Praca oryginalna *Original paper*

Morphometric parameters of canine spermatozoa: Comparison between conventional microscopy and CASA system

RÉDHA BELALA[1,2], SEDDIK KEBBAL[1], CHAOUKI BOUGUETOF[2], MYRA MEDJKOUNE[2], CHOAYB MECHEROUK[1], NORA MIMOUNE[1,2,3]

[1]Laboratory of Biotechnologies related to Animal Reproduction (LBRA), Institute of Veterinary Sciences, Blida University 1, Blida, Algeria
[2]Biotechnologies Platform for Animal Medicine and Reproduction (BIOMERA platform), Blida University 1, Blida, Algeria
[3]Animal Health and Production Laboratory, Higher National Veterinary School of Algiers, Algeria

Received 20.07.2024 *Accepted 01.10.2024*

Belala R., Kebbal S., Bouguetof C., Medjkoune M., Mecherouk C., Mimoune N.
Morphometric parameters of canine spermatozoa: Comparison between conventional microscopy and CASA system

Summary

The present study aimed to evaluate the effect of two cell detection parameters (Head Brightness Minimum „HBM" and head surface „AREA") on the automatic morphometric analysis of canine spermatozoa (spz) by using Hamilton-Thorne (HT)-IVOS II CASA system. Twenty ejaculates were collected from 6 identified dogs and the spz morphology was analyzed by conventional technique (microscopy) and by the HT-IVOS II system in two different situations (mobile and static spz). The 20 videos recorded from the preliminary analysis of the ejaculates were reassessed by modifying the two parameters of the setup studied according to an optimization protocol which made it possible to obtain 1120 IVOS II analyses. Statistical analysis was performed using IBM-SPSS software. Data showed a very significant effect of HBM on normozoospermia and teratozoospermia rates. On the contrary, the AREA factor did not affect spz quality except for the extreme values (14-16 µm²). Indeed, the HBM of 140 and the AREA between 4-10 µm² seemed to be the most suitable cell detection setting for automatic morphometry by the HT-IVOS II system and it can constitute an alternative to microscopy. In addition, Improper parameterization of the HBM could induce contrast artifacts leading to an overestimation of the major anomaly "Proximal Droplet". SPZ immobilizing before analysis by the HT-IVOS II system did not improve the results. To conclude, the authors suggest that manufacturers of CASA systems should ensure complete validation of the settings loaded on their systems in order to prevent random adjustments by users leading to great intra- and inter-laboratory variability in the results of automatic morphometry.

Keywords: dog, semen, CASA, light microscopy, automatic morphometry

Spermatozoa (spz) morphology constitutes an important parameter in the exploration of sperm quality and its ability in fertilizing the egg (17, 25). This parameter is classically evaluated by microscopy after fixation and staining (19). Antoni van Leewenhoek reported microscopic sperm morphology in 1678 for human and dog sperm using an early single-lens microscope (6). This technique is quite precise depending on the experience of the operator, but requires specialized microscopes or stains and involves the mobilization of laboratory personnel, and can induce visual fatigue which is a probable source of error. Moreover, it can also suffer from subjectivity when it is not carried out by highly experienced operators (2, 11, 26).

Computer-Assisted Semen Analysis (CASA) was introduced as an alternative to the conventional method and has the advantage of speed, precision, automation which could help to avoid human errors and better standardize this analysis (20, 23). In the same context, CASMA (for Computer Aided Sperm Morphology Analysis) is performed by a CASA system that analyzes spz morphology separately from motility on fixed and stained cell smear slides. Morphometry therefore uses software different from that of motility and a bright field illumination system (9, 24). CASMA has been widely evaluated and validated for several systems and in different animal species (10, 24).

Furthermore, some recent systems such as the HT-IVOS II allow automatic morphometric analysis at the same time as motility (with same analysis software and same negative phase contrast illumination system). Compared to morphometry on stained slides, automatic morphometry could thus offer the advantage of speed, lower analysis cost and the large number of spz analyzed (3, 24). It also helps avoid fixation and staining already identified as error factors in morphometry on stained slides (8, 9). However, if morphometry on stained slides has been widely studied and validated for different CASA systems compared to the reference technique recommended by the WHO (8, 9, 15), unfortunately, it is not the case for automatic morphometry without staining.

According to the literature review, no study has yet focused on the effect of the cellular detection configuration of the HT-IVOS II system in the automatic morphology analysis of canine spz. Thus, the present work aimed to evaluate the effect of the cellular detection parameters (HBM & AREA) on the morphometric assessment of canine spz by HT-IVOS II system.

Material and methods

Study area. This study was conducted at the level of Animal Medical & Reproductive Biotechnologies Platform (AMRBP), Saad Dahleb Blida University 1 (Blida, Algeria) during the period from 2021 to 2022.

Animals. In this current work, we collected twenty ejaculates from six different healthy identified dogs (Tab. 1), collected at a minimum interval of 48 hours.

Tab. 1. Animals used

Dog's name	Breed	Age (years)
Chopper	Belgian Shepherd Malinois	6
Ascko	Belgian Shepherd Malinois	10
Fidel	Belgian Shepherd Malinois	3
Tyron	Belgian Shepherd Malinois	9
Lucky	Belgian Shepherd Malinois	13
Fax	German Shepherd	11

Methodology

Semen collection. In the current study, we collected the semen of each animal manually, and fractionally on three conical tubes (pre-spermatic fraction, spermatic, and post-spermatic), according to the technique described previously (1). We heated the tubes in an oven and maintained a temperature of 37°C. All the fractions were evaluated, but only the sperm-rich fraction (second) was used.

Initial assessment of the sperm. Each ejaculate was evaluated immediately after collection to assess its quality (individual and mass motility and concentration). Mass and individual motility were assessed under a light microscope (7). Only ejaculates with a motility ≥ 3 were included in our study. Sperm concentration was assessed using a photometer calibrated for canine sperm (SDM Canine, Minitube, Germany). This device had already been the subject of external calibration in the laboratory with regard to the conventional technique recommended by the WHO (Improved Neubauer type cells).

For semen dilution, a commercial buffer was used (Easy Buffer B, IM-Technologies, Aigle, France). Depending on the volume (V1) and the initial concentration value (C1) of the ejaculate, a calculated volume of dilutor is added to have a final concentration (C2) of 100 million sperm/ml according to the relationship $C1 \times V1 = C2 \times V2$.

Slide Preparation for the conventional morphology study. After identification and adding 10 µl of diluted sperm, the slides were then stained using the equivalent Diff-Quik method (RAL kit, CNL®) in accordance with WHO recommendations. After staining and drying, the slides were mounted using a commercial solution (Eukitt®, SIGMA-ALDRICH) and cover slips. Once assembled, these slides were stored in slide storage boxes and kept until observation by an optic microscope (× 40; × 100).

