Received: 20 September 2018 | Revised: 20 November 2018 | Accepted: 21 November 2018

DOI: 10.1111/and.13223

ORIGINAL ARTICLE

WILEY ANDROLOGIA

Combined effect of type and capture area of counting chamber and diluent on Holstein bull sperm kinematics

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Funding information
Ministerio de Economía y Competitividad; CONICIT AND MICITT Costa Rica

Abstract

The evaluation of sperm motion is crucial for processing of seminal doses for artificial insemination. Here, the combined effect of the type and capture area of three counting chambers, together with the type of diluent employed, on sperm motility was analysed. Ejaculates from thirteen Holstein bulls were used for sperm kinematic analysis with the ISAS®v1 CASA-Mot system, using two capillary-loaded counting chambers (Leja® and Cell-Vu®) and one drop displacement chamber (Makler®). Nine fixed positions were analysed per chamber type, considering central and lateral and three longitudinal fields. Independent of the diluent used, differences were found between the three chambers. Independent of the extender, no differences in x-axis were observed with Cell-Vu®, while using Leja®, some parameters showed lower values in the centre than in lateral areas. In both counting chambers, the lowest values were observed in the distal area. Results obtained with the two diluents were highly different with a very low correlation between them. In conclusion, the capture area inside the chambers leads to significant changes in sperm kinematic parameters and different dilution media introduce considerable differences in the motility patterns. It is necessary to optimise sampling methods and specific set-ups to be used with CASA-Mot technology.

KEYWORDS
bull sperm kinematics, CASA-Mot system, counting chamber, sampling

1 | INTRODUCTION

Cattle are a fundamental resource in human economy, basically because of their use for milk and meat products. This has led to an intensive artificial selection throughout human history to obtain high-quality animals in order to meet different purposes (Felius et al., 2014). Currently, cattle breeding involves the use of various forms of assisted reproductive techniques (ART) (Velazquez, 2018). The use of cryopreserved seminal doses for artificial insemination is, right now, the most widely used ART in this and other species (Correa, Pace, & Zavos, 1997; Lyashenko, 2015; Waberski, Petrunika, & Töpfer-Petersen, 2008). Other, more sophisticated techniques, such as intracytoplasmic sperm injection (ICSI) or embryo selection, are also used although to a lesser extent (Ohlweiler et al., 2013; Skrzyszowska et al., 2002).

The accurate evaluation of semen quality is fundamental to maximise the efficient use of seminal doses. However, even today, subjective semen analysis is commonly performed in many bull stud farms, which reduces the number of doses produced. In this context, it is not an uncommon practice to make approximations to the closest 5% value when analysing both sperm concentration and sperm motility (Al Naib, Hanrahan, Lonergan, & Fair, 2011). The progressive introduction of computer-assisted semen analysis (CASA) systems in the production lines has considerably

improved the processing of semen samples, offering higher consistency in results (Broekhuijse, Šoštarić, Feitsma, & Gadella, 2011; Vyt et al., 2004; Yániz, Silvestre, Santolaria, & Soler, 2018). In any case, the correct use of CASA technology must be associated with optimised protocols to provide valuable and reliable information for the final calculations in dose production (Amman & Waberski, 2014; Bompart et al., 2018; Yeste, Bonet, Rodríguez-Gil, & Rivera del Álamo, 2018). There are three main aspects to consider when optimising automated semen analyses, namely, the type and depth of the counting chamber (del Gallego et al., 2017; Gloria et al., 2013; Soler et al., 2012), the dilution media (Awad, 2011; Büyükleblebici et al., 2014) and the frame rate of image acquisition (Castellini, Bosco, Ruggeri, & Collodel, 2011; Valverde et al., 2018).

The aim of the present work was to analyse the differential sperm distribution and motility characteristics within the capture area in three different commercial counting chambers (Cell-Vu®, Leja® and Makler®) and the effect of two different commercial dilution media (Biladyl® and Andromed®) on this distribution, with a view to optimising the use of currently available CASA-Mot technology.

2 | MATERIAL AND METHODS

2.1 | Semen collection and processing

This study was performed on Holstein bulls (n = 13, 1.5–7 years old), regularly employed in artificial insemination (AI) under a regime in which two ejaculates were collected per week. Animals were housed in Xenética Fontao AI Centre, S.A. (Lugo, Spain), following all European Union regulations for animal husbandry.

Within 5–10 min of semen collection by artificial vagina, samples were assessed for volume in a conical tube graduated in 0.1 ml subdivisions and gross motility determined by placing 20 µl of fresh semen on a pre-warmed slide at 37°C, using a cover slide of 20 × 20 mm. All ejaculates were split into two aliquots, one processed with a commercial egg yolk extender (Biladyl®, referred to as BLD) and the other with a soy lecithin-based extender (Andromed®, referred to as ADM) (both from Minitube GmbH, Tiefenbach, Germany). The semen aliquots were diluted in a two-step procedure when using the BLD extender, and in one step when the ADM extender was used, to a final concentration of about 100×10^6 spermatozoa/ml, using as a reference value that was estimated during gross motility analysis. After dilution, samples were slowly cooled to 4°C at a linear rate of −0.3°C/min in a refrigerator and maintained at this temperature during 4–5 hr for equilibration.

The refrigerated samples were packaged in 0.25-ml straws (IMV Technologies, L'Aigle, France) with an automatic straw filling and sealing machine (MRS1; IMV Technologies), and they were immediately frozen by using a programmable freezer (Digitcool 5300; IMV, L'Aigle, France) with the following curve: 4°C to −10°C at −5°C/min, −10°C to −100°C at −40°C/min and −110°C to −140°C at −20°C/min; and then plunged into liquid nitrogen for storage.

