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Combined effects of type and depth of counting chamber, and rate of image frame capture, on bull sperm motility and kinematics

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ABSTRACT

Semen quality assessment requires accurate, reliable and objective methods for examination of sperm variables including sperm motility. For preparation of semen samples for artificial insemination, as a genetic resource, samples that are used for insemination need to have the capacity to result in a highly acceptable fertility rate. Several methods have been developed for evaluation of bull sperm in laboratory conditions and for preparation of doses for artificial insemination. Computer-assisted semen analyses can provide objective information on various sperm variables. Nevertheless, this equipment requires fine-calibrations considering differences among species, breeds and conditions for sample evaluation and data analyses. In the present study, there was examination of the interaction between factors such as image frame rate and type and depth of counting chamber in which sperm were evaluated, together with differences between bulls of four breeds. The use of the Spermtrack® reusable 10 µm-depth chamber provided more reliable results than results obtained using disposable chambers (10 and 20 µm depth). A capture rate of at least 90 fps is required for assessment of sperm motility percentage, whereas a rate of 250 fps is needed for obtaining consistent kinematic data. Differences among breeds in the present study indicate conditions for sperm analyses should include specific equipment calibrations for each breed. These results contribute to development of more precise conditions for assessments of bull sperm quality taking into account breed differences and the requirement each breed has for the adequate evaluation and preparation of samples for artificial insemination.

1. Introduction

Domestic cattle are an important source of protein for human nutrition for both its meat and its milk. This has led to maximising cattle production through the development of assisted reproduction programs which rely substantially on artificial insemination (AI) using cryopreserved semen samples (Donnelly et al., 1998; Velazquez, 2008; Verma et al., 2012; Ombelet and Van Robays, 2015). The large amount of variability in sperm quality and fertility among bulls (Valverde et al., 2016) is one of the problems affecting the extent of use of this biotechnology and an accurate test to assess and predict bull fertility has not been fully developed (Tanghe et al.,

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2002; Flint et al., 2003; Rodríguez-Martínez, 2006). Sperm evaluation is the basic technique for predicting the fertility that might result from use of semen samples and, as a consequence, for the optimization of the production of doses to be used in artificial insemination (AI; Hansen, 2014; Cojkić et al., 2017; İnanç et al., 2018).

Although there are many methods to evaluate semen quality, a subjective approach is still the predominant standard used on most farms. Among semen characteristics, sperm motility is considered one of the most accurate fertility predictors (Rodríguez-Martínez, 2007), but the accuracy of assessments is highly dependent on the technician's experience and ability to evaluate samples, thus leading to major inaccuracies (Christensen et al., 2005). Importantly, a biased assessment leads to incorrect estimates of sperm concentration and motility, which results in suboptimal insemination doses. This is a well-known problem that was identified long ago, when it was realized that different technicians and duplicate counts conducted by the same technician differ significantly in values for total sperm counts (Freund and Carol, 1964). For this reason, it is essential to standardize these analyses with the aim of reducing this variability (Chong et al., 1983; Bompart et al., 2018) and to introduce adequate quality control programs (Cooper et al., 2002)

To overcome these limitations, the computer assisted semen analysis (CASA) technology was developed in the 1980′s (Gallagher et al., 2018). This use of this technology provides accurate and significant information on the concentration, motility, morphology and other variables related to sperm quality, all of these based on objective metric evaluations (see Bompart et al., 2018 and Yániz et al., 2018a, 2018b for review). The CASA systems consist of a video-camera attached to a convenient microscope (phase contrast optics and warm stage) that allows for obtaining and sending of images (or image sequences) to a computer for analysis with a specially designed software (Didion, 2008; Broekhuijse et al., 2011). The output of CASA systems designed for motility analyses (CASA-Mot systems; Soler et al., 2016) includes variables such as percentages of total and progressive motile sperm and the calculation of a suite of kinetic variables of sperm swimming such as curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), wobble (WOB = VAP/VCL), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) (Didion, 2008).