Sperm analysis using CASA system. Computer analysis of the semen was carried out using a Hamilton Thorne IVOS II version 1.11.3 system (USA), and a Léja® analysis slide with four chambers of 20 µl depth. The parameters measured included: total motility (%); progressive motility (%); morphological abnormalities: curved tail, coiled tail (CT), proximal droplet (PD), distal droplet and distal midpiece reflex (DMR); concentration; kinematic parameters: amplitude of lateral head movement (ALH, µm), curvilinear speed (VCL, µm/s), straight line speed (VSL, µm/s), average trajectory speed (VAP, µm/s), linearity (LIN = VSL/VCL, %), wobble (WOB = VAP/VCL, %) and straightness (STR = VSL/VAP, %). The morphometric parameters analyzed by this system and used in our study were: Bent Tail (BT); Coiled Tail (CT); DMR (Distal Midpiece Reflexes); Proximal Dropled (PD); Distal Dropled (DD).

Experimental design summary. In this study, 20 ejaculates were collected from 6 dogs. Each ejaculate was subjected to an initial quality control assessing mass and individual motility as well as sperm concentration. Each ejaculate meeting the conditions for inclusion in the study was diluted to be analyzed by the HT-IVOS II system in two different conditions (motile spz "MOB" and static (immobile) "STA") and by standard microscopy (Diff-Quik® staining). The computer analysis of the ejaculate was carried out according to the settings recommended by the manufacturer and the result was recorded in video form. The latter was subsequently re-analyzed following several experimental setups resulting from the variation of two cellular detection parameters, namely the minimum brightness (HBM: Head Brightness Minimum) as well as the area (µm²) of the head of the spz. For the purpose of optimization and standardization of the computer analysis setup by the HT-IVOS II system, these two factors (HBM and Area) were varied as follows: (HBM: 100, 120, 140, 160, 180, 200; Area: 2, 4, 6, 8, 10, 12, 14). The 20 recorded videos of the 20 ejaculates studied (n = 20) were reanalyzed according to the protocol above, which made it possible to obtain 1120 IVOS II analyses.

Ethical statement. All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the course of the work. All the experiments

were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA. 14).

Statistical analysis. Statistical analysis of the raw data from the 20 ejaculates (n = 20) and 1120 IVOS II analyzes was carried out by IBM software – SPSS version 25, © 2017. After a descriptive analysis, the following comparison tests were applied: ANOVA and Tukey and Duncun tests in post hoc; Pearson correlation; Linear regression. The results were presented as mean ± SD, and the significance level was set at 5%.

Results and discussion

• Effect of cell detection parameters on morphometrics

Head Brightness Minimum (HBM)

Effect on normozoospermia and teratozoospermiarates. The following figures showed the influence of HBM parameter on the rates of normozoospermia and teratozoospermia of the dogs. According to the data, HBM variation between 100-200 presented a significant effect on the sperm. The rate of normozoospermia and teratozoospermia obtained closest to microscopy, corresponded to the value of 140 (HBM) with a rate of 85.91 ± 2.14 versus 92.42 ± 0.97.

Effect on Proximal Droplet (PD) and Coiled Tail (CT) rates. For these two major anomalies, according to our results, the optimum HBM was between 140 and 160 respectively for the PD (1.77 ± 0.52 versus 3.45 ± 1.93) and the CD (5.51 ± 0.77 versus 4.45 ± 0.85%) while the HBM value of 140 corresponded to the CT rate of 2.66 ± 0.50 versus 4.45 ± 0.85% (Fig. 3, 4). Indeed, the low HBM overestimated PD rate and the high HBM overestimated CT rate compared to the reference with respectively 22.61 ± 3.48% versus 3.45 ± 1.93% and 19.99 ± 3.53% versus 4.45 ± 0.85%.

Minimum head size of spz (AREA, µm²)

Effect on normozoospermia and teratozoospermia rates. Data presented in figures 5 and 6, the parameter AREA did not show

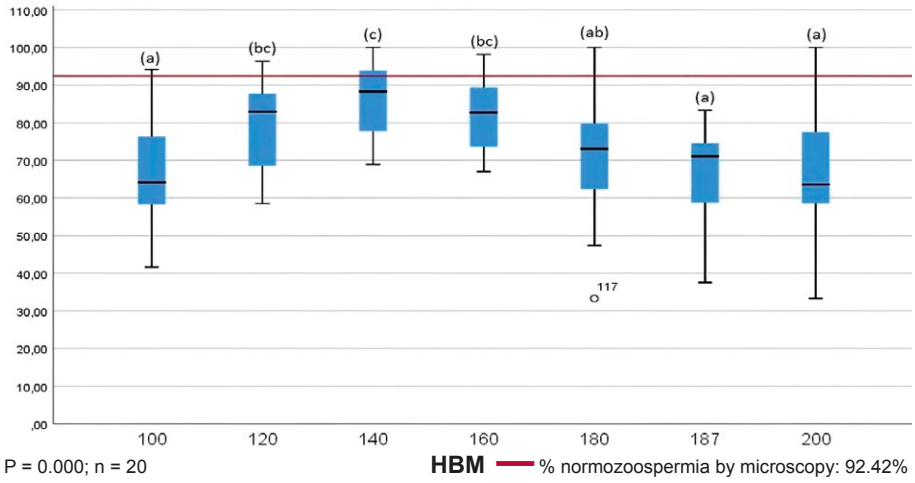

Fig. 1. Effect of HBM variation "100-200" on normozoospermia rate in canine spz analyzed by the HT-IVOS II system with Area set at 16 µm²
Explanations: Different letters mean a very highly significant difference

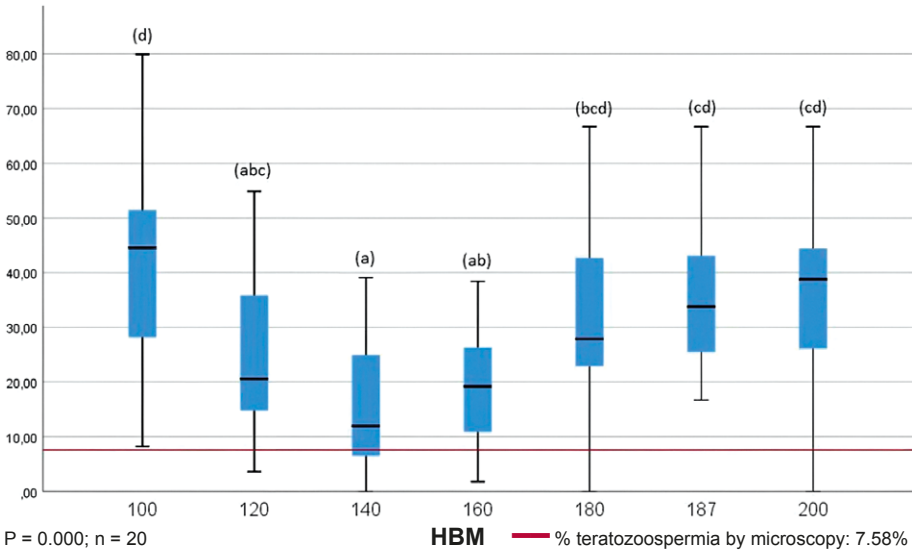

Fig. 2. Effect of HBM variation "100-200" on teratozoospermia rate in canine spz analyzed by the HT-IVOS II system with Area fixed at 16 µm²
Explanations: Different letters mean a very highly significant difference