For the assessment of motility, two straws per sample were thawed in a water bath at 37°C for 30 s, and then, the contents of the straws were emptied in a test tube kept at the same temperature in a dry bath. In order to collect uniform sperm subsamples and avoid inaccuracies, the semen was mixed gently before collecting aliquots for further analyses.

2.2 | Sperm motility evaluation

Samples were analysed for kinematics by using the CASA-Mot system ISAS®v1 (Integrated Semen Analysis System, Proiser R+D, S.L., Paterna, Spain). The equipment consisted of a microscope (Nikon Eclipse E600; Tokyo, Japan) equipped with a heated stage set at 38°C and a 10× negative phase-contrast objective. A video digital camera (Proiser 782M) was mounted on the microscope to capture images and transmit them to a computer. The array size of the video frame grabber was 768 × 576 × 8 bits and 256 grey levels. Resolution of images was 0.84 µm per pixel in both the horizontal and vertical axes. The frame rate used was 30 fps, capture time one second, with the tail detection facility activated for ignoring nonsperm particles, with a particle area between 14 and 80 μm^2 and a connectivity value of 14 µm.

Sperm parameters analysed were concentration and total motility (%), whereas the kinematic parameters were average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), wobble (WOB, %), straightness (STR, %) and linearity (LIN, %).

After dilution, each sample was analysed in three different chambers: Leja® 4 chamber (L4; 20 µm depth; prod. code SC-20-01-04-B; Leja®, IMV Technologies, L'Aigle, France), Cell-Vu® sperm counting chamber (CVD; 20 µm depth; prod. code DRM-600; Millennium Sciences, Inc., NY, EEUU) and Makler® counting chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel). All chambers were pre-warmed at 38°C, and each was loaded with the amount of diluted semen and using the loading procedures recommended by the manufacturer. Each slide was maintained on the heated stage of the microscope for 30 s before analysis to prevent possible passive movement of liquid in the chamber. Nine fields were captured for each analysis of the samples, and all the assessments were completed within 2 min. All the captures followed the same pattern, recording the position in the microscope stage (Figure 1). The order of analysis among counting chambers was randomised.

2.3 | Statistical analyses

Data were examined for normality of distribution and homogeneity of variance and analysed by general linear model (GLM) repeated-measures procedure to determine if there were differences among mean values of the three counting chambers and the two extenders for each kinematic variable, which were tested independently. Mathematically, the model may be expressed as follows: $Y_{ijklm} = \mu$

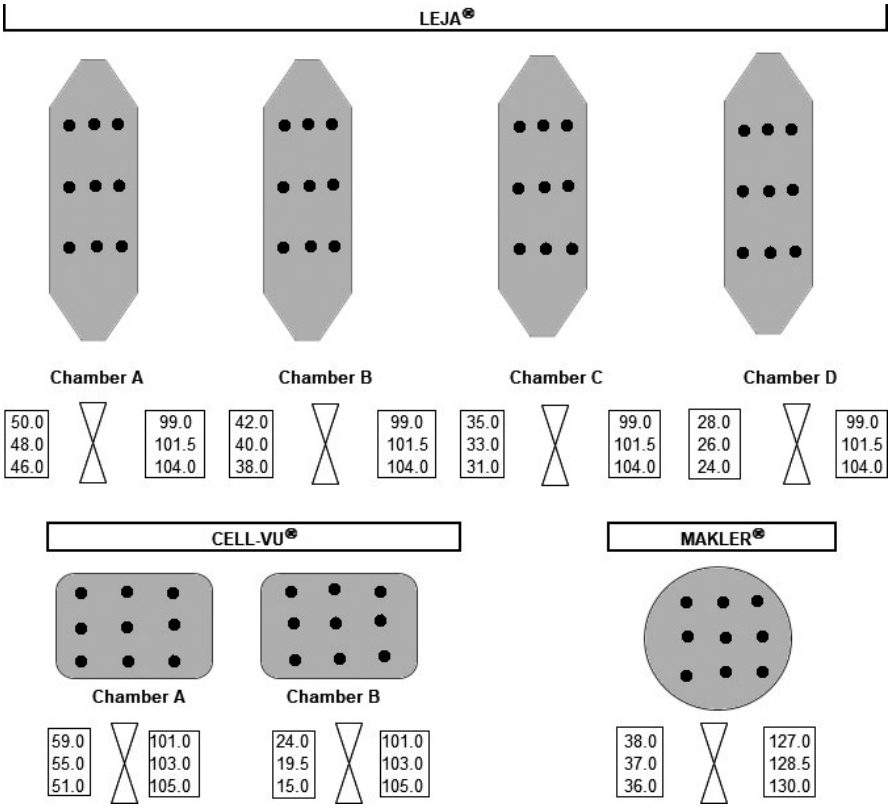

FIGURE 1 Microscope stage micrometric positions analysed in each of the counting chambers used in this study. The left column for each counting chamber indicates the *x*-position and the right the *y*-position of the stage. A total of nine fields were analysed per sample, named in Table 1 for lateral and Table 2 for central x-positions and Tables 1–3 for proximal, central and distal positions regarding the drop deposition for the y-positions