Although CASA-Mot systems facilitate assessments of sperm concentration and sperm motility in comparison to use of the traditional manual method (Krause, 1995; Didion, 2008; Broekhuijse et al., 2011; van der Horst et al., 2018), the usage has some limitations that must be considered in relation to use both in clinics or the production of semen doses to be used for AI (Verstegen et al., 2002; Amann and Katz, 2004; Amann and Waberski, 2014; Simonik et al., 2015). Differences in results can be affected by observer (Gallego et al., 2018), sample dilution, software calibrations (Broekhuijse et al., 2011), video camera configuration and frame rate used for image capture (Valverde et al., 2019b, c), number of analysed fields (Soler et al., 2014) and the type and depth of counting chamber in which sperm are assessed (Le Lannou et al., 1992; Gloria et al., 2013; del Gallego et al., 2017; Soler et al., 2018; Valverde et al., 2019a). Hence, it is very important to define optimized protocols to obtain reliable semen analysis using CASA-Mot systems (Bompart et al., 2918; Holt et al., 2018; Yániz et al., 2018a, 2018b; Yeste et al., 2018).

One additional issue to consider is that related to species differences, which require calibrations adjusted for the species in question. Equally important is the need to consider intraspecific differences because breeds may differ in semen traits and the conditions for evaluation may also vary. It is not unusual to consider that techniques used to assess sperm variables in other species are valid for evaluation of sperm variables in bulls, such as the techniques used to assess dog sperm. It, however, has been reported that there is considerable and significant variability when the same techniques are used to assess sperm characteristics in dogs of different breeds (Soler et al., 2017; Valverde et al., 2019d), so the question arises as to whether it may be necessary to adjust conditions for semen analyses for different breeds of cattle to avoid the possibility of extrapolating inadequate data from one breed to another.

The aim of the present study, therefore, was to determine the optimal conditions regarding frame rate, type of counting chamber (disposable or reusable) and chamber depth (10 or 20 μm) for bull semen analysis using the CASA-Mot technology, and the effect of different breeds on these variables.

2. Materials and methods

2.1. Semen samples

In this study, there was the use of Holstein (n = 3), Rubia Gallega (n = 3), Limousin (n = 2) and Belgian Blue (n = 3) mature bulls regularly used in artificial insemination (AI) with a regimen in which two ejaculates were collected per week. Animals were housed at the Xenética Fontao AI Centre, S.A. (Lugo, Spain), complying with all European Union regulations for animal husbandry. Additional approval from an ethical committee to conduct this study was not required.

Semen samples were collected using a Vaseline-lubricated artificial vagina at 45 °C, these ejaculates were used as fresh semen for the study. Within 5–10 min of semen collection, samples were assessed for volume in a conical tube graduated with 0.1 ml subdivisions and gross motility was determined by placing 20 μL of fresh semen between a microscope slide and a 20 x 20 mm coverslip, both prewarmed to 37 °C. The samples were maintained at 37 °C and diluted in Optidyl (Cryo-Vet, IMV, L'Aigle, France) to a final concentration of about 25 million spermatozoa per millilitre prior to its use in the study.

2.2. Motility evaluation by CASA-Mot

The assessment of sperm motility and kinematics was conducted using the ISAS®v1 CASA-Mot system (Proiser R + D, Paterna, Spain), connected to an ISAS®-UOP200i negative phase contrast microscope equipped with a heating stage at 37 °C and using a 10X

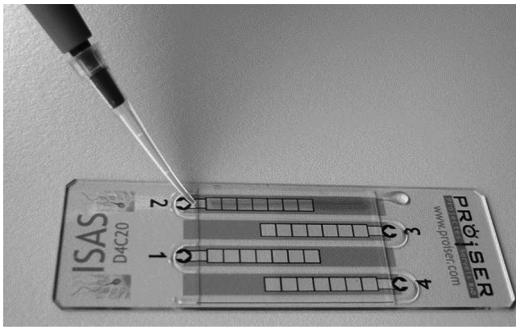

Fig. 1. Filling of disposable chambers.

objective (0.25 NA) (Proiser R + D). A high-speed camera (500 fps; M03-CM, Proiser R + D) was used for obtaining images. The array size of the video frame grabber was 648 × 488 × 8 bits and 256 grey levels. Resolution of images was 0.70 µm per pixel in both the horizontal and vertical axes. The tail detection facility of the system was activated for ignoring non-sperm particles, with a particle area between 20 and 90 μm^2 and a connectivity value of 10 µm. Track recognition mistakes were revised by the same technician (DB) and deleted, when needed, to avoid the introduction of distortions in the final results.

The sperm kinematic variables analysed were curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat/cross frequency (BCF, Hz).