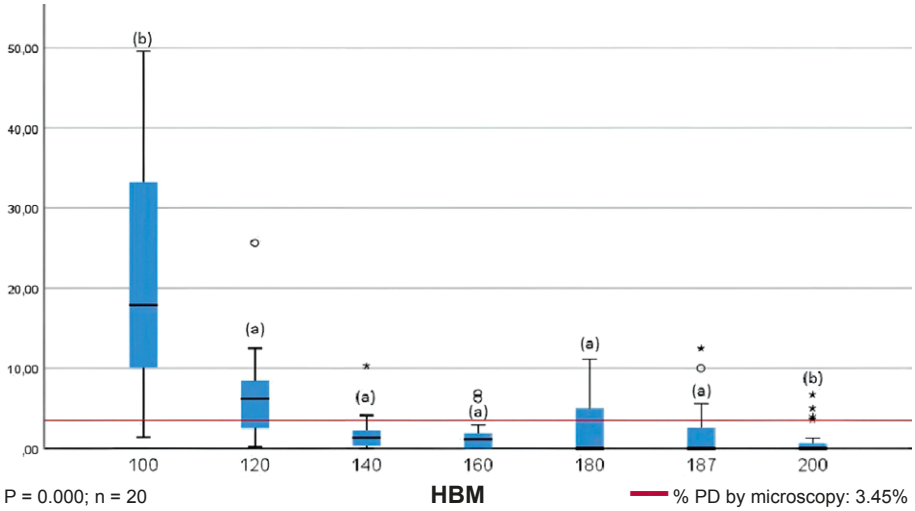

Fig. 3. Effect of variation "100-200" of HBM and Area set at 16 µm² on the rate of canine spz presenting a Proximal Droplet (PD) after analysis by the HT-IVOS II system
Explanations: Different letters mean a very highly significant difference

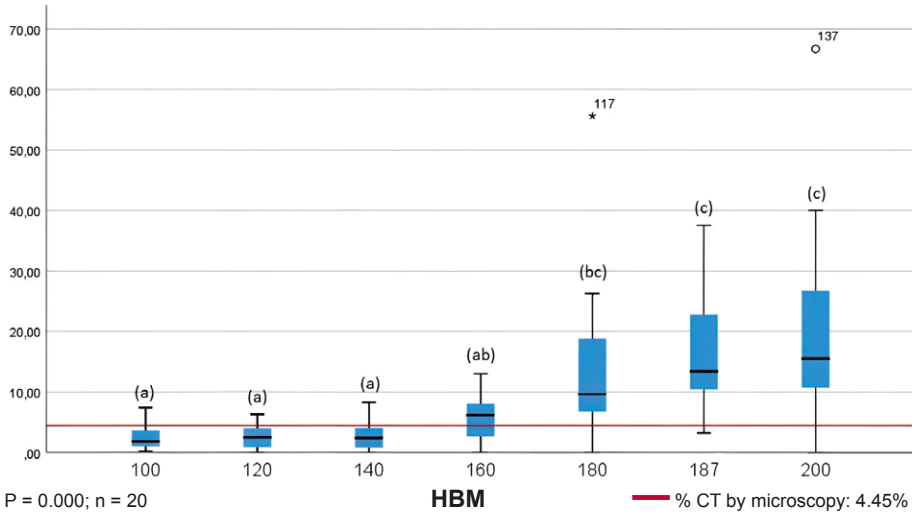

Fig. 4. Effect of variation "100-200" of HBM and Area set at 16 µm² on the rate of canine spz presenting a coiled Tail (CT) after analysis by the HT-IVOS II system
Explanations: Different letters mean a very highly significant difference

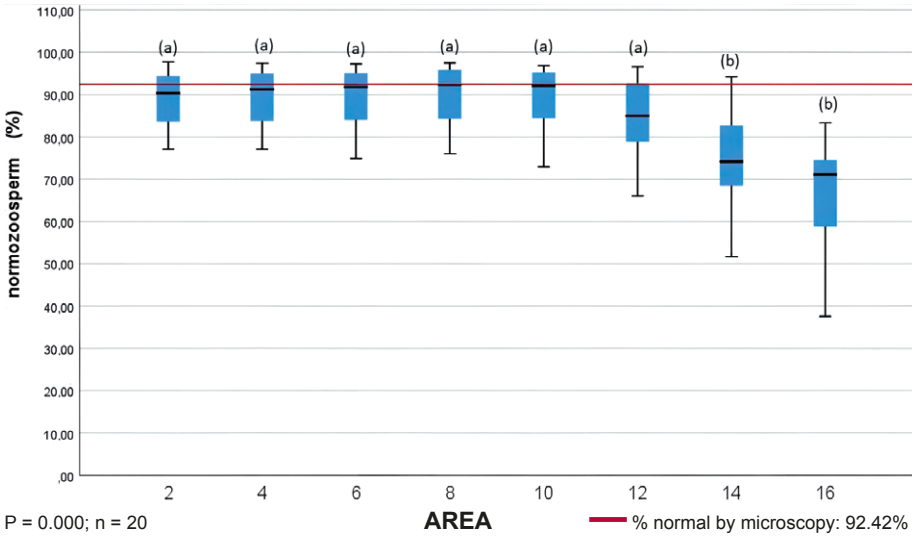

Fig. 5. Effect of variation "2-16 µm²" of the minimum size of the spz "AREA" with the HBM set at 187 on the normozoospermia rate of canine spz analyzed by the HT-IVOS II system
Explanations: Different letters mean a very highly significant difference

