsa were more efficient in differentiating the nucleus and acrosome, although they were also more complex and costly to perform. In conclusion, we recommend the use of the aforementioned three techniques as optimal choices for initial studies of sperm morphology in bats.

Keywords — Spermatozoa, Staining and Labeling, Histocytological Preparation Techniques, Microscopy.

RESUMEN

El estudio de la morfología espermática implica el empleo de diversas técnicas de tinción que permiten la visualización de las diferentes estructuras del espermatozoide y su variabilidad. Cada técnica varía en función de los colorantes empleados y los pasos necesarios para su ejecución. El objetivo de este trabajo fue evaluar distintas técnicas de tinción para el análisis de la morfología y la morfometría espermática en murciélagos. Se recolectaron muestras de epidídimo de 57 ejemplares adultos, que fueron maceradas en solución de Farmer y utilizadas para preparar frotis. Los extendidos fueron coloreados con Azul de Toluidina, Giemsa, May Grünwald-Giemsa, GRAM, Hematoxilina-Eosina, DAPI (4',6-diamino-2-fenilindol), Fucsina Básica y Verde Jano. Los criterios para la comparación de las técnicas fueron: complejidad, tiempo requerido y costos asociados. Para el análisis morfométrico se tomaron microfotografías al microscopio óptico y de epifluorescencia y se midieron 50 espermatozoides por individuo. Solo se obtuvieron espermatozoides de 16 especímenes: *Artibeus planirostris* (n=5), *Sturnira erythromos* (n=3), *Molossus molossus* (n=4), *Molossops temminckii* (n=2), *Histiotus laephotis* (n=1) y *Myotis albescens* (n=1). Entre las técnicas evaluadas, el Azul de Toluidina demostró ser una técnica rápida, sencilla y de bajo costo para determinar la presencia de espermatozoides y describir su morfología general. Las tinciones dicrómicas como Hematoxilina-Eosina y May Grünwald-Giemsa fueron más eficientes en la diferenciación del núcleo y el acrosoma, aunque también resultaron más complejas y costosas en su realización. En conclusión, recomendamos el uso de las tres técnicas mencionadas anteriormente como las opciones óptimas para los estudios iniciales de morfología espermática en murciélagos.

Palabras clave — Espermatozoides, Tinción y Marcado, Técnicas de Preparación Histocitológica, Microscopía.

INTRODUCTION

In mammals, sperm morphology serves as a species-specific trait that offers insights into both the reproductive biology of species and their phylogenetic relationships (Álvarez-Guerrero, Medrano, Moreno-Mendoza, 2014). Additionally, sperm morphology serves as a reliable indicator of seminal quality, enabling the detection of anomalies that can compromise reproductive success and lead to male infertility. Currently, the majority of studies on this topic have focused on humans for medical applications (World Health Organization (WHO), 2010) and on economically important farm animals, including bulls, pigs, and rabbits (Gómez, 2011).

In medium and large mammals, the study of sperm morphology is carried out on fresh semen samples, which are evaluated according to strict criteria (Toro-Montoya, 2009). In the case of small wild mammals, the volume of ejaculate is scarce and difficult to obtain, so samples obtained from previously fixed epididymis macerates are used (Thitipramote, Suwanjarat, Leigh, Breed, 2011). Furthermore, rodents murine and bats, a protocol for taking sperm samples has not yet been standardized.

Many factors, both physical and chemical, influence the normal shape and size of sperm (Yániz, Soler, Santolaria, 2015). Therefore, it must be taken into account that each experimental step does not generate artifacts or modify the dimensions of the spermatozoon. In other words, in order to carry out an accurate evaluation of sperm morphology, it is necessary to choose appropriate techniques for sperm preparation, fixation and staining (García-Herreros, Aparicio, Barón, García-Marín, Gil, 2016).