2.3. Counting chambers

The effects of capillary-loaded, disposable, 10 and 20 µm depth chambers (ISAS® D4C10 and D4C20, respectively, Proiser R + D), and drop-displacement, reusable, 10 µm depth (Spermtrack®, Proiser R + D) counting chambers were assessed for all bulls, with the exception of Limousin bulls for which Spermtrack® chambers were not evaluated. All the chambers were pre-warmed to 37 °C in a heating plate before use. Disposable chambers were loaded by capillarity with a volume of 2 and 3 µl (for 10 and 20 µm depth respectively) maintaining the tip in contact with the coverslip until the fluid reached the end of the counting track (Fig. 1). Whenever possible, seven microscopic fields (one in each chamber square) were analysed. The reusable chamber was loaded placing a 2 µl drop in the centre of the base and immediately placing the cover, with no further movement of the cover. In this chamber nine fields were analysed (one in the centre and eight in the periphery). To avoid possible errors related to biases in examining samples in the different chambers, the counting chambers were assessed randomly. The total number of sperm cells analysed by sample and counting chamber was about 1000.

2.4. Frame rate effect

All semen samples were recorded at 500 fps frame rate (FR) for 1 s. This video was segmented into 25, 50, 100, 150, 200 and 250 FR videos. The command used for video segmentation was: [echo off: set fps = 25, 50, 75, 100, 150: for %%i in (.Ä*.avi) do (set fname = %%˜ni) & call: encodeVideo; goto eof :encodeVideo: ffmpeg.exe -i %fname%.avi -r %fps% -clibx264 -preset slow -qp 0″% fname%_(%fps%fps).avi"; goto eof].

Among kinematic variables, the VCL is the most sensitive to FR changes (Bompart et al., 2018; Valverde et al., 2018) and, therefore, this was the variable used for the calculation of the optimum FR.

2.5. Statistical analysis

Data obtained from the analysis of all sperm variables were first assessed for normality and homoscedasticity by using Shapiro-Wilks and Levene tests. A normal probability plot was used to assess for a normal distribution. When the kinematics variables of the sperm did not satisfy the normality requirement for a parametric analysis of variance, non-parametric analyses were performed using the Kruskal–Wallis test. When statistically significant differences were detected using this test, the non-parametric Mann–Whitney *U* test was used to compare pairs of values directly. The statistical model used was:

$$X_{ijk} = \mu + A_i + B_j + AB_{(ij)} + \varepsilon_{ijk}$$

Where X_{ijk} = Measured sperm kinematic variable; µ = Overall mean of variable x; A_i = Effect of breed B_j = Effect of counting chamber; $AB_{(ij)}$ = Effect of interaction between breed*counting chamber; ε_{ijk} = Residual.

For regression analyses, the effects of FR were assessed using an exponential model, in the form $y = \beta^{*}\alpha \exp(-\beta/x)$, where y is VCL and x is FR, α is the asymptotic level, β is the rate of increase to the asymptote, and exp is the base of natural logarithms. The biological significance of the equation is that the asymptotic values for α represent the maximum achievable when the FR is above the threshold value. The threshold value was calculated as the FR needed to obtain 95% of the maximum value. The rate of the approach

Table 1
Effect of frame rate (fps) on the asymptote of percentage of motility (α), rate of asymptote (β), and percentage of motility of bull sperm for the optimal (α) and other representative frame rates, using three different chambers.

	α^*	SE_α	β*	SE_β	MOT_α	MOT_{25}	MOT_{50}	MOT_{100}	MOT_{200}	MOT_{250}
Belgian Blue										
D4C10	60.80	9.99	1.17	8.81	59.64	58.02	59.39	60.09	60.44	60.52
D4C20	64.06	10.92	1.24	9.15	62.83	60.96	62.49	63.27	63.66	63.74
ST10	76.21	7.39	0.73	5.15	75.48	74.01	75.10	75.65	75.93	75.99
Holstein										
D4C10	69.06	2.02	0.64	1.55	68.42	67.31	68.18	68.62	68.84	68.88
D4C20	65.23	3.87	2.26	3.25	63.01	59.59	62.35	63.77	64.50	64.64
ST10	80.91	3.60	1.64	2.41	79.29	75.77	78.30	79.59	80.25	80.38
Limousin										
D4C10	75.93	0.56	1.19	0.39	74.75	72.40	74.14	75.03	75.47	75.57
D4C20	88.86	1.36	0.45	0.81	88.41	87.27	88.06	88.46	88.66	88.70
Rubia Gallega										
D4C10	45.05	6.94	1.45	8.31	43.62	42.51	43.76	44.40	44.72	44.79
D4C20	45.44	8.49	1.23	10.03	44.23	43.26	44.33	44.88	45.16	45.22
ST10	68.44	8.41	1.83	6.45	67.22	63.61	66.78	67.60	67.82	68.10
Total										
D4C10	61.51	3.87	1.07	3.36	60.45	58.93	60.21	60.86	61.18	61.25
D4C20	63.81	4.66	1.31	3.93	62.51	60.55	62.16	62.98	63.39	63.48
ST10	75.18	3.86	1.39	2.77	73.80	71.11	73.12	74.14	74.65	74.76