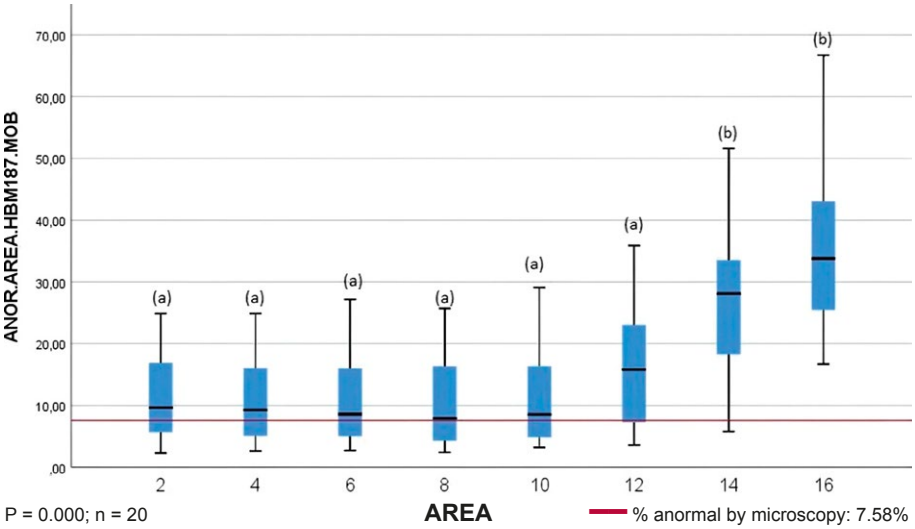

Fig. 6. Effect of variation "2-16 µm²" of AREA with the HBM set at 187 on the rate of teratozoospermia of canine spz analyzed by the HT-IVOS II system
Explanations: Different letters mean a very highly significant difference

a significant effect on normozoospermia and teratozoospermia between values of 2-12 µm² with similar normozoospermia rates between 89.28 ± 1.35 and 90.35 ± 1.47% compared to the reference value of 92.42 ± 0.97%. On the other hand, the values of 14 and 16 µm² deviated downwards from the reference value in a very highly significant manner with underestimated normozoospermia rates of 74.35 ± 2.37 and 66.27 ± 2.74% respectively.

Effect on Coiled Tail rate. Figure 7 showed the effect of AREA varied between 2-16 µm² on CT rate. The latter reached rates of 11.49 ± 1.31% and 16.79 ± 2.11% respectively for sizes of 14 and 16 µm² compared to the reference (4.45%).

• Correlation study between IVOS II and conventional microscopy

A Pearson correlation was carried out for the rates of normozoospermia, the Proximal droplet and the Coiled Tail between the conventional microscopy considered as the reference technique, and the HT-IVOS II with different cell detection settings, namely HBM186-AREA16 (recommended by the manufacturer), HBM140-AREA6, HBM140-AREA4, HBM120-AREA6, HBM120-AREA4. The results were recorded in correlation tables. From these results for normozoospermia and the Coiled Tail rates, a comparison was performed between microscopy and IVOS II with HBM140-AREA4 by comparing the two linear regression curves presented below in figures 8, 9. Data revealed a negative correlation between conventional microscopy and the HT-IVOS II with HBM187 and AREA 16 µm² with a coefficient of –347 and –354 for the rates of normozoospermia and CT respectively. Positive correlation was obtained with HBM140 and AREA 4 µm² (+0.391 and +0.332, respectively).

Effect of spz immobilization on the concentration and morphometrics

Effect on concentration. SPZ immobilization before adjustment made it possible to obtain a con-

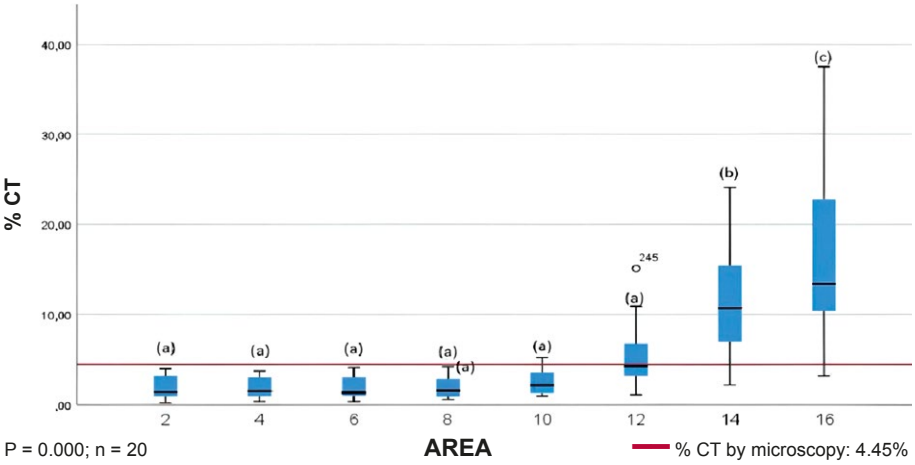

Fig. 7. Effect of variation "2-16 µm²" of the AREA and HBM set at 140 µm² on the rate of canine spz presenting a Coiled Tail (CT) after analysis by the HT-system IVOS II

Explanation: Different letters mean a significant difference

centration value closer to the reference (284.2 versus 286.45) on the overall number of analyzes carried out (n = 1120) with a significant correlation of 0.879 (Fig. 10, 11).

A correlation study between the different HT-IVOS II settings was carried out to better investigate the effect (Tab. 2, 3). Data revealed that the immobilization did not improve the sperm concentration, which was quite underestimated by the settings recommended by the manufacturer (HBM 187 and AREA 16) of the HT-IVOS II compared to the reference technique (19.76 (MOB) and 23.20 (STAT) versus 286.45 million per ml). Even the correlation was not improved with coefficients of 0.792 versus 0.743 respectively for mobile and static spz in this same category or parameterization. This spz immobilization seemed not to have affected the strong correlation existing between the IVOS II with adjusted parameter and the reference technique with coefficients of 0.929 and 0.920 respectively for mobile and static spz in this category. However, if the correla-

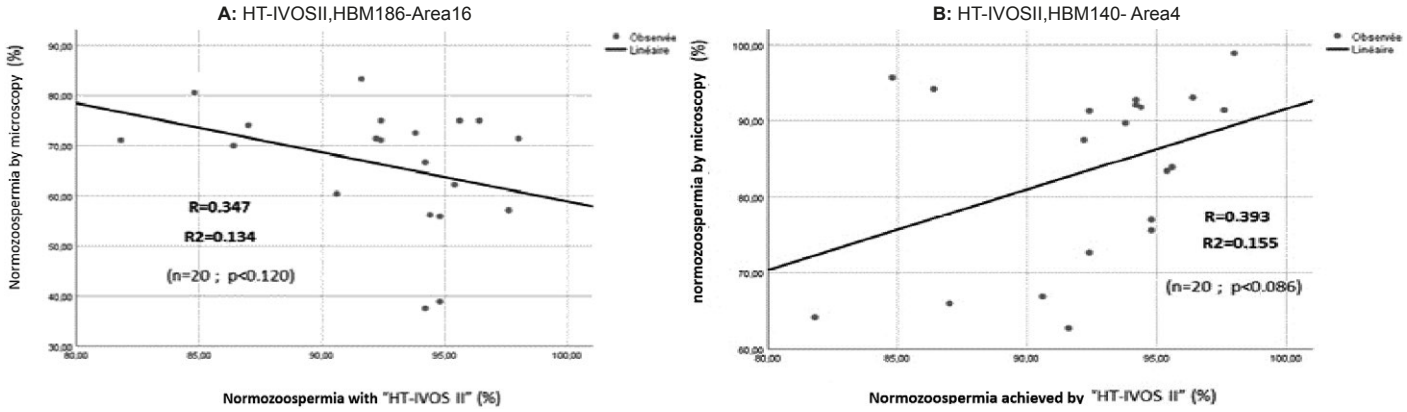

Fig. 8. Linear regression between the normozoospermia rate of canine spz evaluated by conventional microscopy and the HT-IVOS II system with parameters A: "HBM: 187; AREA: 16 µm²; R = –0.347; p = 0.134; n = 20" and B: "HBM: 140; AREA: 4 µm²; R = +0.393; p = 0.134; n = 20"