Staining techniques make possible to differentiate the regions of the spermatozoon, describe its general morphology and identify the presence of ornamentation as well as possible anomalies in the external morphology. A wide range of stains in order to study sperm morphology can be employed (Fig. 1a y 1b) (Nieto Dionisio, 2010). Among the most reported are: Periodic Acid-Schiff (PAS), Toluidine Blue, DAPI (4′,6-diamidino-2-phenylindole), Diff-Quick, Giemsa, Hematoxylin-Eosin, Ferric Hematoxylin, Eosin-Nigrosin, May Grünwald-Giemsa, Orange G, Papanicolaou, Shorr and Janus Green (Forman and Genoways, 1979; Forman, Smith, Hood, 1989; Ding, Leigh, Goodman, Bedford, Carleton, Breed, 2010; Aksoy, Aktan, Duman, Cuce, 2012; Morais, de Paula, de Freitas, da Matta., 2012; Álvarez-Guerrero et al., 2014). Each technique varies in complexity and cost, involving different dyes, reagents, and a number of steps (Aksoy et al., 2012). The choice depends both on the objective of the study and on the animal group with which you are going to work.

Over the past decade, numerous studies have been published on the ultrastructure of bat spermatozoa using electron microscopy (Beguelini, Puga, Taboga, Morielle-Versute, 2011, Beguelini, Taboga, Morielle-Versute, 2012, 2017; Bueno, Beguelini, Comelis, Taboga, Morielle-Versute, 2014). However, the general morphology of spermatozoa has been studied and described in only a few species. Therefore, the aim of this study was to evaluate eight different staining techniques for the analysis of sperm morphology in bats and to compare their impact on morphometric variables.

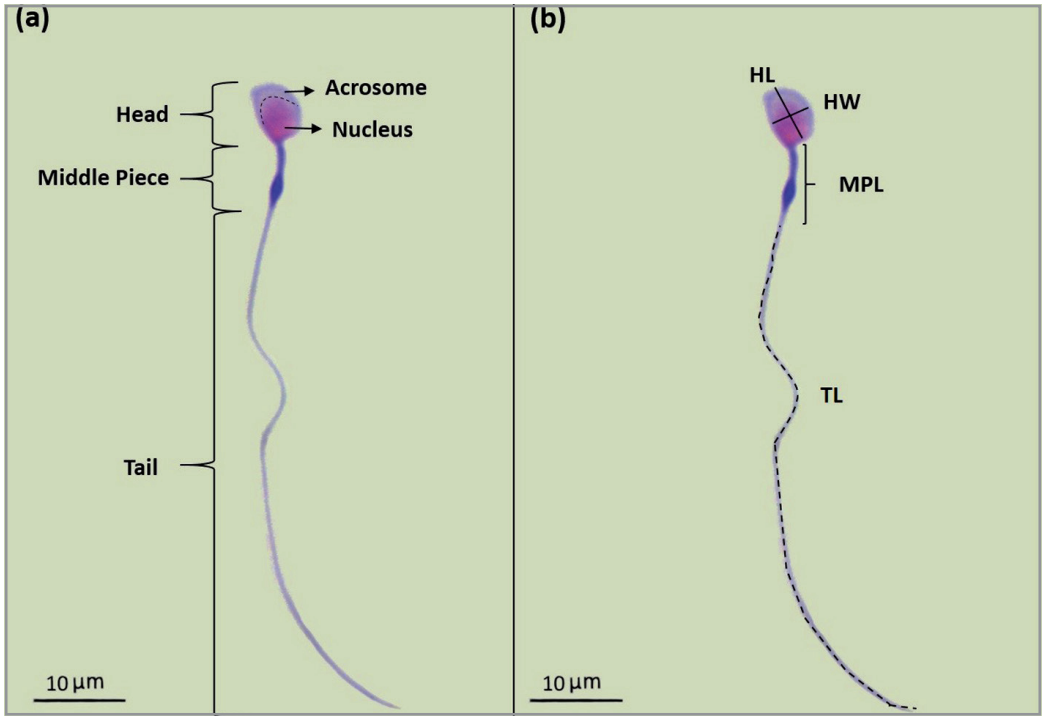

Fig. 1. Sperm morphology and morphometric variables. (a) Part of the spermatozoa, (b) morphometric variables measured in this study: head length (HL), head width (HW), middle piece length (MPL) and tail length (TL).