α = percentage motility; β = rate of increase; SE = standard error; MOT: total motility (%); subindex indicates frame rate; $^*P < 0.05$.
D4C10: disposable chamber; depth, 10 μm. D4C20: disposable chamber; depth, 20 μm. Sp10: Spermtrack® reusable chamber; depth, 10 μm.

to the asymptote represents the dependence of the curve on the FR; that is, a relatively greater value of β indicates a greater increase of VCL as FR increases and vice versa.

The results are presented as mean ± standard deviation (SD). Statistical significance was considered at $P < 0.05$. All data were analysed with IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, Il, USA).

3. Results

The optimal FR for the estimation of percentage of motile sperm was different both among breeds and among counting chambers. Limousin bulls had the greatest values and Rubia Gallega the least with similar conditions, while, in general, Spermtrack® optimal FR was greater than that with use of the disposable chambers. Whereas, in general almost 90 fps are required for assuring a reliable evaluation of bull sperm motility (Table 1).

Regarding kinematic variables, as represented by the values of VCL, the optimum FR also varied among breeds and among counting chambers, the maximum values were those observed for Rubia Gallega bulls using the reusable Spermtrack® (268.32 μm/s) and the minimum for Holstein bulls was with use of the disposable D4C10 (170.02 μm/s). In general terms, the optimal FR was greater with Spermtrack® and lesser with the disposable chambers. A FR of 250 fps, however, was needed for assuring a reliable measurement of values for kinematic variables in bull semen samples (Table 2).

The greatest differences in the optimal FR among breeds were observed when using the D4C10 counting chamber, with the greatest values being for Belgian Blue bulls and the least for Holstein bulls. With the D4C20 reusable chamber only the Holstein bulls had a lesser optimal FR in comparison with the other three breeds and there were similar results for the other breeds. With the Spermtrack® chamber, Rubia Gallega and Belgian Blue bulls had a greater optimal FR than the Holstein bulls (Fig. 2).

When the FR was set to 250 fps for all the breeds, VCL, VSL, VAP, LIN and STR were greater with reusable than with disposable chambers of 10 and 20 μm depths. Only the WOB was greater with use of the disposable chambers. The ALH and BCF was different when there was use of the different chambers (Table 3). For disposable chambers there were no differences among the seven fields (Fig. 1; Results not shown).

The data obtained using the ISAS®D4C20 were used for comparisons among breeds (Table 3). There were the greatest VCL and VSL values for Limousin and the least with Holstein bulls. There were similar results when there was evaluation of BCF values. The most linear sperm movement (LIN and STR) was observed in Rubia Gallega, followed by Limousin and Belgian Blue, with Holstein bulls being the least. In contrast, there was the greatest WOB for Holstein sperm and the least with Rubia Gallega bulls. For the ALH, there were no biological differences (less than 10%) among breeds (Table 3).

4. Discussion

Currently, there are about 12 CASA-Mot commercial systems on the market. Most of these systems are based on the identification of sperm head centroid position (x_1, y_1) in the first image of the sequence that must be linked with the new (x_2, y_2) position in the second one, continuing in the same way until the last image (Bompart et al., 2018). Differences between CASA-Mot systems are based on the fact that for each system there are specific algorithms for identification of the sperm head centroid and susbsequent kinematics

Table 2
Effect of frame rate (fps) on the asymptote of curvilinear velocity (α), rate of asymptote (β) and curvilinear velocity for the optimal (α) and other representative frame rates.