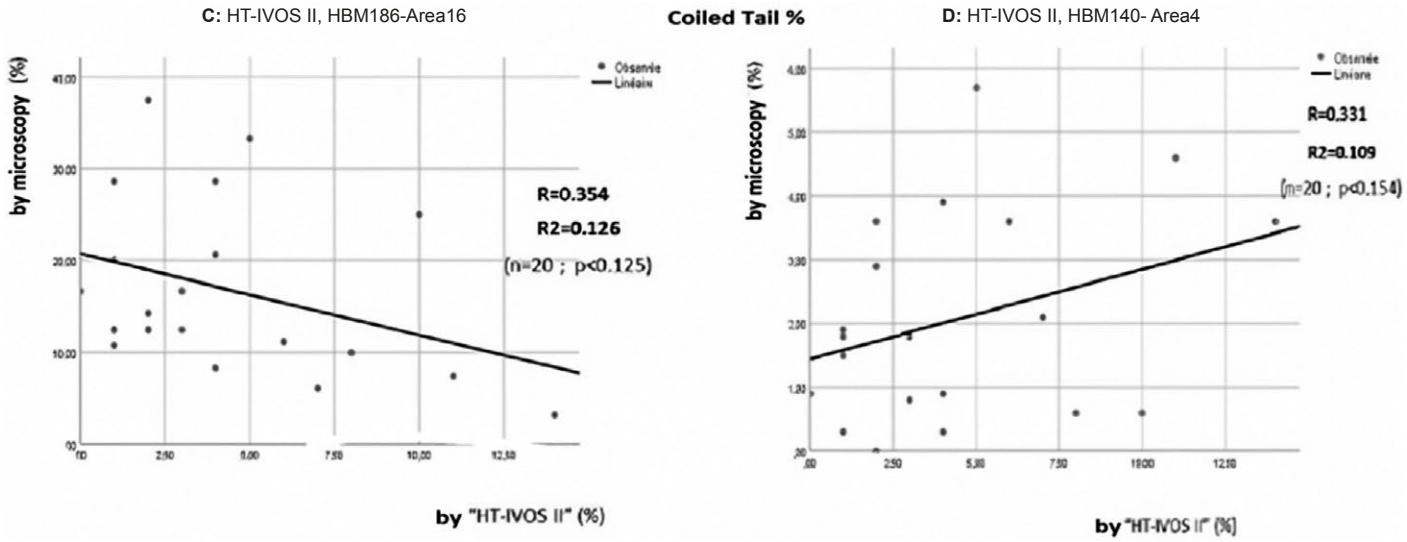

Fig. 9. Linear regression between the Coiled Tail (CT) rate of canine spz evaluated by conventional microscopy and the HT--IVOS II system and parameters C: "HBM: 187; AREA: 16 µm²; R = –0.354; p = 0.126; n = 20" and D: "HBM: 140; AREA: 4 µm²; R = +0.331; p = 0.154; n = 20".D: "HBM: 140; AREA: 4 µm²; R =+0.331; p = 0.154; n = 20"

Tab. 2. Correlations between the concentrations measured by "Neubauer" cell counting and by HT-IVOS II system with several settings (HBM187-AREA16; HBM140-AREA4) with mobile (MOB) and immobile spz before analysis (STAT) (n = 20)

		Correlations				
		CONC.NB	CONC.187.16.MOB	CONC.187.16.STAT	CONC.140.4.MOB	CONC. 140.4.STAT
CONC.NB	Correlation of Pearson	1	0.792**	0.743**	0.929**	0.920**
	Sig (bilateral)		0.000	0.000	0.000	0.000
	NOT	20	20	20	20	20
CONC.187.16.MOB	Correlation of Pearson	0.792**	1	0.938**	0.734**	0.833**
	Sig (bilateral)	0.000		0.000	0.000	0.000
	NOT	20	20	20	20	20
CONC.187.16.STAT	Correlation of Pearson	0.743**	0.938**	1	0.716**	0.797**
	Sig (bilateral)	0.000	0.000		0.000	0.000
	NOT	20	20	20	20	20
CONC.140.4.MOB	Correlation of Pearson	0.929**	0.734**	0.716**	1	0.871**
	Sig (bilateral)	0.000	0.000	0.000		0.000
	NOT	20	20	20	20	20
CONC.140.4.STAT	Correlation of Pearson	0.920**	0.833**	0.797**	0.871**	1
	Sig (bilateral)	0.000	0.000	0.000	0.000	
	NOT	20	20	20	20	20

Explanation: ** Correlation is significant at 0.01 level (bilateral)

Tab. 3. Average concentrations measured by Neubauer cell counting "CONC.NB" and by HT-IVOS II system with several settings (HBM187-AREA16; HBM140-AREA4) with mobile spz (MOB) and immobilized before analysis (STAT) (n = 20)

Descriptive Statistics			
	Average	Standard deviation	NOT
CONC.NB	286.4495	198.34548	20
CONC.187.16.MOB	19.7650	39.99382	20
CONC.187.16.STAT	23.2000	27.98582	20
CONC.140.4.MOB	292.5410	248.19334	20
CONC.140.4.STAT	399.0330	326.90742	20

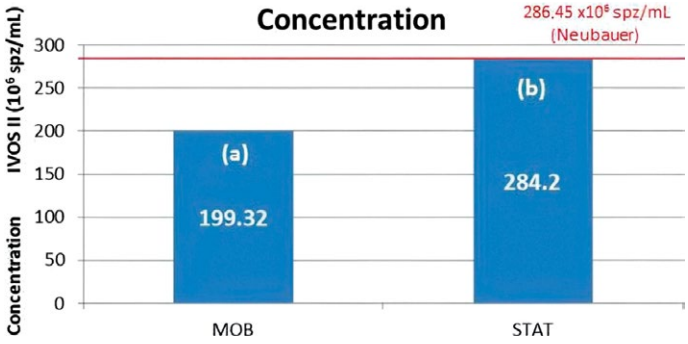

Fig. 10. Concentration analyzed by the HT-IVOS II system with several settings with mobile (1) and immobile spz before analysis (2), P = 0.000 (n = 1120)