MATERIALS AND METHODS

This study was carried out with the recommendations of the Guide for the use of animals in field research and in captivity, established by the Ethics Commission of the Sociedad Argentina para el Estudio de los Mamíferos (SAREM). The research protocol and animal collecting permits were approved by the Dirección de Flora, Fauna Silvestre y Suelos de la Provincia de Tucumán (DFFSyS), Argentina (File Number: 386-330-2021; Resolution Number: 108-2021 DFFSyS). Euthanasia was performed using the physical methods recommended by the American Veterinary Medical Association (AVMA) and the American Society of Mammalogists (ASM) to minimize animal suffering (Underwood and Anthony, 2020).

Specimens Examined

The specimens utilized in this study predominantly originate from the Colección Mamíferos Lillo (CML) at the Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán (UNT). While 11 specimens are housed within the CML, they have yet to be cataloged in the database, thus retaining the numbering system and initials of their original collectors (MDM: María Daniela Miotti; MEM: María Eugenia Montani; MO: Mirna Oviedo). Additionally, two specimens were sourced from the Colección de Mamíferos at the Museo de La Plata (MLP), Buenos Aires, and two from the Museo Provincial de Ciencias Naturales

Florentino Ameghino (MFA), Santa Fe, Argentina. All individuals were initially fixed in 4% formaldehyde and subsequently preserved in 70% ethanol.

Fifty-six adult males belonging to 16 species and 3 different families of bats were selected: Phyllostomidae: *Artibeus planirostris* (n=6): CML 8574, 8575, 8582, 8584, 8609, 8653; *Sturnira erythromos* (n=6): CML 8697, 8703, 8704, 8705, 10306, 10909; Molossidae: *Eumops bonariensis* (n=2): CML 10998, 12003; *Molossops temminckii* (n=9): CML 2533, 2534, 3703, 3708, 3712, 5736, 10999, 12015, 12016; *Molossus molossus* (n=5): CML 8840, 10750, 10762, 11003 and MLP 2136; *Tadarida brasiliensis* (n=7): CML 8756, 8759, 11009, 11938, MDM 848, 849, MO 871; Vespertilionidae: *Dasypterus ega* (n=1): MEM 235; *Eptesicus diminutus* (n=1): MFA 1461, *Eptesicus furinalis* (n=2): MLP 2127, MEM 238; *Histiotus laephotis* (n=5): CML 8847, 8850, 8851, 10672, 10833; *Lasiurus blossevillii* (n=1): CML 10685; *Lasiurus villosissimus* (n=4): CML 8560, 10979, 11922, MO 872; *Myotis albescens* (n=1): CML 11923; *Myotis dinellii* (n=2): CML 10842, 10984; *Myotis nigricans* (n=3): MEM 250, 251, MFA 1425; *Myotis riparius* (n=1): CML 10848. Each male underwent an orchiectomy and the gonads were preserved in 70% ethyl alcohol.

Sperm Staining

The epididymis was separated and macerated in Farmer's solution (3:1 absolute ethyl alcohol – glacial acetic acid) (Barth and Oko, 1989; Thitipramote et al., 2011). Smears were made using a combination of drop and air dry techniques. Since spermatozoa are translucent and invisible under an optical microscope, the preparations were colored with different stains: Toluidine Blue (TB) (Bancroft and Gamble, 2008); Giemsa (G) (Vitullo, Roldan, Merani, 1988); May Grünwald-Giemsa (MG-G) (Solis, 1996); GRAM stain (Gr) (Toro-Montoya, 2009); Hematoxylin-Eosin (HE) (McManus and Mowry, 1968); DAPI (Ding et al., 2010); Basic Fuchsin (BF) and Janus Green (JG) (McManus and Mowry, 1968).