	α^*	SE_α	β^*	SE_β	VCL_α	VCL_{25}	VCL_{50}	VCL_{100}	VCL_{200}	VCL_{250}
Belgian Blue										
D4C10	243.98	3.03	17.33	0.91	231.00	121.98	186.89	213.53	223.73	231.31
D4C20	223.26	2.27	17.18	0.75	206.72	112.29	158.34	188.02	204.88	208.43
ST10	258.45	1.74	19.21	0.46	239.94	119.86	176.00	213.28	234.78	239.33
Holstein										
D4C10	170.02	2.16	13.31	0.86	157.21	99.83	130.28	148.83	159.07	161.20
D4C20	194.81	2.45	13.19	0.82	182.06	114.94	149.64	170.74	182.38	184.80
ST10	231.82	1.88	17.07	0.58	215.36	117.11	164.77	195.44	212.85	216.52
Limousin										
D4C10	231.38	2.65	15.54	0.80	216.35	124.27	169.57	198.08	214.08	217.43
D4C20	231.98	1.96	16.63	0.61	215.93	119.28	166.34	196.44	213.47	217.05
Rubia Gallega										
D4C10	199.42	3.81	17.36	1.42	182.79	99.58	140.92	167.64	182.84	186.04
D4C20	218.57	3.11	16.31	1.01	202.85	113.83	157.73	185.68	201.45	204.76
ST10	268.32	1.34	20.37	0.37	248.70	118.79	178.53	218.87	242.33	247.32
Total										
D4C10	213.26	1.59	15.80	0.53	198.03	113.35	155.48	182.09	197.06	200.20
D4C20	218.18	1.24	16.17	0.40	202.59	114.26	157.89	185.60	201.23	204.51
ST10	255.73	0.93	19.14	0.26	237.29	118.92	174.39	211.18	232.39	236.88

α = curvilinear velocity; β = rate of increase; SE = standard error; VCL = curvilinear velocity, μm/s; subindex indicates frame rate; $^*P < 0.05$. D4C10: disposable chamber; depth, 10 μm. D4C20: disposable chamber; depth, 20 μm. Sp10: Spermtrack® reusable chamber; depth, 10 μm.

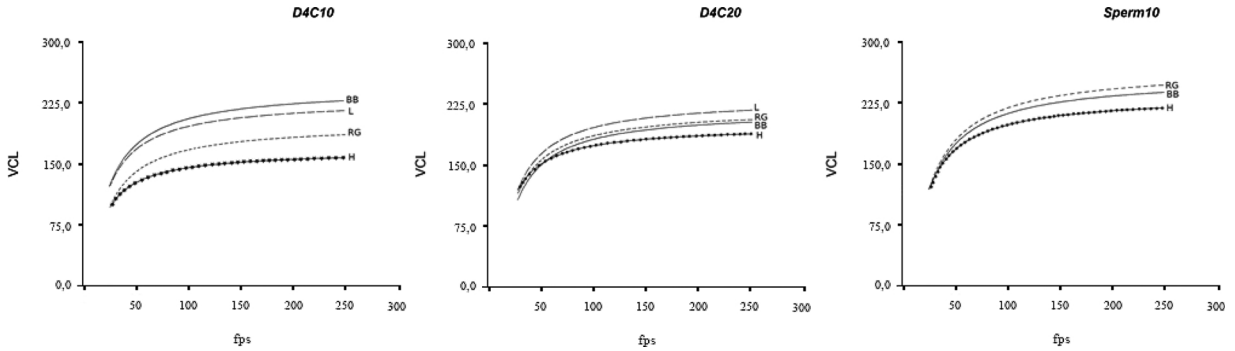

Fig. 2. Effect of frame rate on bull sperm curvilinear velocity (VCL, μm/s) in three different counting chambers. D4C10: disposable chamber; depth, 10 μm. D4C20: disposable chamber; depth, 20 μm. Sp10: Spermtrack® reusable chamber; depth, 10 μm. Breeds were: BB, Belgian Blue; L, Limousin; RG, Rubia Gallega; H, Holstein.

calculations (Holt et al., 2007). Of particular importance is the method used for resolving sperm track crossings, which is affected by estimates of sperm concentration and also depends on counting chamber depth (Lu et al., 2013; Bompart et al., 2018; Caldeira and Soler, 2018; Valverde et al., 2019a).

