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Original Article

Canine semen evaluation using a new configuration of the CASA system

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Abstract

Background: For many years, the computer-assisted sperm analysis (CASA) has been one standard in the laboratory for semen evaluation, with no clear description of a species-specific setup. **Aims:** This study aimed to adjust the cell detection parameters (Head Brightness Minimum "HBM" and Head Size Minimum "AREA") of the CASA system and to assess their effects on evaluating canine semen, specifically concentration and motility. **Methods:** For this purpose, 20 ejaculates were collected from six dogs and subjected to microscopic assessment of motility, concentration evaluation using the SDM1 photometer, cell counting using a Neubauer chamber, and kinetic and morphometric analysis using the HT-IVOS II CASA system. Each CASA analysis was recorded, and then the video was re-analyzed by changing the two cell detection parameters (HBM and AREA). A total of 27 settings were used, and 560 CASA assessments were performed. Data were statistically analyzed using IBM SPSS. **Results:** The results showed that the HT-IVOS II CASA system provided an average concentration value (287.17 million spermatozoa (SPZ)/ml) much closer to that obtained by the reference method, Neubauer counting (286.45 million SPZ/ml), compared to the photometer (260.35 million SPZ/ml), which deviated from the gold standard technique by 26 million. In contrast, the IVOS with the adjusted setting (HBM140, AREA4) only presented a difference of 0.5. The IVOS II with the adjusted showing a strong correlation with the Neubauer count and the SDM photometer, with a correlation coefficient of R= 0.94. A perfect concordance was recorded between total and progressive motility evaluated by conventional microscopy and the percentages of motile and progressive sperm analyzed by HT-IVOS at values 120 and 140 for the HBM and 4 and 6 µm² for the AREA. **Conclusion:** An optimal cell detection parameter setting (HBM: 140 and AREA: 4 µm²) was selected and proposed for the HT-IVOS II CASA system.

Key words: Analyzer, Cell detection, Concentration, Dog semen, Motility

Introduction

Spermatozoa (SPZ) motility and concentration measures are crucial for evaluating male reproductive ability (Tanga *et al*., 2021; Önder *et al*., 2023). Motility is traditionally assessed subjectively by microscopy, resulting in significant imprecision and variability in the results (Arokia *et al*., 2016; Bulkeley *et al*., 2021; Mazzuchini *et al*., 2024). For concentration, the reference technique is cell counting using the Neubauer chamber (Meena and Manchiwal, 2023). The conventional methods are accurate when performed by an experienced operator, but are time-consuming and restrictive due to visual fatigue and staff mobilization. They can also suffer from subjectivity due to inexperienced manipulators (Yurdakök-Dikmen *et al*., 2017; Jorge-Neto *et al*., 2020; Zhang *et al*., 2020; Hussein *et al*., 2024).

Computer-assisted sperm analysis (CASA) offers an alternative to the conventional method, with the advantages of speed and precision, which could help avoid human errors and better standardize this analysis (Tanga *et al*., 2021; Belala *et al*., 2024). It is considered the gold standard in motility assessment (Jorge-Neto *et al*., 2024). However, while the CASA system is reliable for motility analysis compared to the conventional technique, there is no consensus on its reliability in measuring concentration (WHO, 2010; Belala *et al*., 2025b). The values obtained are often overestimated or underestimated compared to the reference method (Schubert *et al*., 2019).

CASA system limitations are attributable to several

pre-analytical factors related to human errors in handling and preparing the semen, as well as factors linked to CASA analysis conditions such as chamber type and depth, temperature, and concentration of analysis and dilution (Iguer-Ouada and Verstegen, 2001; Rijsselaere *et al*., 2003; Rijsselaere *et al*., 2012; Van der Horst and du Plessis, 2017; Van der Horst *et al*., 2021). However, the factors related to the technical configuration of the analyzer (pre-established setting of the image analysis software) have not been sufficiently explored since the validation studies of the CASA system for canine SPZ, which led to the proposed setup still considered as the standard recommended by the manufacturer (Iguer-Ouada and Verstegen, 2001; Rijsselaere *et al*., 2003; Rijsselaere *et al*., 2004). Recently, O'Meara *et al*. (2022) worked to adjust the kinematic and morphometric setup of the HT-IVOS II analyzer used for frozen bovine SPZ. These authors reported that IVOS II detects sperm cells according to the contrast (brightness) of sperm cells to the background, sperm head area, and elongation. The transparency of the extender affects the sperm contrast and head brightness and needs to be adjusted to correctly identify sperm cells. An incorrect setup will lead to misidentification of sperm cells and over-/underestimation of the normal sperm population.

To our knowledge, no study has yet addressed the effect of the cell detection parameters of the HT-IVOS II system on canine SPZ analysis. Thus, the present work aimed to optimize the setup of two parameters (HBM and AREA) and evaluate their effect on canine SPZ concentration and motility.

Materials and Methods

Study area

This study was conducted at the level of the Biotechnologies Platform for Animal Medicine & Reproduction (BIOMERA), Saad Dahleb Blida University 1 (Blida, Algeria) from 2021 to 2022. 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 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).

Animals

In this study, we collected twenty ejaculates from six different identified dogs (Table 1), collected at a minimum interval of 48 h.

Table 1: Breed and age of animals included in the study

Dog	Breed	Age (years)
1	Belgian Shepherd Malinois	6
2	Belgian Shepherd Malinois	10
3	Belgian Shepherd Malinois	3
4	Belgian Shepherd Malinois	9
5	Belgian Shepherd Malinois	13
6	German Shepherd	11

Methodology

Semen collection

Semen was collected from each animal by digital manipulation and fractionally in three conical tubes (pre-spermatic fraction, spermatic, and post-spermatic) (Fig. 1), according to the technique described previously (Belala *et al*., 2025c). The tubes were heated in an oven and maintained at a temperature of 37°C. 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 Phase Contrast Light Microscope with a heated stage at 37° (Nikon Eclipse E100, China) by using an objective ×100 (Belala *et al*., 2025c). The different movements (wave, gathering, and dispersion) of spermatozoa were assessed, and a score of 0 to 5 was then attributed according to the MILOVANOV scale (Milovanov, 1962). Only ejaculates with motility ≥3 were included in our study.