In selecting the staining techniques, several factors were taken into consideration. Firstly, we prioritized stains commonly employed in sperm morphology studies, including Toluidine Blue, Hematoxylin-Eosin, DAPI, and Janus Green (Aksoy et al., 2012). Secondly, we explored other coloration methods frequently used in cytology, albeit not specifically tailored for spermatozoa, such as GRAM, Giemsa, and May Grünwald-Giemsa staining. Each technique underwent refinement through experimentation with various staining time sequences, dye concentrations, and modifications to standard protocols outlined in existing literature. For instance, pretreatment with acid hydrolysis was employed for Basic Fuchsin staining, and baths in citrate buffer solutions were incorporated for the DAPI technique.

Four criteria were established so as to compare the techniques: the total time required for each stain, the number of steps involved, the associated costs, and the efficacy of visualizing spermatozoa regions. A comprehensive assessment was conducted considering the advantages and disadvantages of each technique based on these criteria.

Sperm morphology (Fig. 1a) was evaluated through a Zeiss AxioLab optical microscope and the preparations were photographed with an AxioCam ERc5s digital camera. Additionally, an Olympus BX3 Clinical Microscope with a DP 21 digital camera was used to visualize the smears stained with DAPI. Photomicrographs were captured at 100X magnification using oil immersion.

Sperm Morphometric Analysis

For sperm morphometry analysis, 50 spermatozoa per individual were measured using the Zen 2 Blue edition (2014) software. The measurements included head length (HL), head width (HW), middle piece length (MPL), tail length (T), and total length (TL) of each spermatozoon (Fig. 1b). Subsequently, a matrix was constructed in order to facilitate statistical analyses.

Statistic Analysis

To assess the impact of staining techniques on sperm morphometry, mean and standard deviation (±SD) were calculated for each variable per species. The data obtained from the Toluidine Blue (TB) and Hematoxylin-Eosin (H-E) staining methods were compared. A table summarizing the data was constructed, and statistical significance was determined using a T-test. All statistical analyses were conducted using the freely available InfoStat program, version 2008 (Di Rienzo, Casanoves, Balzarini, González, Tablada, Robledo, 2008).

RESULTS

From the 56 specimens studied, spermatozoa were recovered only in the epididymis of 16 individuals belonging to six species (Table 1). In the remaining 10 species, spermatozoa were not obtained in the macerates.

The staining times, the concentrations of the dyes, the application of pretreatments and the results observed in each of the cases are presented in Table 2.

Table 1. Specimens with mature spermatozoa in their epididymis. N: number of individuals per species, N° CML: collection number of the Colección Mamíferos Lillo (CML).

Family	Species	*N*	N° CML
Phyllostomidae	*Artibeus planirostris*	5	8574, 8575, 8582, 8584, 8653
	Sturnira erythromos	3	8703, 10306, 10909
Molossidae	*Molossops temminckii*	2	3708, 12016
	Molossus molossus	4	8840, 10750, 10762, 11003
Vespertilionidae	*Histiotus laephotis*	1	10833
	Myotis albescens	1	11923
Total		**16**	

Table 2. Optimization of staining techniques used for studies of sperm morphology in bats. Modifications made with respect to time, dilutions and/or pretreatments. The time was expressed in minutes (’). Hematoxylin (H), Eosin (E).