From the early stages in the development of CASA-Mot technology, the accuracy in measurements for some kinematics variables was directly related to the FR used, that is, the number of images taken during a defined time (Davis and Katz, 1992; Mortimer and Swan, 1995, 1999, 2000), because some variables, particularly VCL, have marked changes between relatively lesser and greater FR and capture time (Contri et al., 2010; Castellini et al., 2011; Gallego et al., 2013; Caldeira et al., 2019; Valverde et al., 2019b,c). For the optimal FR in a variety of species, the optimum FR is species-specific and depends on the speed and kind of sperm movement. In general, there are important differences between the optimal FR value for assessments of percentage of motile cells in the sample and that for the kinematics variables based on analyses of VCL. Regarding estimates of percentage of motile sperm, a rate of 25 fps is sufficient for boar sperm (Valverde et al., 2019b), while for several fish species it varies according to the depth of the counting chamber and lens magnification: 42–62 fps for the eel, 82–94 fps for salmon and 97–100 fps for sturgeon (Caldeira et al., 2019). With regard to VCL values, the optimal FR for mammalian species was between 97 (Castellini et al., 2011) and 139 fps for human sperm (unpublished results), with there being 200 fps for rams (Castellini et al., 2011), 212 fps for boars (Valverde et al., 2019b), 150 and 250 fps for mice (Soler et al., personal communication), and 292 fps for rabbits (Catellini et al., 2011). In fish, and depending again on chamber depth and lens used, the ranges were 179–203 fps for eels, 253–260 fps for salmon and 208–228 fps for sturgeon (Caldeira et al., 2019).

The values obtained in the present study for percentages of motile bull sperm (62–75 fps) were greater for the optimal FR when compared with those for boar samples. In reference to VCL values, the results in the present study (255 fps) were much greater than those previously reported for this species (160 fps, Catellini et al., 2011) using a reusable chamber in both the present and previous

Table 3
Kinetic variables of bull sperm from different breeds when assessed with three different counting chambers and with a frame rate capture of 250 fps.

	n	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Belgian Blue									
D4C10	332	233.0 ± 4.6^{ax}	47.4 ± 2.0^{ax}	199.0 ± 4.3^{bx}	22.3 ± 0.8^{ax}	27.6 ± 1.0^{ax}	83.7 ± 0.6^{ax}	1.2 ± 0.01^{ax}	44.4 ± 0.9^{ax}
D4C20	571	208.2 ± 3.5^{bx}	47.2 ± 1.5^{ax}	175.8 ± 3.3^{ax}	23.7 ± 0.6^{ax}	29.3 ± 0.8^{ax}	82.0 ± 0.4^{ax}	1.1 ± 0.01^{bx}	40.6 ± 0.7^{bx}
ST10	385	252.4 ± 4.2^{cx}	55.7 ± 1.5^{bx}	202.6 ± 4.2^{bx}	22.6 ± 0.8^{ax}	29.8 ± 0.9^{ax}	78.2 ± 0.6^{bx}	1.3 ± 0.01^{cx}	42.3 ± 0.7^{abx}
Holstein									
D4C10	257	171.9 ± 5.2^{ay}	44.0 ± 2.4^{ax}	141.7 ± 4.3^{ay}	25.6 ± 0.9^{aby}	31.9 ± 1.1^{ay}	81.4 ± 0.6^{ay}	1.1 ± 0.02^{ay}	38.5 ± 1.0^{ay}
D4C20	376	188.9 ± 4.3^{by}	42.9 ± 2.0^{ax}	161.1 ± 3.6^{by}	23.5 ± 0.7^{ax}	28.0 ± 0.9^{bx}	84.4 ± 0.5^{by}	1.1 ± 0.01^{ax}	40.3 ± 0.8^{ax}
ST10	312	227.3 ± 4.3^{cy}	62.9 ± 2.0^{bx}	182.8 ± 3.7^{cy}	27.6 ± 0.8^{by}	34.5 ± 0.9^{ay}	79.9 ± 0.6^{ax}	1.3 ± 0.01^{bx}	41.1 ± 0.9^{ax}
Limousin									
D4C10	257	225.5 ± 4.7^{ax}	67.4 ± 2.2^{ay}	188.3 ± 4.2^{ax}	31.6 ± 0.9^{az}	38.8 ± 1.1^{az}	82.5 ± 0.6^{axy}	1.2 ± 0.02^{ax}	45.2 ± 0.9^{ax}
D4C20	474	220.8 ± 3.4^{ax}	59.4 ± 1.6^{by}	182.9 ± 3.1^{ax}	27.4 ± 0.7^{by}	34.0 ± 0.8^{by}	81.5 ± 0.4^{ayz}	1.2 ± 0.01^{ay}	43.1 ± 0.7^{ax}
Rubia Gallega									
D4C10	150	189.3 ± 6.4^{ay}	59.7 ± 2.9^{az}	158.2 ± 5.3^{ay}	32.8 ± 1.0^{az}	40.8 ± 1.2^{abz}	81.7 ± 0.7^{axy}	1.1 ± 0.02^{ay}	38.9 ± 1.2^{ay}
D4C20	249	207.1 ± 5.1^{ax}	60.1 ± 2.3^{ay}	169.1 ± 4.2^{axy}	30.2 ± 0.8^{az}	38.8 ± 1.0^{az}	79.7 ± 0.5^{az}	1.2 ± 0.02^{by}	41.2 ± 0.9^{abx}
ST10	205	252.9 ± 5.3^{cx}	79.1 ± 2.2^{by}	193.2 ± 4.8^{bxy}	31.1 ± 0.8^{az}	41.0 ± 1.1^{az}	75.7 ± 0.7^{by}	1.3 ± 0.01^{cx}	43.1 ± 1.1^{bx}
Total									
D4C10	996	208.7 ± 2.7^{b}	53.5 ± 1.2^{a}	175.3 ± 2.4^{a}	27.1 ± 0.5^{a}	33.6 ± 0.6^{a}	82.5 ± 0.3^{a}	1.1 ± 0.01^{a}	42.2 ± 0.5^{a}
D4C20	1670	207.3 ± 2.1^{b}	51.6 ± 0.9^{a}	173.5 ± 1.8^{a}	25.7 ± 0.4^{b}	31.7 ± 0.4^{b}	82.1 ± 0.3^{a}	1.1 ± 0.01^{a}	41.3 ± 0.4^{a}
ST10	902	243.8 ± 2.6^{a}	63.5 ± 0.6^{b}	193.6 ± 2.4^{b}	26.3 ± 0.5^{ab}	34.0 ± 0.6^{a}	78.3 ± 0.3^{b}	1.3 ± 0.01^{b}	42.0 ± 0.5^{a}