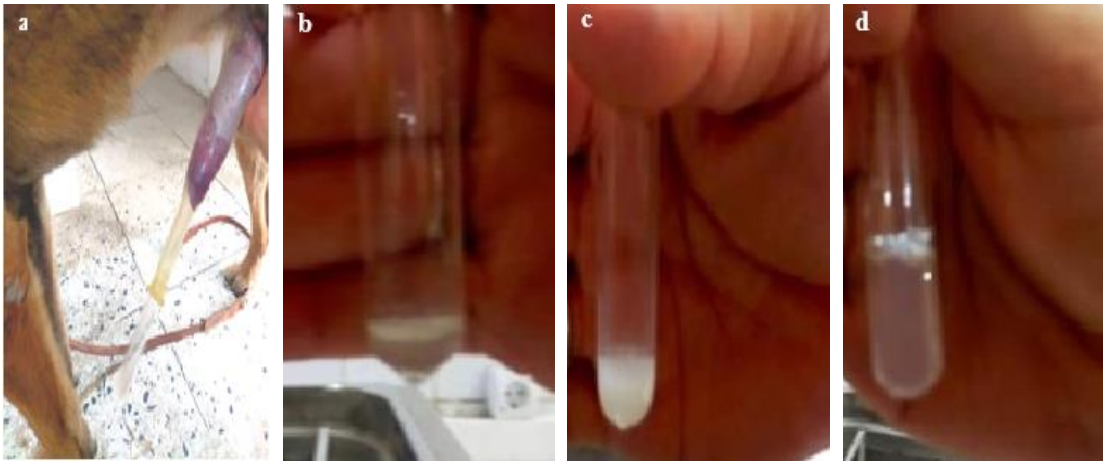


Fig. 1: Semen collection (a) showing the different fractions (pre-spermatic (b), sperm (c), post-spermatic (d), respectively)

Sperm concentration was assessed using a photometer calibrated for canine sperm (SDM Canine, Minitub, Germany). This device had already been subject to external calibration in the laboratory with regard to the conventional technique recommended by the WHO (Improved Neubauer type cells).

Cell counting

The sample must be prepared with an adequate concentration for counting; the range of concentrations that allowed hemocytometer counting was between 250,000 and 2.5 million cells per 1 ml of cells. A hypertonic diluent (4% NaCl) was used to make a dilution of (1:100, v/v), (40 µL of semen and 3960 µL of 4% NaCl) (WHO, 2021). The formula to calculate the concentration is: N/n^x (1/20) x dilution factor (number of SPZ counted in both chambers (N) divided by the volume in which they were found, the volume of the total number of rows examined (n) in both chambers).

Semen dilution

For semen dilution, a commercial buffer was used (Easy Buffer B, IMV Technologies, L'Aigle, France) up to an analysis concentration of between 20 to 30 million SPZ/ml, according to the manufacturer's recommendations.

Sperm analysis using the HT-IVOS II CASA system

Computer analysis of the semen was carried out using the Hamilton-Thorne IVOS II image analyzer (version 1.11.3 system, USA) belonging to BIOMERA Platform, and a Léja® analysis slide with four chambers of 20 µL depth (Ref. 025107, IMV Technologies, France). This system consists of a negative phase contrast and hot stage microscope and a camera, connected to HT-IVOS II image analysis software. The steps for sample preparation, slide filling, and system analysis were as follows: Dilution of the ejaculate with a commercially available buffer solution (Easy Buffer B, IMV-Technologies, France) to an analysis concentration of 20 to 30 million SPZ/ml, according to the manufacturer's recommendations; Preheating the Léjà slide (histological stage at 37°C; System preparation (stage temperature at 37°C; selection of canine setup: HBM 187; AREA 16); Placement of the slide in the system's slide holder; Filling of the slide with 3 µL of diluted semen in a chamber (A, B, C, D) using a micropipette and a suitable tip; For each sample, 8 fields were recorded and analyzed.

The IMSI Strict™ software (Intracytoplasmic Morphologically Selected Sperm Injection) allowed the acquisition of the following parameters: Motility (MOT), progressive motility (PROG), rapid motility (RAP), slow motility (SLOW), median motility (MED), 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, %).

Experimental design summary

Before starting our research experiment, a preliminary study was conducted on three ejaculates from three dogs. The objective was to evaluate the adequacy of the cell detection setup recommended by the manufacturer and its effect on the ability of the system to capture SPZ. In this study, 20 ejaculates were collected from six dogs. Each sample underwent an initial quality control assessing mass and individual motility, as well as sperm concentration. Those meeting the inclusion criteria were then diluted for analysis using both the HT-IVOS II system and the standard Neubauer counting method. The computer analysis of the ejaculate was carried out according to the configuration 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 cell detection parameters, namely HBM and AREA. For 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 were reanalyzed according to the protocol above, which made it possible to obtain 560 IVOS II analyses.

Statistical analysis

Statistical analysis of the data from the 20 ejaculates (n=20) and 560 IVOS II analyses was carried out using IBM SPSS version 25, 2017. After a descriptive analysis, the following comparison tests were applied: ANOVA, Tukey, and Duncan tests in post hoc, Pearson correlation, and linear regression. The results were presented as mean ± SD, and the significance level was set at 5%.

Results

Preliminary study (Selection of cell detection parameters to evaluate)

The preliminary study focused on three ejaculates collected from three dogs. It was conducted using the "auto-configuration" function of the image analysis software, which controls the illumination system before analysis as well as the cell detection parameters (HBM 187; AREA 16). It allows the detected SPZ to be covered with blue color while the undetected SPZ remains white (Fig. 2). The different parameters were changed, and the corresponding cell detection was appreciated on the photos taken by screenshot systematically. This study highlighted the effect of two parameters: HBM (contrast) and AREA (SPZ head surface/size, µm²) on the capacity of the image analysis software to detect these cells.