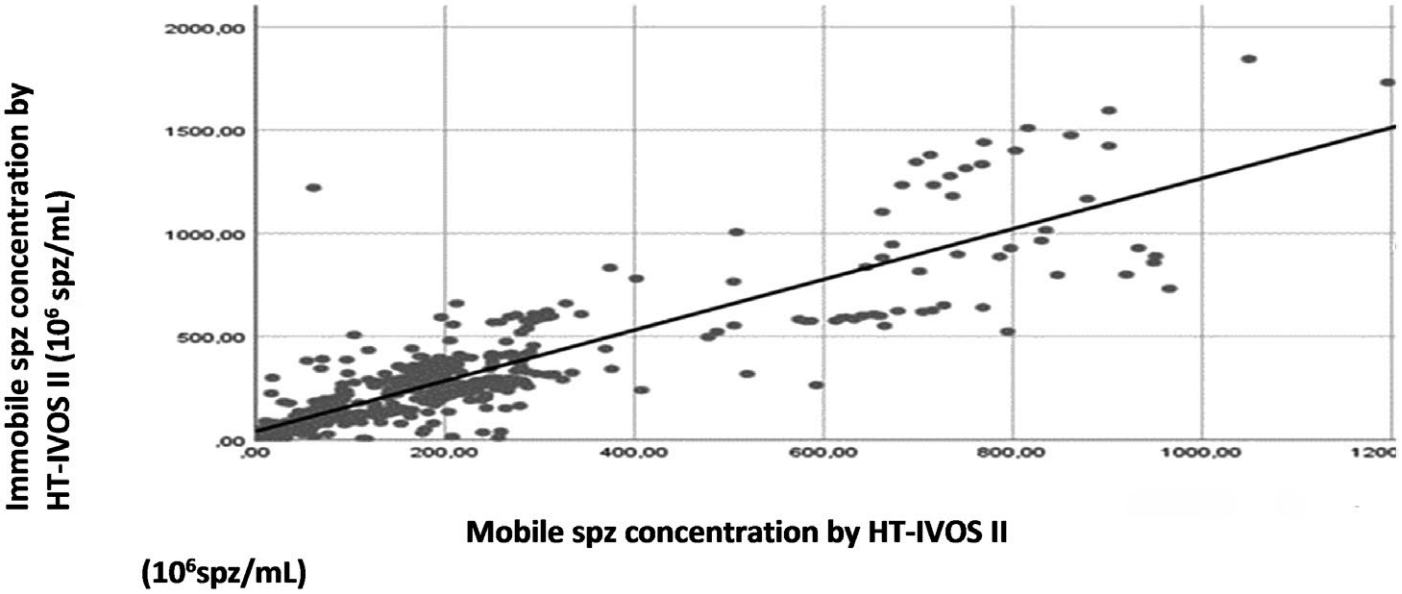

Fig. 11. Linear regression between the concentration of canine spz evaluated by cell count (Neubauer) and HT-IVOS II with different settings

tion was not changed, the concentration value raised from 292.54 to 399.03 million spz/mL.

• Effect on morphometrics

Regarding the effect of spz immobilization on morphometrics, our data showed a slight underestimation of normozoospermia with rates of 64.71 versus 58.68% for the HBM187-AREA16 parameterization; 87.45 versus 76.15% for the optimal configuration in our study (HBM140-AREA4) compared to a reference value of 92.42% (Tab. 4). On the other hand, a slight improvement in the correlation with the microscopy was recorded after immobilization of the spermatozoa for the two parameters with respectively –0.251 versus 0.237 and 0.230 versus 0.542 before and after immobilization (Tab. 5).

According to our data, the cellular detection parameters studied in the present work (HBM and AREA) had a high significant effect on normozoospermia, teratozoospermia and in particular on the major morphological anomalies, namely the proximal droplet (PD) and the coiled tail (CT).

The normozoospermia rate obtained by CASA system closest to light microscopy (considered in our study to be the reference), corresponded to the value of 140 (HBM). This HBM value would represent the optimum contrast allowing optimal analysis of the morphology by HT-IVOS II system. Any deviation on either side of this optimum revealed a rate of normozoospermia or teratozoospermia far from the reference. It should be noted that the value recommended by the manufacturer (IMV-Technologies; 187 for HBM) corresponded in this study to a rate of normozoospermia of 66.27 ± 2.74 which deviated considerably from the reference (92.42 ± 0.97). It means that this set-up is unsuitable. This observation could possibly be explained by the direct HBM effect on the ability of IVOS II morphometry software to accurately and precisely evaluate the morphology of canine spz, because the increase in HBM implies low illumination and low contrast of the spz compared to the image background. Furthermore, it could also be explained by an indirect effect of brightness resulting from poor detection of spz, which implies proportion errors in the morphometry.

Concerning the HBM effect on teratozoospermia rate, it inversely reflected that of normozoospermia, with an optimum compared to the reference technique corresponding to the value of 140. On either side of this optimum, a minimum brightness that was too low (HBM = 100) "too high

Tab. 4. Average normozoospermia levels analyzed by "TOTAL.NORM.MIC" microscopy and by HT-IVOS II system with several settings (HBM187-AREA16; HBM140-AREA4) with mobile spz (MOB) and immobilized before analysis (STAT)

	NOT	Minimum	Maximum	Average	
	Statistics	Statistics	Statistics	Statistics	Error standard
NORM.MOB	560	33.30	100.00	80.6830	0.53453
NORM.STAT	560	0.00	95.30	68.4957	0.74751
NORM.187.16.MOB	20	38.90	80.60	64.7100	2.59343
NORM.187.16.STAT	20	35.70	82.60	58.6800	3.00350
NORM.140.4.MOB	20	64.10	140.00	87.4650	3.60668
NORM.140.4.STAT	20	54.50	92.20	76.1450	2.45434
TOTAL.NORM.MIC	20	81.80	98.00	92.4200	0.96898

Tab. 5. Correlations between normozoospermia rates analyzed by microscopy and by HT-IVOS II system with several settings (HBM187-AREA16; HBM140-AREA4) with mobile spz (MOB) and immobilized before analysis (STAT)

		Correlations				
		TOTAL.NOR M.MIC	NORM.187.16.MOB	NORM.187.16.STAT	NORM.140.4.MOB	NORM.140.4.STAT
TOTAL.NORM.MIC	Pearson Correlation	1	–0.251	0.237	0.230	0.542*
	Sig. (bilateral)		0.286	0.314	0.329	0.013
	NOT	20	20	20	20	20
NORM.187.16.MOB	Correlation of Pearson	–0.251	1	0.327	0.324	0.127
	Sig. (bilateral)	0.286		0.160	0.164	0.594
	NOT	20	20	20	20	20
NORM.187.16.STAT	Correlation of Pearson	0.237	0.327	1	0.080	0.623**
	Sig. (bilateral)	0.314	0.160		0.736	0.003
	NOT	20	20	20	20	20
NORM.140.4.MOB	Correlation of Pearson	0.230	0.324	0.080	1	0.477*
	Sig. (bilateral)	0.329	0.164	0.736		0.033
	NOT	20	20	20	20	20
NORM.140.4.STAT	Correlation of Pearson	0.542*	0.127	0.623**	0.477*	1
	Sig. (bilateral)	0.013	0.594	0.003	0.033	
	NOT	20	20	20	20	20

Explanations: * Correlation is significant at 0.05 (bilateral); ** Correlation is significant at 0.01 (bilateral).

illumination" or too high (HBM = 180-200) "too low illumination" overestimated the rate of teratozoospermia.