Staining	Modification	Results
Toluidine Blue (Bancroft y Gamble, 2008)	Staining time: a) 10’ b) 20’	Similar results were obtained with both times.
Giemsa (Vitullo et al., 1988)	Staining time: a) 3’ b) 5’ c) 10’ Colorant concentration: a) Pure b) Diluted (1:10)	The staining time of 10 minutes showed better results. Pure Giemsa was chosen for use, as the diluted Giemsa was slightly colored.
May Grünwald – Giemsa (Solís, 1996)	Colorant concentration: a) Pure Giemsa b) Diluted Giemsa (1:10)	Pure Giemsa was chosen for use, as the diluted Giemsa was slightly colored.
GRAM (Toro-Montoya, 2009)	Colorant concentration: a) Pure Lugol b) Diluted Lugol (1:10)	Better results were obtained using diluted lugol 1:10.
Hematoxylin-Eosin (Mc Manus y Mowry 1968)	Staining time: a) 4’ H, 4’ agua y 1’ E; b) 4’ H, 4’ agua y 1,5’ E; c) 4’ H, 4’ agua y 2’ E; d) 3’ H, 3’ agua y 1,5’ E	Better results were achieved by coloring with combination b.
DAPI (Ding et al., 2010)	Staining time: a) 5’ b) 10’	Better results were obtained with the 5-minute protocol, since leaving it for longer resulted in overstaining of the preparations.
Basic Fuchsin (McManus y Mowry, 1968)	Pretreatment: a) with acid hydrolysis (hydrochloric acid): b) without acid hydrolysis	Identical results were obtained with and without acid hydrolysis, so we decided to use the dye without pretreatment.
Janus Green (McManus y Mowry, 1968)	Staining time: a) 20’ b) 30’	The same results were obtained at both times. We continue using 20 minutes.

The comparison between the different techniques in terms of the total time taken by each one, the number of steps involved in each protocol and the total cost of reagents and dyes is presented in Table 3. The technique with the highest number of steps was considered to be more complex. More steps, takes more overall time, and is more expensive. An error of 2 minutes was added to each technique, except for DAPI staining, in which 5 minutes were added based on its complexity.

Regarding the effectiveness of each technique in selectively staining various regions of the spermatozoon, we observed that:

Toluidine Blue (Fig. 2a) was ideal for determining the presence or absence of spermatozoa in the preparations. As it is a monochromatic stain, the spermatozoa were completely colored blue: intensely, the nuclear region and the middle piece, and more lightly, the acrosome and the flagellum. It provides a good contrast for morphometric analysis.

The Giemsa stain (Fig. 2b) uniformly colored the head of the spermatozoa in blue-violet, preventing the differentiation between the nucleus and the acrosome. Occasionally, the flagella were not stained. However, when May Grünwald stain

Table 3. Assessment of staining techniques. Time is expressed in minutes (') and costs are in US dollars (U$S). **In the case of Janus Green, no costs are reported since the dye is not currently marketed.

Stains	Time	Number of steps	Costs
TB	12'	2	35
G	7'	2	26
MG-G	22'	4	45,6
Gr	4'	7	28,4
HE	13'	6	61,1
DAPI	100'	20	96,80
JG	22'	2	**
BF	22'	4 o 6 +	66,6

was applied as a contrast (Fig. 2c), it was possible to distinguish the main regions in different shades: the nucleus was colored blue, the acrosome pink, the middle piece deep purple, and the tail light violet.

GRAM (Fig. 2d) allowed to distinguish two areas in the cephalic region: the nucleus in light blue (region related to crystal violet) and the acrosome in pale pink (related to safranin). However, it was not able to differentiate the limits between middle piece and the flagellum.

Hematoxylin–Eosin (Fig. 2e) stained the sperm structures differentially. Hematoxylin stained the nucleus violet-blue (basophilic), while Eosin stained the cephalic apex pink (acidophilic). The middle piece acquired an intense violet color and the tail a light purple. This staining provided a good contrast for morphometric analysis of the spermatozoa.

DAPI staining (Fig. 2f) uniformly colored the entire sperm, exhibiting a stronger affinity for the nuclear region. This dye produces a blue fluorescence by binding to the adenine and thymine-rich regions of the genetic material (DNA). While the coloration is visually striking, it did not always facilitate the differentiation of the head regions (nucleus and acrosome).