$+ A_i + C_j + E_k + P_l + CE_{(jk)} + \varepsilon_{ijkl}$. Here, Y_{ijklm} is the "m"th value of individual "*i*" measured with counting chamber "*j*," on the extender "*k*" and the (x_i, y_i) position "*l*"; "μ" is the overall mean; "A_i" is a random effect describing variation between individuals; "C_j" is a fixed effect of counting chamber; "E_k" is a fixed effect of the extender; "P_l" is a fixed effect of the x_i, y_i position in counting chamber area describing variation between x_i, y_i positions; "$CE_{(jk)}$" is a interaction effect between counting chamber and extender; and "ε_{ijkl}" is the residual variation. If differences were detected among factors for each kinematic variable, Bonferroni post hoc tests were used to determine the pairwise directional differences between counting chambers and extenders. Results are reported as the mean ± standard error of the mean (*SEM*). Data were considered to differ at $p < 0.05$ (i.e., Type I error was set at $\alpha = 0.05$). Pearson correlation was calculated for VCL values between diluents for counting chamber and between counting chambers for dilution media. All statistical analyses were performed with IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

TABLE 1 Effect of sperm counting chamber on concentration and total motility of bull spermatozoa as assessed by computer-assisted semen analysis (mean ± *SD*)

	Cell-Vu®	Leja®	Makler®
Concentration (×10^6)	30.6 ± 7.3[a]	26.9 ± 5.7[ab]	21.3 ± 7.2[b]
Total motility (%)	60.1 ± 14.9[a]	62.2 ± 14.2[ab]	67.1 ± 14.1[b]

Note. Values with different superscripts (a, b) differ significantly within row ($p < 0.05$), $N = 13$.

3 | RESULTS

Although the total sperm count in each bull sperm straw was, in theory, 25 × 10^6 spermatozoa, we found departures from this expected value when different chambers were used to estimate concentration of straws after thawing. The actual concentration was significantly higher with the Cell-Vu® chamber and lower with the Makler® one. Total sperm motility was significantly higher with the Makler® chamber and lower with the Cell-Vu® chamber, whereas the Leja® chamber showed no differences with regard to the other two chambers (Table 1). When comparing the field positions inside the chambers (for Leja® and Cell-Vu®), concentration and total motility showed no differences between positions, in both the vertical and horizontal axes (Table 2). Regarding the Makler® chamber, no differences were observed both for concentration and for total motility between the eight peripheral positions and the central position.

All the kinematic parameters were significantly lower, but with higher coefficient of variation (CV), when the BLD diluent was used, independently of the counting chamber used (Table 3). After dilution with the ADM diluent, the highest values for VCL and BCF and the lowest for LIN and WOB were observed when using the Makler® chamber, indicating an increment in the oscillatory movement with regard to the chambers loaded by capillarity. Other parameters showed no differences between counting chambers (Table 3). The use of BLD introduced much more variability in the three counting chambers. In this case, the highest VCL was observed with the Leja®, while all the other parameters were higher in the Makler® and lower in the Cell-Vu® chamber (Table 3). The effect of the interaction extender × counting chamber

TABLE 2 Mean (±*SD*) values for the concentration and total sperm motility from Holstein bulls on planar (x_i, y_i) chamber coordinates

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
Concentration					
Cell-Vu®	29.7 ± 10.23	31.6 ± 10.2	31.3 ± 7.0	30.1 ± 11.4	29.1 ± 10.0
Leja®	27.8 ± 6.35	27.2 ± 7.0[a]	27.7 ± 6.7	27.1 ± 7.6	26.3 ± 7.0
Total motility					
Cell-Vu®	58.4 ± 14.5	64.2 ± 16.0	61.1 ± 14.8	60.4 ± 15.7	58.8 ± 14.5
Leja®	63.3 ± 15.7	61.8 ± 13.2	64.8 ± 13.5	63.7 ± 13.4	58.2 ± 15.2

Note. No differences were observed.

TABLE 3 Effect of semen diluent and three sperm counting chambers on kinematic parameters of bull spermatozoa as assessed by computer-assisted semen analysis (mean ± *SD*)

	Andromed®			Biladyl®		
	Cell-Vu®	Leja®	Makler®	Cell-Vu®	Leja®	Makler®
n	3,862	4,269	4,079	4,073	4,032	3,963
VCL	105.8 ± 41.7[ab]	103.8 ± 43.5[b]	107.2 ± 39.2[a]	92.6 ± 40.9[b*]	101.0 ± 42.1[a*]	98.8 ± 39.2[a*]
VSL	52.3 ± 24.9	51.6 ± 26.3	51.3 ± 22.9	43.3 ± 25.6[b*]	49.2 ± 25.7[a*]	50.4 ± 24.6[a]
VAP	62.8 ± 23.5	62.6 ± 25.6	62.7 ± 21.6	55.0 ± 24.6[b*]	60.6 ± 24.5[a*]	61.6 ± 23.6[a*]
LIN	49.7 ± 18.6[ab]	50.0 ± 19.0[a]	48.8 ± 18.0[b]	46.1 ± 19.8[c*]	48.9 ± 19.2[b*]	51.8 ± 20.3[a*]
STR	80.0 ± 20.8	79.4 ± 21.0	79.6 ± 20.5	74.2 ± 23.3[c*]	77.6 ± 21.8[b*]	79.1 ± 20.5[a]
WOB	60.7 ± 12.8[b]	61.4 ± 12.9[a]	59.8 ± 12.2[c]	60.2 ± 13.2[c*]	61.2 ± 13.0[b]	63.6 ± 14.2[a*]
ALH	3.5 ± 1.4[a]	3.3 ± 1.4[b]	3.5 ± 1.3[a]	3.4 ± 1.5[b*]	3.5 ± 1.5[a*]	3.3 ± 1.4[b*]
BCF	13.1 ± 5.5[b]	13.8 ± 5.4[a]	14.0 ± 5.3[a]	10.8 ± 5.1[c*]	11.7 ± 5.1[b*]	12.3 ± 5.1[a*]

Note. Values with different superscripts (a, b, c) differ significantly within row and sperm diluent.
ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble.
*Indicates differences between diluents for chamber ($p < 0.05$).