PD is associated with a considerable decrease in sperm fertilizing capacity. A high and permanent rate of this anomaly is considered a reliable indicator of spermatogenesis disorder and often associated with decreased semen quality (4, 18). It is therefore essential that HT-IVOS II system can detect this major anomaly with precision and accuracy, particularly in the context of quality control.

Our data regarding the PD overestimation by HT-IVOS II system is consistent with previous report on bovine spz (16). It could be explained by an artifactual effect of brightness or contrast. Indeed, during strong illumination following a drop in the brightness threshold to around 100 (HBM), the normal structure of the neck (connective part) of the spz could illuminate and shine in an exaggerated manner and be detected by the image analysis software as a proximal droplet. An ultrastructural exploration could explain the possible causes of increased brightness in the neck region inducing these artifactual images (14).

As for the CT anomaly overestimated by low illumination, it did not seem to be directly affected by the parameter studied, „HBM", but rather by another detection parameter which was not targeted in our study, namely the minimum brightness of the tail "TBM: Tail Brightness Minimum". Thus, the explanation of the overestimation of this anomaly remains dependent on further exploration including the study of other cellular detection parameters, notably TBM.

Unlike brightness, statistical analysis did not reveal any significant effect of the minimum head size (AREA of 2-12 μm²) on normozoospermia and teratozoospermia rates while the values of 14 and 16 μm² deviated downwards from the reference value significantly. It should be noticed that the value recommended by the manufacturer (16 μm²) deviated from the reference of around 28.23% for the rate of teratozoospermia, which is significantly different in terms of morphology evaluation. This doubtfully high teratozoospermia rate was mainly represented by the CT which reached high rates compared to the reference while the PD rates all remained below the reference value for all AREA rates. This is explained by the opposite effect of brightness mentioned above. Indeed, a weak illumination following a high brightness threshold (HBM = 187) induced PD underestimation. Even a minimum size set at 16 μm² did not make it possible to obtain half of the reference rate of the PD (4.45%) in these low illumination conditions. This shows the benefit of good brightness settings for automatic morphometry without staining carried out by the HT-IVOS II system.

Regarding the AREA effect at minimum brightness values (HBM) of 140 and 120, the results showed no significant difference for normozoospermia, teratozoospermia, PD and CT rates. This result confirmed again that the AREA did not significantly affect morphometrics under the conditions of the present study. It should be emphasized here that at these brightness values, even a minimum size of 16 μm² did not cause an exaggerated overestimation of normozoospermia rates compared to the reference technique (85.91 ± 2.14 versus 92.42 ± 0.97%).

Although the correlation did not show many high coefficients, it made it possible to highlight a negative relationship between conventional microscopy and the HT-IVOS II with the parameterization (set-up) recommended by the manufacturer (HBM187 and AREA 16 μm²) for the rates of normozoospermia and CT respectively. Adjusting the cellular detection setting HBM140 and AREA 4 μm² enabled reversing the direction of this correlation by making it positive. However, further explorations and the optimization of other settings seem necessary for a better adaptation of this automatic morphometry technique by HT-IVOS II system (13).

Furthermore, the low correlation with microscopy could be due to a lack of optimization of the parameterization, but on the other to a lack of standardization of the conventional microscopy which may represent a bias in our study (5). The ideal would have been to make two observations by two experienced researchers, and if more than 10% difference is recorded between the results, a third reading is necessary to decide (21).

For many years, the CASA analysis has been one standard in the laboratory for motility and kinetic parameters (22), with no clear description of a species-specific setup (14). In the current study, the concentration rate in immobile spz obtained was closer to the reference before any adjustment. This represents a compensation effect of the different settings of the CASA system included in these 1120 CASA analyses. The correlation study between the different HT-IVOS II settings confirmed that the immobilization did not allow for improving the sperm concentration, which was very underestimated by the settings recommended by the manufacturer. This result revealed that this unsuitable parameter setting cannot be compensated by immobilizing the spz before analysis, a measure previously proposed (12). The overestimation of the concentration following the spz immobilization before analysis could be explained by the fact that the parameterization proposed in our study as an adjustment was adapted for moving and not static spz. Concerning the influence of spz immobilization on morphometrics, it seems to be associated with a slight underestimation of normozoospermia for the optimal configuration (HBM140-AREA4) compared to the reference.

The cellular detection settings of the HT-IVOS II system recommended by the manufacturer (IMV-Technologies: HBM 187 and AREA 16 μm²) seemed to be completely unsuitable for the analysis of canine spz morphology using the system's automatic morphometry. Adjusting the HBM to 140 and the AREA between 4-10 μm² represented the cell detection setting

best suited to automatic morphometric analysis without staining using the HT-IVOS II system. It can represent an acceptable alternative to the conventional technique (microscopy) offering the advantage of speed and automation. Incorrect HBM setting could induce contrast artifacts leading in particular to an overestimation of the major anomaly (PD). Immobilizing spermatozoa before analysis by the HT-IVOS II system did not improve the results but rather to the overestimation of the concentration and underestimate the morphology. Finally, the authors suggest that the manufacturers of the CASA technique should ensure complete validation of the settings loaded on their systems in order to standardize and to prevent random adjustments by users leading to great intra- and inter-laboratory variability in the results of automatic morphometry (taking into consideration variable factors: breed, genetic, species, age, climate, and nutrition).