Janus Green staining (Fig. 2g) uniformly colored the spermatozoa in blue-green. This technique exhibited a more intense staining of the middle piece owing to its specificity for mitochondria. Remarkably, it was the only method that strongly stained the acrosome while weakly staining the nucleus.

Basic fuchsin (Fig. 2h) similarly colored the nucleus and the middle piece in an intense magenta tone, due to the presence of nucleic acids (DNA and RNA) in both regions; while the acrosome and tail were stained pale pink.

In order to confirm that the techniques used do not produce artifacts or alterations in the morphometry of the sperm studied, measurements of the different sperm regions with two different techniques were taken. Hematoxylin-Eosin staining presented higher values than Toluidine Blue for almost all morphometric variables. In exceptional cases the values with Toluidine Blue were slightly higher, but they were within the standard deviation. Only in *Molossus molossus* the variables "head length", "head width" and "middle piece length" were significantly different and in *Artibeus planirostris* and *Myotis albescens was* for "head width" ($P<0.001$).

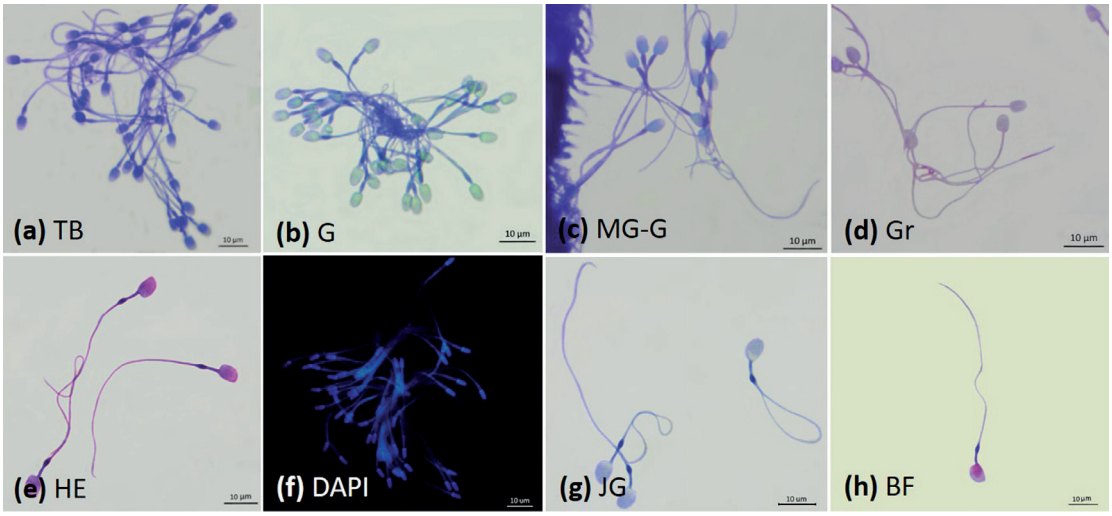

Fig. 2. Microscopic images of spermatozoa stained with (a) Toluidine Blue (TB), (b) Giemsa (G), (c) May Grünwald-Giemsa (MG-G), (d) GRAM (Gr), (e) Hematoxylin-Eosin (HE), (f) DAPI, (g) Janus Green and (JG) (h) Basic Fuchsin (BF), b=10 μm.

DISCUSSION

Several physical and chemical factors significantly influence the normal shape and size of sperm, which must be considered when studying sperm morphology. In medium and large mammals, the analysis typically starts with extracting a semen sample (Toro-Montoya, 2009). However, for bats, obtaining seminal fluid samples necessitates electroejaculation (Fasel, Helfenstein, Buff, Richner, 2014), a complex and expensive technique that poses risks such as internal burns and is impractical for smaller bats like those from the *Myotis* genus (weighing only 5 grams).