TABLE 4 Mean (±*SD*) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of the Cell-Vu® chamber when using Andromed® diluent

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2,367	1,495	1,324	1,387	1,151
VCL	106.0 ± 42.1	105.2 ± 41.1	109.0 ± 43.3[a]	106.1 ± 40.7[a]	101.7 ± 40.8[b]
VSL	52.5 ± 24.7	51.5 ± 25.1	56.1 ± 27.0[a]	52.1 ± 23.7[b]	47.8 ± 23.1[c]
VAP	62.9 ± 23.5	62.4 ± 23.6	66.9 ± 25.6[a]	62.5 ± 22.3[b]	58.5 ± 21.8[c]
LIN	49.9 ± 18.2	49.3 ± 19.1	51.2 ± 18.4[a]	49.8 ± 18.7[a]	47.8 ± 18.6[b]
STR	80.4 ± 20.1	79.3 ± 21.9	80.2 ± 20.6	80.5 ± 20.9	78.8 ± 21.0
WOB	60.8 ± 12.7	60.5 ± 13.0	62.4 ± 12.3[a]	60.3 ± 13.1[b]	59.1 ± 13.0[b]
ALH	3.5 ± 1.4	3.4 ± 1.3	3.4 ± 1.3	3.5 ± 1.4	3.5 ± 1.5
BCF	12.9 ± 5.6	13.2 ± 5.5	13.5 ± 5.6[a]	13.2 ± 5.5[a]	12.6 ± 5.3[b]

Note. Values with different superscripts (a, b, c) differ significantly within row and X_i or Y_i coordinates ($p < 0.05$; values are mean ± *SE*).
ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble.

was significant for all kinematic parameters ($p < 0.05$) except for ALH ($p > 0.05$).

Regarding the location for the analysis in the chambers loaded by capillarity (Figure 1), independently of the dilution medium used,

TABLE 5 Mean (±*SD*) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i,y_i) coordinates of the Cell-Vu® chamber when using Biladyl® diluent

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2,639	1,434	1,331	1547	1,195
VCL	93.5 ± 40.8	92.5 ± 41.3	94.4 ± 42.6[a]	94.1 ± 39.6[a]	90.5 ± 40.6[b]
VSL	44.0 ± 25.7	43.1 ± 25.5	44.5 ± 27.0[a]	44.3 ± 25.1[a]	41.9 ± 24.5[b]
VAP	55.5 ± 24.5	54.7 ± 24.7	56.4 ± 26.2[a]	55.9 ± 23.8[a]	53.1 ± 23.8[b]
LIN	46.5 ± 20.1	46.1 ± 19.5	46.1 ± 20.3	46.7 ± 19.9	46.0 ± 19.2
STR	74.7 ± 23.3	74.6 ± 23.3	73.9 ± 23.3	75.3 ± 23.6	74.6 ± 22.9
WOB	60.3 ± 13.3	59.9 ± 13.0	60.3 ± 13.5	60.2 ± 13.1	59.8 ± 12.9
ALH	3.4 ± 1.5	3.4 ± 1.4	3.4 ± 1.5	3.4 ± 1.4	3.4 ± 1.5
BCF	10.8 ± 5.1	10.7 ± 5.1	10.7 ± 5.0[b]	11.0 ± 5.1[a]	10.4 ± 5.2[b]

Note. Values with different superscripts (a, b) differ significantly within row and X_i or Y_i coordinates ($p < 0.05$; values are mean ± *SE*).
ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble.