Values are means ± standard error.
D4C10: disposable chamber; depth, 10 µm. D4C20: disposable chamber; depth, 20 µm. Sp10: Spermtrack® reusable chamber; depth, 10 µm.
VCL = curvilinear velocity; VSL = straight-line velocity; VAP = average path velocity; LIN = linearity (LIN = VSL/VCL); STR = straightness (STR = VSL/VAP); WOB = wobble (WOB = VAP/VCL); ALH = amplitude of lateral head displacement; BCF = beat cross frequency; $P < 0.05$.

study. This inconsistency can be explained because, even following the same distribution of the sample inside the counting chamber, the brands used were different. In addition, the video cameras were also different: that used in the previous study had a FR of 200 fps (Catellini et al., 2011) whereas in the present study there was a 500 fps camera used, thus increasing the capabilities in the present study. The results from the present study for bull sperm were similar to that for boar samples when there was use in both species for sperm analysis the same disposable counting chamber of a 20 µm depth (Valverde et al., 2019a), but the values were greater than the corresponding values in human samples (unpublished results).

There are three primary variables related to the counting chamber effect, namely the design of the disposable chamber, way the chamber is loaded and chamber depth. Disposable chambers have the coverslip attached to the slide and are loaded by capillary action, whereas with the reusable chambers there is distribution of the semen drop by displacement when the weighted coverslip is placed on the base (Bompart et al., 2018). Most of the commercially available disposable chambers have different shapes, with variations in the calibre of the counting space, which results in differences in sperm distribution (Bompart et al., 2018; Valverde et al., 2019a), in part due to the Segré-Silberberg effect (Douglas-Hamilton et al., 2005a,b). When using the same type of disposable counting chamber used in the present study, sperm from several species, including bulls, were not different from the site of sample deposition to the end of the chamber [(human, Soler et al., 2012; goat, del Gallego et al., 2017)], whereas in other species such as the fox, there was a decrease in kinematic values in the last compared with the first part of the chamber (Soler et al., 2014). These differences among species, therefore, do not result from the chamber design but are related to species differences.