Effect of cell detection parameters

Effect of HBM & AREA on concentration analysis

This section included the evaluation of HBM & AREA effects on SPZ concentration, first by the analysis of variance, then by the correlation study (linear

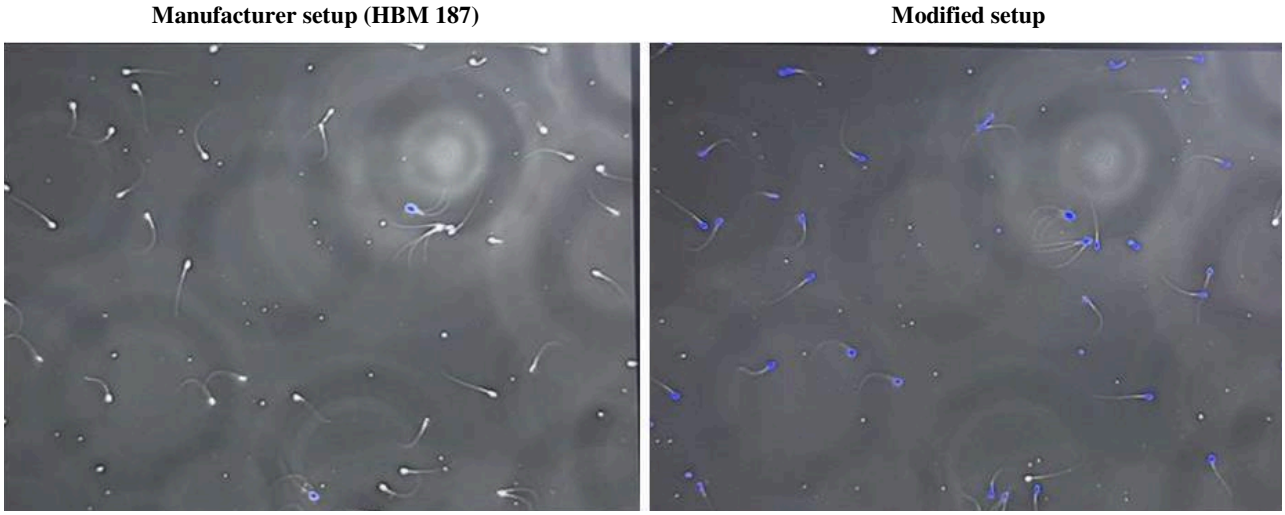


Fig. 2: Effect of HBM on canine SPZ detection by HT-IVOS II system "using auto-configuration tool" for a dog

regression) between the concentration measured by HT-IVOS II and that carried out by cell counting with the Neubauer hemocytometer.

Effect of HBM variation "100-200" with AREA set at 16

Statistical tests highlighted a very highly significant difference (P<0.000) between the groups, and the post hoc multiple comparison by the Tukey test is represented by the letters of significance in Fig. 3. According to the HBM parameter setup, the optimum would be 120, because this value (201.58 × 10^6 SPZ/ml) was the closest to the concentration evaluated by the reference technique (286.45 × 10^6 SPZ/ml). The value recommended by the manufacturer (HBM 187) revealed a concentration value (19.77 × 10^6 SPZ/ml) very low compared to that obtained by the Neubauer count (286.45 × 10^6 SPZ/ml).

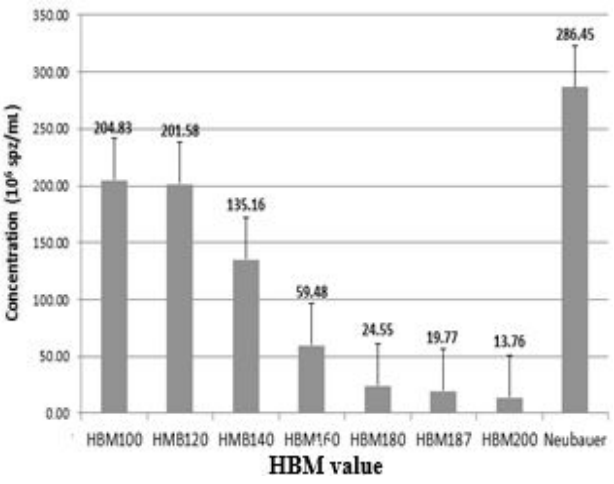


Fig. 3: Effect of HBM variation "100-200" on canine SPZ concentration analyzed by HT-IVOS II system in comparison with Neubauer technique, P<0.05 (n=20)

Data presented in Fig. 4 showed that the HBM setup appeared to have an overall significant effect (P=0.000) on the concentration measured by the HT-IVOS II system. The multiple comparison carried out by the Tukey test in post hoc between the different values of HBM "100-200" and the concentration value obtained by the reference technique (Neubauer counting) showed that the latter differed very significantly (P=0.000) from the IVOS II system set to the HBM values: 160-180-200, including the value recommended by the manufacturer (187), highly significant at the value of 140 (P=0.003) but revealed no statistical difference at values: 100-120 with P=0.414 and 0.362, respectively.

Effect of "AREA" variation for 3 values of HBM

This section is devoted to studying the second factor, "AREA, µm²," by setting the values of the first factor, "HBM" to three values, namely 187, the control value (recommended by the manufacturer), and the two optimal values revealed in the previous study of this factor.