In the present study, the maceration technique to obtain sperm was employed following Thitipramote et al. (2011), who successfully applied it in studies of sperm morphology in murine rodents. Nevertheless, this method had limitations. Mechanical pressure during the process led to flagellar separation, tail cuts, and even decapitated sperm, rendering it impossible to measure variables like tail length and total length accurately.

Chemical fixation is another crucial factor that can distort sperm morphology (Ariagno and Mormandi, 2016). Formaldehyde is commonly used in histology due to its excellent penetration and cost-effectiveness (Meschede, Keck, Zander, Cooper, Yeung, Nieschlag., 1993; Mortimer and Menkeld, 2001; García-Herreros et al., 2006). A previous study by Hirth (1960) found no significant differences between formalin-fixed samples and fresh semen samples concerning sperm shape and size. Even though we used previously fixed material with 4% formaldehyde in our study, a notable advantage was that it preserved sperm morphology and facilitated cellular component staining. However, it also caused tissue hardening, contributing to subsequent sperm rupture.

The selection of staining techniques significantly influences the visualization of sperm under a microscope. While numerous studies in the literature compare various staining methods in humans (Van der Horst and Maree, 2009; Nieto Dionisio, 2010)

Table 4. Comparison of the influence of two techniques on morphometric variables: Toluidine Blue (TB) and Hematoxylin-Eosin (HE): head length (HL); head width (HW); middle piece length (MPL); tail length (LT); M(TB): means Toluidine Blue; M(HE): means Hematoxylin-Eosin; M(TB-HE): means Toluidine Blue- Hematoxylin-Eosin; THV: test for homogeneity of variances; *T*: Student's T-test value; p: p value; test: being in this case of bilateral type (P<0.001).

Species	Variable	M(TB)	M(HE)	M(TB-HE)	THV	*T*	*P*
Artibeus planirostris	HL	4,38	4,49	-0,11	0,1464	-4,02	**0,0001**
	HW	3,14	3,23	-0,09	0,0003	-5,01	<0,0001
	MPL	9,09	9,18	-0,09	0,5842	-3,28	0,0012
	LT	60,19	60,75	-0,56	0,0451	-1,20	0,2376
Sturnira erythromos	HL	4,82	4,87	-0,05	0,5345	-2,04	0,0430
	HW	3,23	3,24	-0,01	0,5821	-0,36	0,7181
	MPL	9,43	9,51	-0,08	0,8923	-2,28	0,0240
	LT	48,18	48,32	-0,14	0,6022	-0,55	0,5824
Molossus molossus	HL	6,38	6,54	-0,16	0,0010	-4,25	<0,0001
	HW	4,64	4,81	-0,17	0,0056	-4,92	<0,0001
	MPL	6,58	6,78	-0,20	0,1783	-4,61	<0,0001
	LT	58,21	58,58	-0,37	0,5299	-1,06	0,2888
Molossops temminckii	HL	6,54	6,5	0,04	0,8658	0,67	0,5029
	HW	4,78	4,88	-0,11	0,5233	-2,10	0,0385
	MPL	6,79	6,91	-0,11	0,1874	-1,83	0,0701
	LT	57,53	58,46	-0,93	0,3850	-0,69	0,4990
Histiotus laephotis	HL	5,88	5,99	-0,10	0,0234	-3,38	0,0016
	HW	2,31	2,37	-0,06	0,4698	-2,20	0,0325
	MPL	8,98	8,97	0,01	0,6162	0,37	0,7156
	LT	65,93	65,81	0,13	0,8806	0,44	0,6598
Myotis albescens	HL	5,15	5,25	-0,10	0,1298	-3,46	0,0011
	HW	1,77	1,86	-0,09	0,8039	-4,58	<0,0001
	MPL	12,78	12,74	0,04	0,4018	0,85	0,3981
	LT	45,37	45,73	-0,36	0,0667	0,88	0,3875

and domestic as well as wild animals (Beletti and Mello, 2004; Beletti, da Fontoura Costa, Guardieiro, 2005; Soler, Gadea, Soler, Fernández-Santos, Esteso, Núñez, Moreira, Núñez, Gutierrez, Sancho, Garde, 2005; García-Herreros et al., 2006; Xu, Jin-Chun, Tang, 2022), there is a lack of comparable research focused on bats.