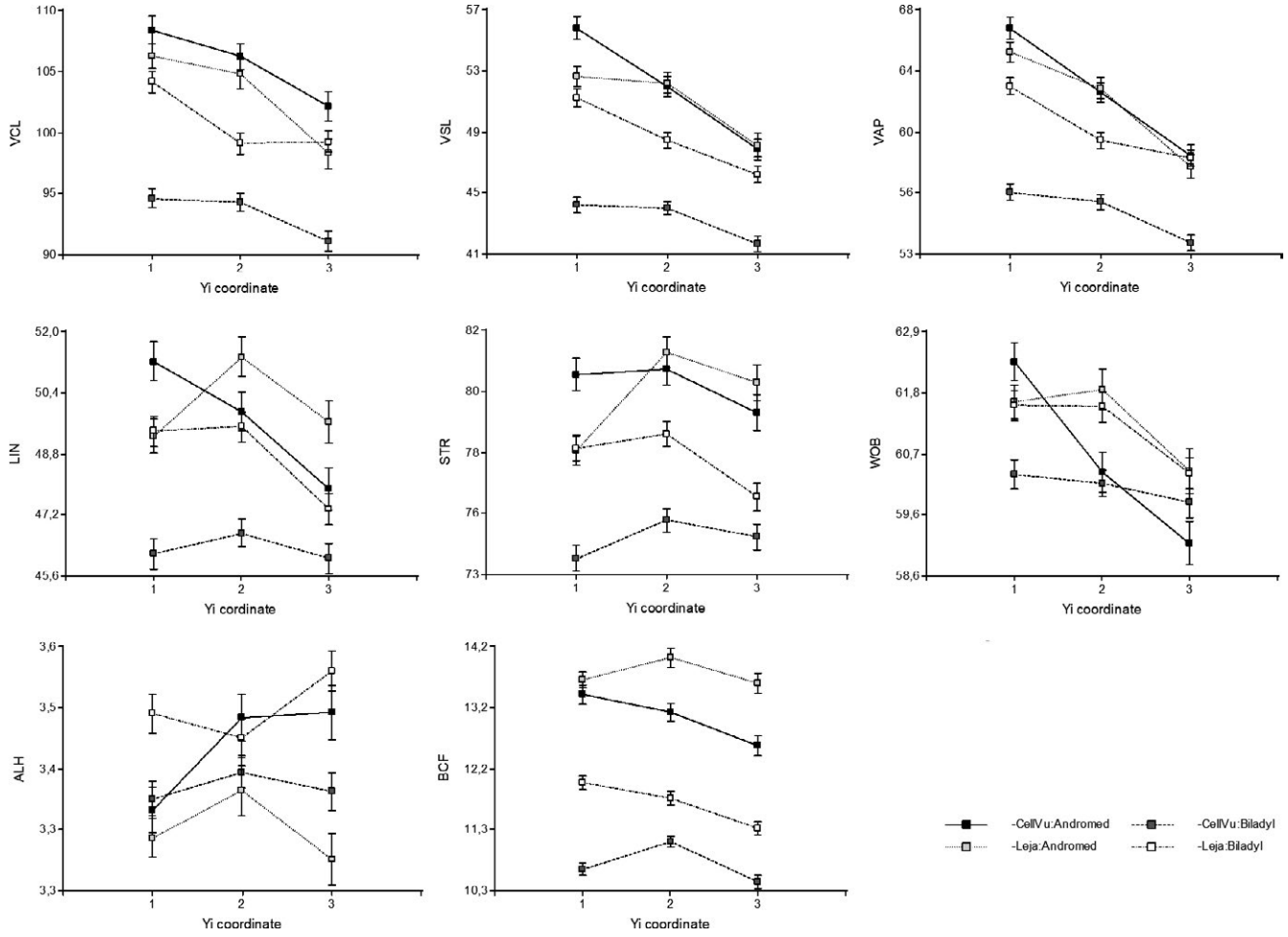

FIGURE 2 Kinematic parameters along a longitudinal distribution in the counting chambers loaded by capillarity. VCL: curvilinear velocity (μm/s); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz)

Cell-Vu® showed no differences between lateral and central areas, even though all the values were slightly higher in the lateral than in the central positions. Most of the kinematic parameters (VCL, VSL, VAP, LIN, WOB and BCF for ADM, and VCL, VSL and VAP for

TABLE 6 Mean (±*SD*) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of a Leja® chamber when using Andromed® diluent

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2,680	1589	1842	1,317	1,110
VCL	104.0 ± 43.5	102.5 ± 43.5	107.3 ± 43.9[a]	104.8 ± 44.0[a]	97.6 ± 41.7[b]
VSL	52.1 ± 26.3	50.7 ± 26.3	53.2 ± 28.3[a]	52.5 ± 24.6[a]	48.4 ± 24.7[b]
VAP	61.3 ± 25.4[b]	63.1 ± 25.7[a]	65.7 ± 27.4[a]	62.9 ± 24.0[b]	58.0 ± 23.7[c]
LIN	50.6 ± 19.1	49.8 ± 19.0	49.2 ± 19.6[b]	51.5 ± 18.5[a]	49.9 ± 18.8[ab]
STR	79.5 ± 21.0	79.9 ± 20.9	77.6 ± 21.9[b]	81.3 ± 19.7[a]	80.2 ± 20.8[a]
WOB	61.0 ± 13.0	61.8 ± 12.7	61.6 ± 12.9	61.9 ± 12.8	60.7 ± 13.1
ALH	3.3 ± 1.4	3.3 ± 1.3	3.4 ± 1.3	3.4 ± 1.5	3.3 ± 1.4
BCF	13.7 ± 5.4	13.9 ± 5.2	13.7 ± 5.3	14.0 ± 5.4	13.6 ± 5.5

Note. Values with different superscripts (a, b) differ significantly within row and X_i or Y_i coordinates ($p < 0.05$; values are mean ± *SE*).
ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble.

TABLE 7 Mean (±*SD*) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of a Leja® chamber when using Biladyl® diluent

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2,651	1,381	1,427	1,347	1,258
VCL	100.1 ± 42.0[b]	102.2 ± 42.2[a]	104.2 ± 43.8[a]	99.8 ± 41.7[b]	99.5 ± 40.3[b]
VSL	49.1 ± 25.9	48.9 ± 25.4	51.4 ± 27.7[a]	48.9 ± 24.7[b]	46.5 ± 24.5[c]
VAP	60.9 ± 24.5	60.2 ± 24.3	63.0 ± 25.9[a]	59.9 ± 23.9[b]	58.6 ± 23.2[b]
LIN	49.0 ± 19.3	48.2 ± 18.9	49.4 ± 19.8[a]	49.3 ± 18.2[a]	47.2 ± 19.5[b]
STR	77.5 ± 21.9	77.2 ± 21.5	77.8 ± 22.3[a]	78.3 ± 20.4[a]	76.0 ± 22.6[b]
WOB	61.4 ± 13.2[a]	60.7 ± 12.8[b]	61.6 ± 13.2[a]	61.4 ± 12.6[a]	60.3 ± 13.3[b]
ALH	3.5 ± 1.5[b]	3.6 ± 1.5[a]	3.5 ± 1.5	3.5 ± 1.5	3.6 ± 1.5
BCF	11.8 ± 5.1	11.6 ± 5.2	12.1 ± 5.3[a]	11.8 ± 5.0[a]	11.3 ± 5.1[b]

Note. Values with different superscripts (a, b) differ significantly within row and X_i or Y_i coordinates ($p < 0.05$; values are mean ± SE).
ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble.