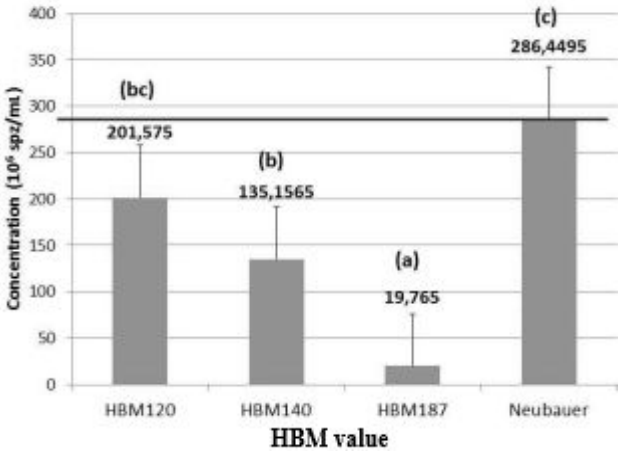


Fig. 4: Effect of 3 values of HBM: "187, 140 and 120" on SPZ concentration analyzed by HT-IVOS II system. Different letters mean a significant difference at P<0.05 (n=20)

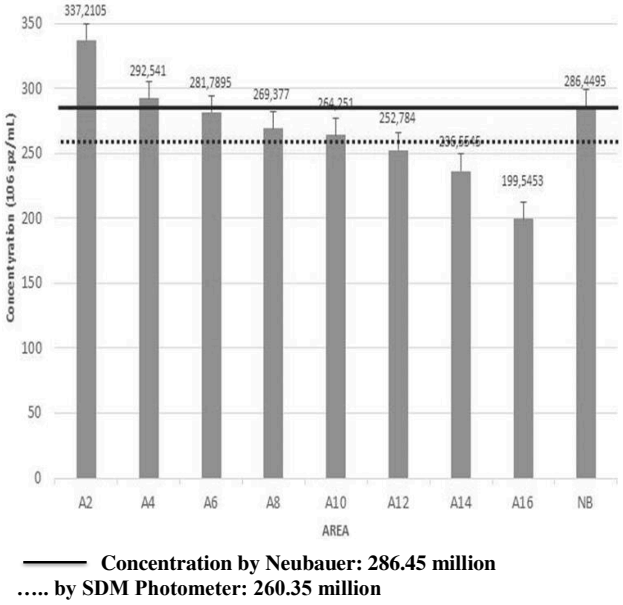


Fig. 5: Effect of AREA variation "2-16, µm²" with HBM 187 on SPZ concentration analyzed by HT-IVOS II system. No statistical difference (SD), P=0.803 (n=20)

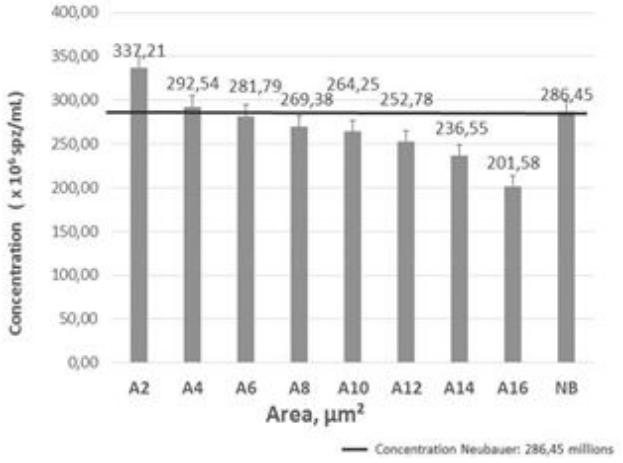


Fig. 6: Effect of area variation "2-16, µm²" with HBM 120 on SPZ concentration analyzed by the HT-IVOS II system. No statistical difference (SD), P=0.802 (n=20)

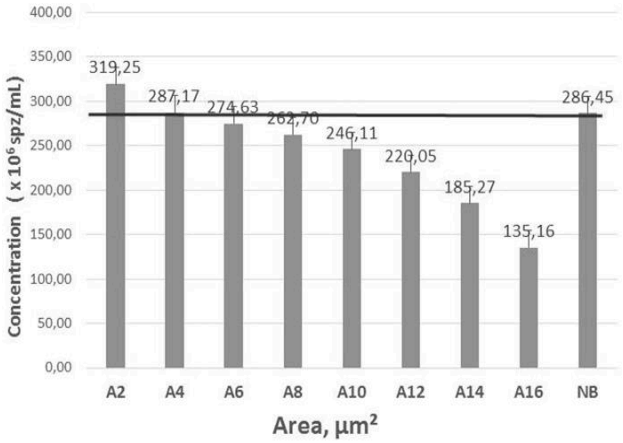


Fig. 7: Effect of AREA variation "2-16, µm²" with HBM 140 on SPZ concentration analyzed by the HT-IVOS II system. No statistical difference (SD), P=0.149 (n=20)

Effect of AREA variation with HBM 187 on the concentration by HT-IVOS II

The AREA did not have a significant effect (P=0.803) on the concentration measured by the HT-IVOS II system (Fig. 5). Given that the ANOVA was not significant, the post hoc multiple comparison cannot be carried out statistically between the different values of AREA "2-16" and the concentration rate obtained by the reference technique (Neubauer counting). However, it is easily noticed that the values closest to the reference were those of 4 and 6 µm² with concentrations of 292.54 and 281.79, respectively, compared to the reference value (286.45 million SPZ/ml), with 6.09 and 4.66 million ranges. Thus, a new analysis of variance will be carried out on AREA variation, but only associated with HBM of 120 and 140.