In our study, Toluidine Blue was a simple and fast technique to corroborate the presence or absence of sperm in the preparations and to describe the morphology of each region. Consistent with findings by Aksoy et al. (2012), it provided excellent contrast for morphometric evaluation. In contrast, Giemsa was the most cost-effective and rapid stain; however, it often failed to distinguish the various regions of the spermatozoa, occasionally leaving the flagella unstained. This observation aligns with the conclusions of Nieto Dionisio (2010) regarding this staining method. When used in combination with GRAM staining, Giemsa provided the least informative results.

Dichromic stains such as May Grünwald-Giemsa and Hematoxylin-Eosin entailed greater complexity and cost. However, when Gram was incorporated with May Grünwald as a counterstain, the differentiation of nucleus and acrosome was dramatically improved. Therefore, we suggest the May Grünwald-Giemsa stain as a good technique that has not been used before to study sperm morphology in bats.

The Hematoxylin-Eosin is a routine technique in histology that plays a critical role in the diagnosis and investigation of human tissues and organs. In the present work on bats, it allowed to clearly differentiate the three regions of the spermatozoon and also distinguished the acrosome and the nucleus in the cephalic region. These results also accord with those obtained by Xu et al. (2022) in their study on sperm morphology in humans.

DAPI fluorescent staining was the one that yielded the most visually striking results. However, it did not provide relevant information regarding the differentiation of the spermatozoon regions, and it is also the most expensive and complex technique with a long processing time. Until now, the spermatozoa of the bat species studied present an external morphology without ornamentation, for which reason staining with DAPI does not provide relevant results such as those found by Ding et al. (2010) for rodents.

Lastly, consistent with the findings of Aksoy et al. (2012), the Janus Green and Basic Fuchsin stains intensely colored the middle piece but failed to differentiate between the nucleus and acrosome in the cephalic region.

There are fully automated systems such as CASA (Computer-assisted Sperm Analysis) to perform morphometric measurements of spermatozoa. These programs are precise and reproducible and have been successfully used in humans, horses, bulls, wild boars, and mice (García-Herreros et al., 2006). However, we resorted to a semi-automated manual measurement with the Zen 2 Blue edition program, since CASA has not yet been standardized for bats.

The morphometric results of the present study are in agreement with previous works carried out on related species (Hirth, 1960; Forman and Genoways, 1979) being within the same range of values. However, they differ markedly from the work of Álvarez-Guerrero et al. (2014) on sperm morphometry of *Artibeus jamaicensis* and *Sturnira lilium* with values that double our results for head length measurements, for example. This discrepancy could be due to the fact that different software was used for the measurements (Image Pro Express 4.5 vs. Zen 2 Blue edition). In future investigations, it might be possible to standarize the automated CASA software so as to test the reliability of the morphometric variables.

The results of this work can serve as a future reference on staining techniques and measurement methods that are recommended for studies on sperm morphology and morphometry of bats, thus allowing the results to be comparable.

CONCLUSIONS

– Epididymal maceration offers a straight forward and practical method for obtaining sperm in bats, despite its limitations.
– Toluidine Blue staining is ideal for swiftly and effectively assessing the presence or absence of sperm on slides.
– Dichromic stains, such as Hematoxylin-Eosin and May Grünwald-Giemsa, were more effective in distinguishing between the nucleus and acrosome in the cephalic region.

– Both Toluidine Blue and Hematoxylin-Eosin stains provided excellent visualization and contrast for studying sperm morphometry.

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PARTICIPATION OF AUTHORS

All authors contributed equally to data collection, sample analysis, interpretation of the results, and writing of the work.

CONFLICTS OF INTEREST

The authors confirm that there are no conflicts of interest associated with this publication.

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