BLD) were significantly higher in the area closest to the site of drop deposition and lower in the place far away from where the drop was placed. In the case of BLD, BCF was higher in the central position than in both the proximal and distal ones (Tables 4 and 5, Figure 2).

Similar results were obtained regarding central and lateral positions when the Leja® chamber was used. Nevertheless, when the ADM diluent was employed, VAP was significantly higher in the central position, whereas when using BLD diluent, VCL and ALH were higher in the central position and WOB was higher in the lateral one. Concerning the direction in which the drop progresses, highest values for VCL, VSL and VAP were observed in the proximal area with the lowest values in the distal one. For LIN and STR, the highest values were found in the central area and the lowest ones in the proximal area. A different pattern was observed with BLD diluent, with which the highest values for VCL, VSL, VAP, LIN, WOB and BCF were observed in the proximal areas and the lowest in the distal ones. Only STR had the highest values in the central area and the lowest ones in the distal area (Tables 6 and 7, Figure 2).

In the case of the Makler chamber, correlation values for VCL when assessed using different counting chambers were higher for the use of ADM diluent (0.99–0.96) than with BLD diluent (0.82–0.73). The highest correlation values were observed between the Leja® and Makler® chambers (Figure 3). Values of VCL correlated poorly between two diluents (0.23–0.10), independently of the counting chamber used (Figure 4).

4 | DISCUSSION

Although fertility is multifactorial and involves not only male effects, but others related to females, such as oocyte quality, oviductal

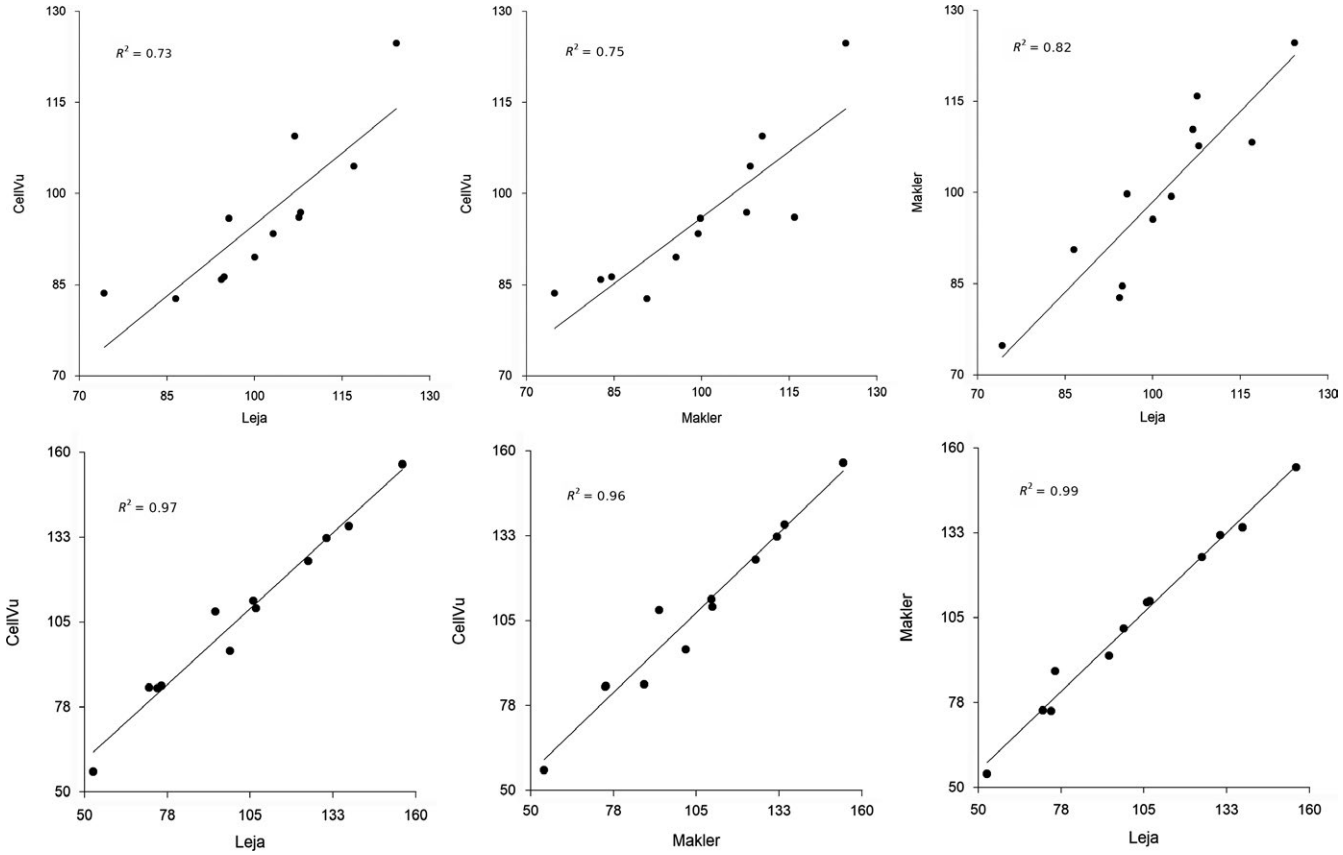

FIGURE 3 Correlation and regression analysis of VCL values between chambers. Upper row corresponds to Biladyl medium and lower row to Andromed