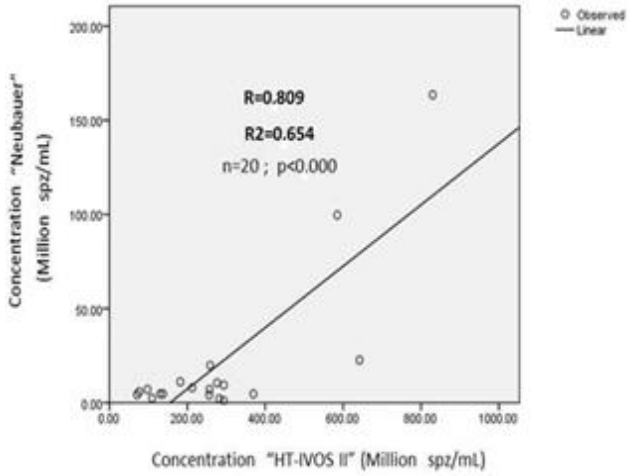
Effect of AREA variation with HBM 120 and 140 on the concentration by HT-IVOS II

The following figures (Figs. 6 and 7) show the effect of AREA setup with HBM 120 and 140 on the concentration obtained by the CASA system compared to the reference technique. Data showed that the AREA analyzed separately in the HBM 120 and HBM 140 classes did not show any statistical difference between the different values of "2-16" and the concentration value obtained by the reference technique. However, we noticed that the values closest to the reference were those of 4 and 6 µm² with concentrations of 292.54 and 281.79 million SPZ/ml for HBM 120 and 287.17 and 274.63 million SPZ/ml for HBM 140, respectively, compared to the reference value of 286.45 million SPZ/ml, with 6.09 and 4.66 million deviations for HBM 120 and 0.5 and 11.83 million SPZ/ml deviations for HBM 140, respectively. It should be noted that the optimal AREA value was 4 µm² for HBM 120 and 6 µm² for HBM 140. Furthermore, the AREA recommended by the manufacturer (16 µm²) resulted in concentration values farthest from the reference value, namely 201.58 and 135.16 million SPZ/ml, respectively, for HBM 120 and HBM 140, with 84.87 and 151.29 million SPZ/ml respective differences.

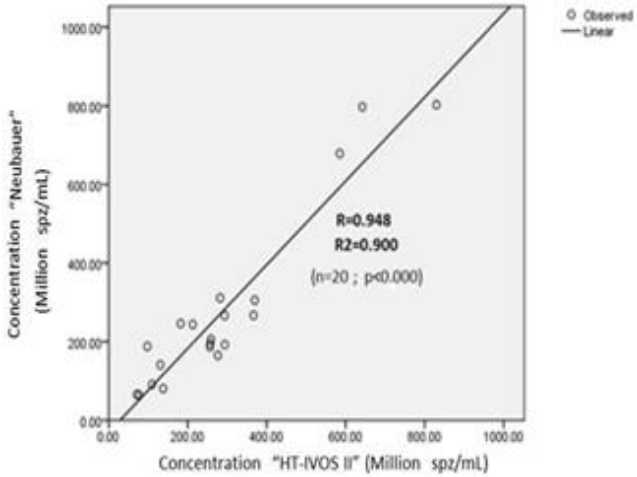
Correlation study (linear regression) between HT-IVOS II and the reference method

This involves the study of correlation and linear regression between the concentration analyzed by the HT-IVOS II system with five different settings "HBM-AREA: 187-16, 140-6, 140-4, 120-6, and 120-4," and the Neubauer cell count (Fig. 8, Table 2).

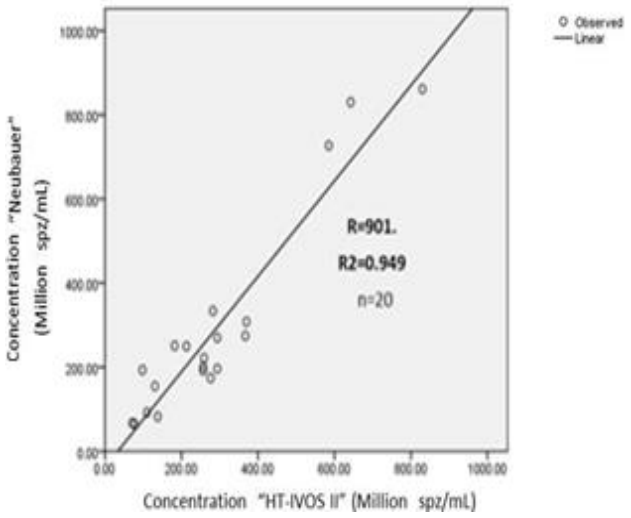
According to our data, the two pairs of parameters "HBM140-AREA6 and HBM140-AREA4" were the most strongly and significantly correlated with the reference technique (P=0.000; Pearson coefficients of 0.948 and 0.949, respectively). The concentration averages obtained by these two settings were 287.17 million SPZ/ml and 274.63 million SPZ/ml, with deviations from the reference value of 0.5 and 11.82 values, respectively.



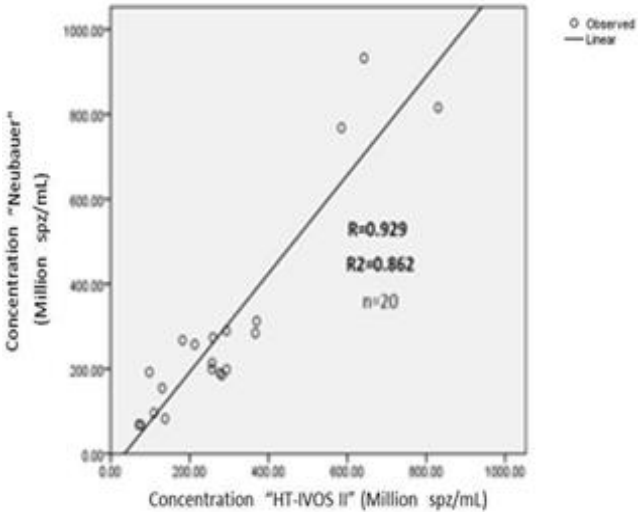
Linear regression between SPZ concentration analyzed by HT-IVOS II system "HBM: 187; AREA: 16 µm²" and Neubauer cell count. P=0.149