References

1. *Arokia R., Jayaprakash G., Mayur P., Tamilmani T., Sathiyabarathi M.*: Collection and evaluation of canine semen – A review. International Journal of Science, Environment and Technology 2016, 5 (3), 1586-1595.
2. *Auger J., Eustache F., David G.*: Standardisation de la classification morphologique des spermatozoïdes humains selon la méthode de David modifiée. Androl. 2000, 10, 358-373, doi: 10.1007/BF03034491
3. *Augustinus A., Pakpahan C.*: Computer assisted sperm analysis: A review. Indonesian Andrology and Biomedical Journal. 2020, 1, 60-66, doi: 10.20473/iabj.v1i2.35.
4. *Boe-Hansen G. B., Fortes M. R. S., Satake N.*: Morphological defects, sperm DNA integrity, and protamination of bovine spermatozoa. Andrology 2018, 6, 627-633, doi: 10.1111/andr.12486.
5. *Broekhuijse M. L. W. J., Šoštarić E., Feitsma H., Gadella B. M.*: Additional value of computer assisted semen analysis (CASA) compared to conventional motility assessments in pig artificial insemination. Theriogenology 2011, 76, 1473-1486, doi: 10.1016/j.theriogenology.2011.05.040.
6. *Bulkeley E., Collins C., Foutouhi A., Gonzales K., Power P., Meyers S.*: Assessment of an iPad-based sperm motility analyzer for determination of canine sperm motility. Translational Animal Science 2021, 5, 2, doi: 10.1093/tas/txab066.
7. *Domain G., Banchi P., Ali Hassan H., Eilers A., Lannoo J., Wydooghe E., Niżański W., Van Soom A.*: Sperm gone smart: A portable device (iSperm®) to assess semen concentration and motility in dogs. Animals 2022, 12 (5), 652, doi: 10.3390/ani12050652.
8. *Hidalgo M., Rodriguez I., Dorado J.*: Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. Theriogenology 2006, 66, 996-1003.
9. *Horst G. van der, du Plessis S.*: Not just the marriage of Figaro: but the marriage of WHO/ESHRE semen analysis criteria with sperm functionality. Postepy Androl. (Online) 2017, 4, 6-21.
10. *Horst G. van der, Maree L. Du Plessis S.*: Current perspectives of CASA applications in diverse mammalian spermatozoa. Reproduction, Fertility and Development 2018, 30, doi: 10.1071/RD17468.
11. *Hussein R. S., Mohamed E. E. M., Mohamed R. R., Abdelbasset W. K., Morsy W. E., Elsayed S. H.*: Assessment of sperm morphometry in evaluating male infertility. International Journal of Biomedicine 2024, 14 (1), 93-98, doi: 10.21103/Article14(1)OA14.
12. *Iguer-Ouada M., Verstegen J.*: Evaluation of the hamilthon thorn computer based automated system for dog semen analysis. Theriogenology 2001, 55, 733-749.
13. *Jorge-Neto P. N., Araújo G. R., Silva M. C. C., Salom˜ao-Jr J. A., Csermak-Jr A. C., Pizutto C. S.*: Description of the CASA system configuration setup for jaguar (Panthera onca). Anim. Reprod. Sci. 2020, 220, 40-41, doi: 10.1016/j. anireprosci.2020.106436.
14. *Jorge Neto P. N., Francisco F., Carneiro M. D., Santos S., Requena L. D. R. S., Goés M., Valle R., Padilha F., Colbachini H., Gutierrez R., Schneider Brandão S. L., Manoel V., Reisfeld L., Deco-Souza T., Leite R., Pizzutto C.*: Specific setup and methodology for Computer-Assisted Sperm Analysis (CASA) in evaluating elasmobranch sperm. Theriogenology Wild 2024, 4, doi: 10.1016/j.therwi.2024.100091.
15. *Maroto-Morales A., Ramón M., García-Álvarez O., Soler A. J., Esteso M. C., Martínez-Pastor F., Pérez-Guzmán M. D., Garde J. J.*: Characterization of ram (Ovis aries) sperm head morphometry using the Sperm-Class Analyzer. Theriogenology 2010, 73, 4, 437-448, doi: 10.1016/j.theriogenology.2009.10.003.
16. *O'Meara C., Henrotte E., Kupisiewicz K., Latour C., Broekhuijse M., Camus A., Gavin-Plagne L., Sellem E.*: The effect of adjusting settings within a Computer-Assisted Sperm Analysis (CASA) system on bovine sperm motility and morphology results. Anim Reprod. 2022, 4, 19 (1), e20210077, doi: 10.1590/1984-3143-AR2021-0077.
17. *Öztürkler Y., Baran A., Evecen M., Ak K., İleri İ. K.*: Comparison of ovine spermatozoa morphological features after staining of fixation and assessment of morphological abnormalities in dead/live spermatozoa. Turkish Journal of Veterinary & Animal Sciences 2001, 25, 5.
18. *Pena A. I., Barrio M., Becerra J. J., Quintela L. A., Herradon P. G.*: Infertility in a dog due to proximal cytoplasmic droplets in the ejaculate: investigation of the significance for sperm functionality in vitro. Reprod. Dom. Anim. 2007, 42, 471-478.
19. *Polesso M., Novello G., Segabinazzi L.*: Modified diff-quick staining for canine sperm morphology. Clinical Theriogenology 2024, 16, doi: 10.58292/ct.v16.10243.
20. *Rijsselaere T., Soom A., Maes D., Niżański W.*: Computer-Assisted Sperm Analysis in dogs and cats: an update after 20 years. Reproduction in domestic animals = Zuchthygiene 2012, 47 Suppl 6, 204-207, doi: 10.1111/rda.12057.
21. *Schubert B., Badiou M., Force A.*: Computer-aided sperm analysis, the new key player in routine sperm assessment. Andrologia 2019, 51, e13417, doi: 10.1111/a d.13417.
22. *Şengül E., Dayanıklı C., AlpcanKünyeli B., Çoban İ., Bülbül B., Üstüner B., Nur Z.*: Effect of equex on ram semen in different freezing extenders. Kafkas Univ. Vet. Fak. Derg. 2024, 30 (2), 207-214, doi: 10.9775/kvfd.2023.30789.
23. *Tanga B. M., Qamar A. Y., Raza S., Bang S., Fang X., Yoon K., Cho J.*: Semen evaluation: methodological advancements in sperm quality-specific fertility assessment – A review. Anim. Biosci. 2021 Aug; 34 (8), 1253-1270, doi: 10.5713/ab.21.0072. Epub 2021 Apr 23. PMID: 33902175; PMCID: PMC8255896.
24. *Van der Horst G., Du Plessis S., Maree L.*: Have we conquered sperm morphology analysis in different mammalian species as analysed by CASMA? 2021, doi: 10.1007/978-3-030-66292-9_38.
25. *Wysokińska A., Wójcik E., Chłopik A.*: Evaluation of the morphometry of sperm from the epididymides of dogs using different staining methods. Animals 2021, 11 (1), 227, doi: 10.3390/ani11010227.
26. *Yurdakök-Dikmen B., Tekin K., Tırpan M. B., Daşkın A., Filazi A.*: In vitro toxicity of some pesticides on goat and dog spermatozoa. Kafkas Univ. Vet. Fak. Derg. 2017, 23 (2), 297-303, doi: 10.9775/kvfd.2016.16501.

Corresponding author: Prof. Dr. Nora Mimoune; e-mail: nora.mimoune@gmail.com, n.mimoune@ensv.dz