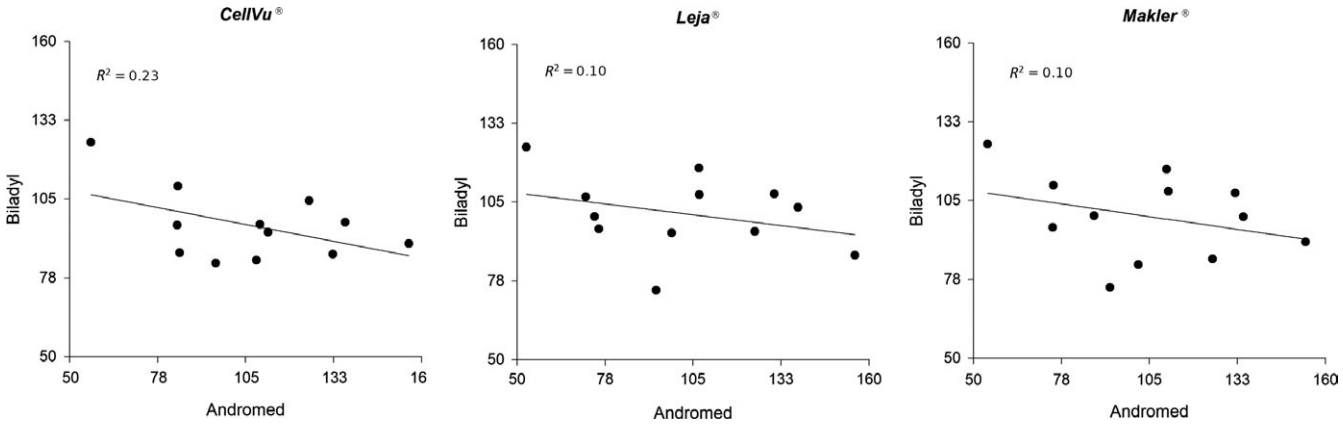

FIGURE 4 Correlation and regression analysis of VCL values between media (Biladyl and Andromed) in the different counting chambers

environment or time of insemination/fertilisation, among others (Utt, 2016), the correct evaluation of seminal characteristics is the first essential step in the preparation of seminal doses for ART (Amman & Waberski, 2014; Broekhuijse, Šoštarić, Feitsma, & Gadella, 2012). Two basic parameters have been considered as the best indicators of semen fertility, concentration and motility of spermatozoa. For a long time, the most popular technique for sperm counting involved the use of a haemocytometer (Eliasson, 1971). The improved Neubauer chamber has been accepted as the gold standard for the estimation of sperm concentration (Tomlinson et al., 2001; World Health Organization, WHO, 2010). On the other hand, it is still common to use wet preparations, placing sperm suspensions between a slide and a coverslip, for the assessment of motility (Del Gallego et al., 2017; Gloria et al., 2013). The introduction of the Makler® chamber resulted in the opportunity for faster sperm counting (Makler, 1978), even if there are discrepancies about its reliability (Bompart et al., 2018; Cardona-Maya, Berdugo, & Cadavid, 2008; Matson, Irving, Zuvela, & Hughes, 1999). The main problems with this chamber are related to the elapsed time between drop and cover placement, because it has been established that any delay in cover

placement causes great variations in the final results (Matson et al., 1999). Also, it is needed to consider the depth of this chamber is only 10 μm, what makes difficult a natural sperm movement and reduces the sampled volume. These reasons have led WHO to recommend the use of the haemocytometer for the estimation of sperm counts (WHO, 2010) and chambers of almost 20 μm depth for motility evaluation (Bompart et al., 2018).

The subjective analysis of motility in semen samples generates considerable variability in results (Al Naib et al., 2011), which has been largely overcome by the development of CASA technology during the 1980s (see Yániz, Soler, & Santolaria, 2015, Soler, Cooper, Valverde, & Yániz, 2016, Bompart et al., 2018, Gallagher, Smith, & Kirkman-Brown, 2018, and Yániz et al., 2018, for reviews of CASA history). CASA systems afford high accuracy, repeatability and large amounts of quantitative data but, nonetheless, they require a strict setting to achieve reliable and comparable results (Contri, Valorz, Faustini, Wegher, & Carluccio, 2010; Verstegen, Iguer-Ouada, & Onclin, 2002). The effect of repeated collection from the same animal on semen quality has been well documented in previous work and is not considered here (Valverde et al., 2016).

Regarding the evaluation of sperm quality, two of the main factors affecting concentration, motility and kinematic parameters are the counting chamber and the dilution media used. With regard to counting chambers, there are two main physical principles that are relevant for their use, capillarity (Cell-Vu® and Leja®, in the present study) and drop displacement (Makler®, as used here). In the first case, the differential capillary forces along the track and the different resistance close to the borders (lateral positions) regarding the centre are the reasons for dissimilar distribution that is dependent on the species (Bompart et al., 2018). Another factor particularly important in chambers loaded by capillarity is related to the shape of the counting area, because some of them can introduce turbulence in the fluid, due to their design, as in the case of the Leja® chamber (Bompart et al., 2018). These two factors are not so relevant in drop displacement chambers (as Makler® or Spermtrack®), but taking into account, in the case of the Makler chamber, the limitations aforementioned. In addition, chambers vary in their depths, being 10 μm (Makler®) or 20 μm (Cell-Vu® and Leja®), with greater depths being unavailable because of the optical limitations of microscopes. Both aspects, loading principle and depth, can thus affect the final results of motility analysis, including some of the differences we have found here (Bompart et al., 2018; Del Gallego et al., 2017; Gloria et al., 2013).