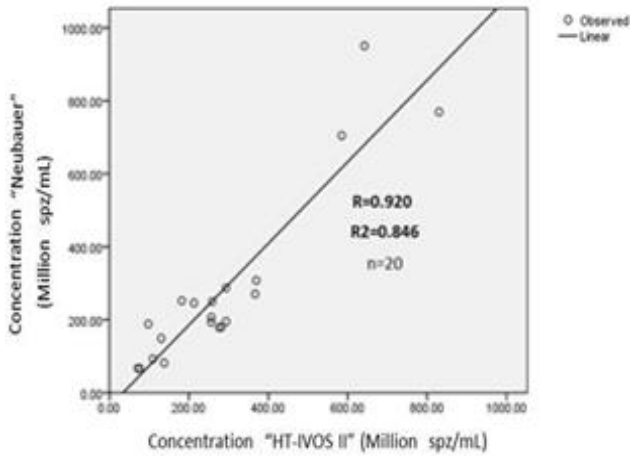
Linear regression between SPZ concentration analyzed by HT-IVOS II system "HBM: 140; AREA: 6 µm²" and Neubauer cell count. P>0.05



Linear regression between SPZ concentration analyzed by HT-IVOS II system "HBM: 140; AREA: 4 µm²" and Neubauer cell count. P>0.05



Linear regression between SPZ concentration analyzed by HT-IVOS II system "HBM: 120; AREA: 4 µm²" and Neubauer cell count. P>0.05



Linear regression between SPZ concentration analyzed by HT-IVOS II system "HBM: 120; AREA: 6 µm²" and Neubauer cell count. P>0.05

Descriptive Statistics

	Mean	Std. Deviation	N
CONC.HBM187A16	19.7650	39.99382	20
CONC.HBM140A6	274.6265	222.63613	20
CONC.HBM140A4	287.1665	237.23673	20
CONC.HBM120A6	281.7895	241.14032	20
CONC.HBM120A4	292.5410	248.19334	20
CONC.NB	286.4495	198.34548	20

Sperm concentration analyzed by HT-IVOS II system with 5 different setups "HBM-AREA: 187-16, 140-4, 140-6, 120-4 and 120-6" and by Neubauer cell count

Fig. 8: Correlation between the concentration by HT-IVOS II with different setups and by "Neubauer" counting

Table 2: Pearson correlation between SPZ concentration analyzed by HT-IVOS II system with 5 different settings "HBM-AREA: 187-16, 140-4, 140-6, 120-4, and 120-6" and the Neubauer cell count (n=20)

Correlations		CONC. HBM187A16	CONC. HBM140A6	CONC. HBM140A4	CONC. HBM120A6	CONC. HBM120A4	CONC. NB
CONC. HBM187A16	Pearson Correlation	1	.763**	.777**	699**	.734**	.792**
	Sig. (2-tailed)		.000	.000	.001	.000	.000
	N	20	20	20	20	20	20
CONC. HBM140A6	Pearson Correlation	.763**	1	1.000**	.981**	.985**	948**
	Sig. (2-tailed)	.000		.000	.000	.000	.000
	N	20	20	20	20	20	20
CONC. HBM140A4	Pearson Correlation	.777**	1.000	1	978**	.983**	949**
	Sig. (2-tailed)	.000	.000		.000	.000	.000
	N	20	20	20	20	20	20
CONC. HBM120A6	Pearson Correlation	.699**	.981**	.978**	1	.998**	.920**
	Sig. (2-tailed)	.001	.000	.000		.000	.000
	N	20	20	20	20	20	20
CONC. HBM120A4	Pearson Correlation	.734**	985**	.983**	998**	1	.929**
	Sig. (2-tailed)	.000	000	.000	.000		.000
	N	20	20	20	20	20	20
CONC. NB	Pearson Correlation	.792**	.948**	.949**	.920**	.929**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	
	N	20	20	20	20	20	20

** Correlation is significant at the level 0.01 (2-tailed)

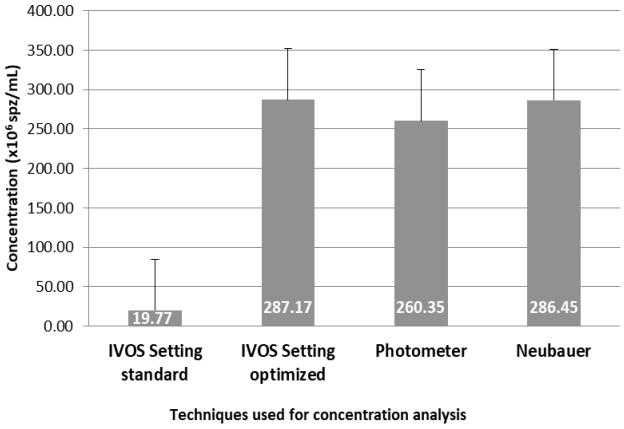


Fig. 9: SPZ concentration analyzed by HT-IVOS II system with standard and adjusted settings; HBM-AREA: 187-16, 140-4, and by SDM photometer calibrated for canine semen and by Neubauer cell count (n=20)

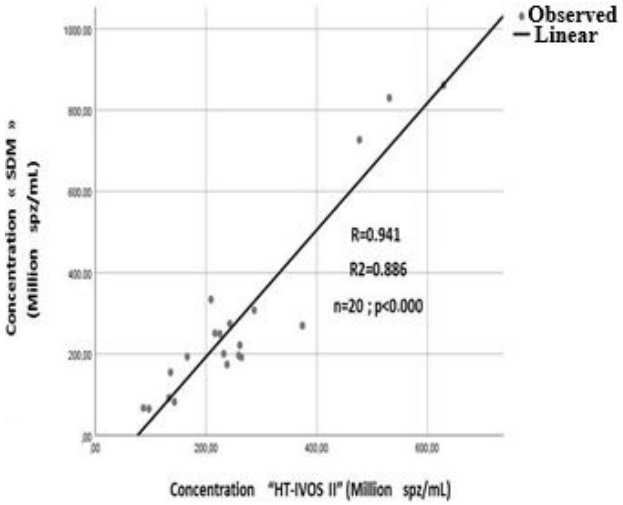


Fig. 10: Linear regression between SPZ concentration analyzed by HT-IVOS II

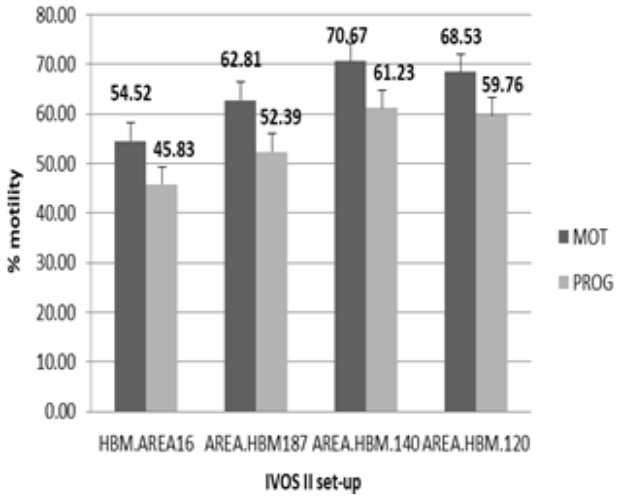


Fig. 11: Effect of 3 IVOS II setups on the percentages of total motility (MOT, %) and progressive motility (PROG, %) of canine SPZ

Comparison between HT-IVOS II "optimized setting" and "SDM" photometer for measuring concentration compared to the reference technique

Our data presented in Fig. 9 showed that the HT-IVOS II CASA system allowed obtaining an average concentration value (287.17 million SPZ/ml) much closer to the reference method (286.45 million SPZ/ml) than the photometer (260.35 million SPZ/ml), which deviated from the gold standard technique by 26 million, while the IVOS with adjusted settings only presented a difference of 0.5. The correlation study (Table 3) revealed that the IVOS II with an adjusted setup correlated perfectly with the reference technique "Neubauer count", and also highlighted a strong correlation between the adjusted HT-IVOS II and the "SDM" photometer with R = 0.981 and an R^2 = 0.886 (Fig. 10).

Table 3: Pearson correlation between SPZ concentration analyzed by HT-IVOS II system with "2 standard and adjusted settings; HBM-AREA: 187-16, 140-4", by SDM photometer calibrated for canine semen, and by Neubauer cell count (n=20)

Correlations		CONC.SDM	CONC.NB	CONC.HBM187A16	CONC.HBM140A4
CONC.SDM	Pearson correlation	1	.966**	.765**	.941**
	Sig.(bilateral)		.000	.000	.000
	N	20	20	20	20
CONC.NE	Pearson correlation	.966**	1	.792**	.949**
	Sig.(bilateral)	.000		.000	.000
	N	20	20	20	20
CONC.HBM187A16	Pearson correlation	.765**	.792**	1	.777**
	Sig.(bilateral)	.000	.000		.000
	N	20	20	20	20
CONC.HBM140A4	Pearson correlation	.941**	.949**	.777**	1
	Sig.(bilateral)	.000	.000	.000	
	N	20	20	20	20

** Correlation is significant at the level 0.01 (bilateral)

Effect of adjusted cell detection parameters (3 HT-IVOS II settings) on motility

Data in Fig. 11 showed that the percentages of motile "MOT, %" and progressive "PROG, %" SPZ analyzed by HT-IVOS at HBM values 120 (68.53%; 59.76%, respectively) and 140 (70.67%; 61.23%, respectively) and AREA 4 and 6 µm² agree with the total and progressive motility evaluated by conventional microscopic technique (70.5%; 59%, respectively) "according to WHO recommendations (2010, 2021)".

Discussion

For many years, CASA analysis has been a standard in the laboratory for motility and kinetic parameters (Şengül *et al*., 2024), with no clear description of a species-specific setup (Jorge-Neto *et al*., 2024). Despite advances in automation technology, CASA systems still require manual intervention to rectify errors and provide reliable results (Agarwal *et al*., 2021). In many andrology laboratories and research centers worldwide, the Hamilton-Thorne IVOS CASA is a popular device validated in several experimental works and proven effective in clinical use to reduce operator subjectivity and uncertainty in results (Lammers *et al*., 2014; Mortimer *et al*., 2015; Agarwal *et al*., 2021). This study aimed to optimize the cell detection parameters to correct errors related to SPZ concentration and motility analysis by the HT-IVOS II system. A preliminary study was conducted using the "auto-configuration" function of the CASA system, revealing the effect of two cell detection parameters (HBM & AREA).

HBM is the minimum light intensity with which the head of the SPZ must shine to be detected by the image analysis software. It is the contrast of SPZ head compared to the background of the image. For HBM, the manufacturer recommends a value of 187 for canine SPZ detection and 180 for bovine and equine SPZ. The minimum size of the SPZ head (AREA), also called "surface," is calculated as the area of an ellipse, which is the shape closest to the SPZ head. It is equal to the product of the two axes (small and large) of the ellipse multiplied by 3.14 (π). For AREA, also called "surface," the recommended value is 16 µm². This preliminary study suggested that the cell detection configuration recommended by the manufacturer (IMV Technologies) is questionable, as it is associated with poor detection, leading to an underestimation of the concentration. This raised interest in exploring these two cell detection parameters more closely to optimize them for analyzing canine SPZ by the HT-IVOS II system.

According to the HBM parameter, the optimum would be 120, as this value was closest to the concentration evaluated by the Neubauer method. However, given that this rate was obtained by setting the AREA at 16 µm² as recommended by the manufacturer, and by hypothetically admitting that these two factors can interact, the optimal brightness value for a size of 16 µm² might not be optimal for another AREA value. Therefore, it was useful to study the interaction of these two parameters. The value recommended by the manufacturer (187) for canine SPZ is, under our work conditions, unsuitable, as it is associated with a concentration value far from that obtained by the reference technique. Recently, O'meara *et al.* (2022) recommended a value of 168 for frozen bovine SPZ, although the initial recommended value was 180. Our result seemed to agree with these authors in the context of adapting the HBM value recommended by the manufacturer in the standard setting.