Concerning concentration, in a previous work on bull semen in different CASA system, no differences were observed between the three chambers used (Gloria et al., 2013). This is in contrast to the differences observed here and in other previous studies (Bailey et al., 2007; Hansen et al., 2006; Hoogewijs, et al., 2012), which can be attributable to the dissimilar sampling areas considered in the different studies.

With regard to total motility, other studies in diverse CASA systems agree with our results, showing higher motility with the Makler® chamber than with the Leja® slides (Contri et al., 2010; Gloria et al., 2013; Lenz, Kjelland, VonderHaar, Swannack, & Moreno, 2011). Furthermore, other studies in goat (Del Gallego et al., 2017), human (Soler et al., 2012), ram (Palacín, Vicente-Fiel, Santolaria, & Yániz, 2013) and stallion (Hoogewijs et al., 2012) spermatozoa, based on different CASA-Mot systems and counting chambers, showed that the motility and kinematic parameters observed in capillary chambers presented lower values than those observed in their drop displacement counterparts. These results appear to indicate that the drop distribution principle is more important than species differences, or the actual brand of the counting chambers or the CASA-Mot system. In this sense, it is possible that loading by capillarity disrupts in some way sperm motility as a consequence of the resultant fluid flow, because capillary action may damage the sperm tail and thus affect sperm movement (Lenz et al., 2011; Palacín et al., 2013) and vitality (Gloria et al., 2013) in comparison with drop displacement counting chambers (del Gallego et al., 2017; Hoogewijs et al, 2012). Nevertheless, the highly significant regression of VCL values observed here between capillary and droplet displacement chambers suggests that a possible toxic effect of the adhesive or the paint used for the serigraphy of the chambers is not a likely explanation for the differences in kinematic parameters as was previously proposed (Gloria et al., 2013). Furthermore, it was interesting that different depths of the counting chambers (10 μm for Makler® and 20 μm for Leja® and Cell-Vu®) showed high correlation for VCL values, which is not in agreement with results obtained with the use of a 3D lensless microscopy and CASA-Mot for boar semen (Soler et al., 2018).

It is necessary to point out that most of the earlier work developed on potential effects of counting chambers has not considered the area in which counts were performed. In the work of Gloria et al. (2013), only the centre and the edges of the Leja® chamber were taken into consideration, but what exactly these positions refer to is not clear. Another study using slides and coverslips, and analysing sperm motility along the equatorial area of the preparation with another CASA system, showed differences just in the fields close to the border, but not in the other sampling areas (Nöthling & dos Santos, 2012). A study considering differences in ram sperm motility between central and peripheral areas, when a slide and a coverslip were used, revealed higher values in the central area for total and progressive motility, VCL and VAP (Palacín et al., 2013). In the present work, the coordinates of the microscope stage were well defined and used repeatedly to obtain a strict sampling model for analyses, revealing no differences in motility and kinematics between the edge and the centre but showing variation along the length of the chamber capture area. The highest values were obtained close to the place where the drop was deposited and the lowest at the end of the fluid movement, which cannot be completely explained by Poiseuille flow and the consequent Segre–Silberberg effect (Kuster, 2005) or by the possible effect of surface tension on the perimeter of the coverslip (Lenz et al., 2011), thus requiring alternative explanations. When counting chamber design allowed a defined linear sampling, some species showed no motility differences along the counting

area (human, Soler et al., 2012), but others agreed with the results observed here (goat, Del Gallego et al., 2017; fox, Soler, García, Contell, Segervall, & Sancho, 2014), indicating that these differences are species-specific and require a biological explanation.

Regarding the effect of diluents on sperm kinematics, previous studies have shown that the increase in the percentage of egg yolk in the diluent elevates the viscosity inducing a decrease in sperm velocity and progressive motility (Aires et al., 2003; Hirai et al., 1997). Our results agree with these observations because the use of Biladyl (egg-based medium) showed lower velocity and linearity than when Andromed (lecithin-based medium) was used. In an apparent contradiction, it has been reported that VCL is higher with the use of egg yolk (Triladyl) than with egg yolk-free media (TCM-199 and Ham's F-10), but these results were obtained using a different bull breed (Raseona et al., 2017).

In conclusion, under the conditions used in the present work, the use of different counting chambers leads to significant changes in estimation of sperm kinematic parameters. In addition, the use of different dilution media introduces differences in the motility patterns. All these results indicate the necessity of repeatable and representative sampling and to define specific set-ups to be used with CASA-Mot technology when different counting chambers or dilution media are used for obtaining reliable results in the calculation of seminal doses for artificial insemination programmes.

ACKNOWLEDGEMENTS

The CONICIT and MICITT, Costa Rica, is acknowledged for supporting AV. MINECO, Spain, supported DB. The authors acknowledge Eduardo R.S. Roldán for his critical revision of the manuscript.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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How to cite this article: Valverde A, Areán H, Fernández A, et al. Combined effect of type and capture area of counting chamber and diluent on Holstein bull sperm kinematics. *Andrologia*. 2019;51:e13223. https://doi.org/10.1111/and.13223