The AREA varied separately with HBM 120 and 140 did not reveal any statistical difference between the different values of "2-16" and the concentration value obtained by the reference technique. However, we noticed that the values closest to the reference were those of 4 and 6 µm² for HBM 120 and 140, respectively. It should be noted that this AREA value is associated with a significant underestimation of the concentration, accentuated by the HBM setup. Indeed, as part of our experiment, when this parameter was set to the lowest possible, i.e., 2 µm², the morphometric values (including AREA) systematically generated by our HT-IVOS II system, thanks to its

automatic analysis program morphology (without coloring) and viewable in playback on the photos captured and analyzed, showed that many canine SPZ detected as such by the IVOS II system have measured values of (AREA) lower than 16 µm². These observations were repeated so many times that it became almost obvious that this threshold of 16 µm² is too high and deserves optimization. Moreover, unlike the HBM parameter, which is a purely physical notion, the AREA is a morphometric entity that should be defined by explorations on fertile dogs SPZ. Several values have been reported in the literature, namely 5±2 µm² with an interval of 2-11 µm² (Iguer-Ouada and Verstegen, 2001), 8 µm² (Rijsselaere *et al*., 2005), and 6 pixels (Domoslawska *et al*., 2013; Belala *et al*., 2025a). No author, to the best of our knowledge, has reported or used a value as high as 16 µm² as a minimum detection threshold in the CASA system. The consequence of such a setting is the rejection by the system of all SPZ with an area (AREA) of less than 16 µm².

For the correlation study between the IVOS II and the Neubauer technique, given that the analysis of variance did not show a significant difference between the AREA values "2-16" (HBM187) and the Neubauer counting value included (despite the large differences observed between the rates), it would be concluded that ANOVA was not the appropriate test in this situation, as it was not very sensitive to the differences between these groups. In this case, it was necessary to apply correlation tests comparing the data in pairs. This correlation study showed that the two pairs of parameters most strongly correlated with the reference technique were HBM140-AREA6 and HBM140-AREA4. This last setup made it possible to obtain a concentration that perfectly overlaps the reference value with a 0.5-point difference. It seemed, under our work conditions, the optimal setup to recommend for the best cell detection of fresh canine SPZ in a clear environment by the HT-IVOS II system. According to Jorge-Neto *et al*. (2024), the correct use of the CASA system, coupled with a detailed description of the setup and procedure employed, facilitates the replication of methodologies and comparisons of studies.

In certain laboratories and canine reproduction centers, where the CASA system cannot be used as a reliable means of measuring concentration, the use of the photometer for rapid analysis is inevitable. Under such conditions, cell counting using a hemocytometer would be restrictive and time-consuming (Zhang *et al*., 2020), especially if it has to be carried out exactly as recommended in the 2010 WHO manual for the sperm analysis laboratory (WHO, 2010). Thanks to the adjustment of the cell detection settings carried out, the IVOS II analyzer was able to give an average concentration value much closer to the reference technique than the photometer. The correlation study, which has already shown that the IVOS with an adjusted configuration correlated perfectly with the reference technique "Neubauer count", highlighted in the current work a strong correlation between the adjusted IVOS II and the "SDM" photometer. In the same context, Brito *et al*. (2016) reported that sperm head detection is based on user-defined parameters (brightness and number of pixels); therefore, modifying these parameters might significantly impact concentration estimates.

Perfect concordance was recorded between total and progressive motility evaluated by conventional microscopy and the percentages of motile "MOT, %" and progressive "PROG, %" SPZ analyzed by HT-IVOS at values 120 and 140 for the HBM and 4 and 6 µm² for the AREA. This is in agreement with our results on concentration, which favored these same values of HBM and AREA. This established effect of "HBM and AREA" on the kinetic analysis carried out by the HT-IVOS II confirmed our initial hypothesis that the CASA system must have good cell detection "concentration" to analyze motility with precision and accuracy. Indeed, poor cell detection affects not only concentration assessment but also motility analysis. This could be explained by the next paragraph.

Cell detection errors by excess when using thresholds that are too low or by default when thresholds are too high for AREA or HBM lead to an overestimation of the concentration in the first case and an underestimation in the second. The SPZ detected, due to analytical errors (Jorge-Neto *et al*., 2024), are no longer representative of the microscopic fields in which they were analyzed, violating the principle of SPZ homogeneous distribution in a cell suspension. This results in concentration and motility evaluation errors. If the system detects non-SPZ particles (with the size of a SPZ head), it will probably overestimate the population of static SPZ (Brito *et al*., 2016). Agarwal *et al*. (2021), reported in humans that CASA systems can have difficulties distinguishing between immotile sperm, non-sperm cells, and debris. This lack of distinction causes inaccurate evaluation of sperm motility as well as counting of spermatozoa, which also affects the evaluation of sperm concentration. It should therefore be noticed that the cell detection configuration has a certain effect on the kinetic parameters generated by the HT-IVOS II system. This finding is in agreement with what was recently reported for frozen bovine SPZ (O'meara *et al*., 2022).

The technical cell detection configuration (HBM-AREA) of the HT-IVOS II system significantly influenced SPZ concentration and motility analysis in dogs. The optimal HBM and AREA values allowing the best cell detection of canine SPZ by the HT-IVOS II system were: HBM: 140, AREA: 4 µm². Thanks to this adjustment, the HT-IVOS system would be a good alternative to the reference technique "Neubauer cell counting" in evaluating sperm concentration and also to the "SDM" photometer calibrated for canine semen. The IVOS II system could thus be used in the calibration of benchtop photometers commonly used in laboratories and canine artificial insemination centers. It therefore saves time and effort compared to cell counting. Finally, the authors suggest that the manufacturers of the CASA technique should ensure complete validation of the settings loaded on their systems to standardize and prevent random adjustments by users, leading to significant intra- and

inter-laboratory variability in the results, taking into consideration variable factors such as breed, genetics, species, age, climate, and nutrition.

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Conflict of interest

The authors declare that they have no conflict of interest.

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