In the present study, there was a greater motility and kinematic values with use of reusable chambers, in comparison to results obtained with disposable chambers, which is consistent with what has been observed for bull semen analysis using another CASA-Mot system and counting chamber brands, assessing samples at 30 fps (Contri et al., 2010). In another study with bull semen using different counting chamber brands, and at 30 fps, there were differences but these were much less (Valverde et al., 2019a). Comparable results were obtained in research with goat sperm using a similar experimental design (del Gallego et al., 2017), and for stallion sperm with a variety of counting chambers (Hoogewijs et al., 2012), although in both studies with a lesser FR of 25 fps. There were no differences in sperm motility and progressiveness with use of both types of chambers in a study with ram semen analysed at 25 fps (Palacín et al., 2013). There were similar results for eel sperm when there was a study conducted at 30 fps (Gallego et al., 2013). Taken together, these results indicate the loading approach has an effect on sperm motility patterns, maybe due to capillary passive fluid displacement in a closed space (for disposable chambers, Douglas-Hamilton et al., 2005a; Valverde et al., 2019a) and/or interactions with the surface ions of the glass (particularly in the reusable chambers, Matson et al., 1999). It is difficult to conclude which conditions allow for determination of the actual sperm motility, and results of the present study indicate that it is not possible to translate the results obtained with use of one type of camera to results when there is use of another camera type and that this factor should be considered when producing semen doses.

The second aspect to consider is the depth of the counting chamber, which is related to the optical quality and magnification of the objective. With a 10X objective (0.25 NA) the theoretical depth of the field is about 14.4 µm, which is reduced to 5.2 µm with a 20X objective (0.4 NA). This implies a limitation in the depth of counting chambers, and, for this reason, the chambers have been built for a long time with a depth 10 µm to assure that all cells are in focus. This shallow depth constrains the movement of the cells that need more than 100 µm to have a natural motility pattern (Kraemer et al., 1998). This is particularly important for the correct

evaluation of hyperactivated spermatozoa (Morales et al., 1988; Shivaji et al., 1995). Unfortunately, it is not possible to effectively evaluate sperm motility with an optical microscope when there is a chamber depth and the consequence is that the counting chamber depth that can be effectively used for sperm motility evaluation is 20 μm. This results in some cells not being in focus, which is a limitation in the quality of the final analysis using CASA systems. The recent development of laser microscopy for sperm analysis allows for overcoming of this impediment (Soler et al., 2018), this in addition with the use of specifically designed microfluidic chambers can advance the new frontiers in sperm motility evaluation.

In the present research, analyses at 250 fps considering all the breeds together, indicated there were no differences in results when there was use of disposable chambers of 10 and 20 μm for VCL, VAP, WOB, ALH and BCF evaluations with only VSL, LIN and STR being greater with use of the 10 than 20 μm chambers. When considering the results of a previous study when there was use of the same type of counting chambers, but with capturing sequences at 25 fps, goat sperm had greater values of VCL, VAP and LIN when using 10 μm in comparison with those with use of a 20 μm chamber, whereas the value for VSL was greater with the 20 than 10 μm chamber (del Gallego et al., 2017). In boars, using a laser (at 100 fps) instead to an optic microscope, the increase in chamber depth (10, 20 or 100 μm) resulted in an increase in values of VCL, VSL, WOB and BCF, whereas there were no differences in LIN, STR and ALH with the use of the different chambers (Soler et al., 2018). All these data when considered together indicate sperm from different species have different motility patterns when placed in spaces with different depths. When chambers with greater depths become available, perhaps with greater chamber depths than 100 μm, it may become possible to differentiate sperm motility characteristics in different species in relation to cell size, tail flexibility, cell metabolism and other functional aspects (Soler et al., 2018).

5. Conclusion

The results of the present study lead to the recommendation that with bull sperm analysis using the CASA-Mot systems and reusable counting chambers there should be use of a frame rate of almost 90 fps for assessments of percentage of motility and of 250 fps for kinematic analyses. The results of this study also indicate, for the first time, that important differences exist in sperm kinematic variables among bull breed. With future investigations, there should be designing of research protocols and a breed-by-breed cut-off value should be considered for the production of seminal doses for artificial insemination taking into account the breed differences that were ascertained in the present study.

Overall, results of the present study indicate that there should be caution when considering results of previous studies where there was use of CASA-Mot technologies that relied on less advanced cameras because of the lesser frame rate values, and the conclusions drawn from some of these results should be re-evaluated. Perhaps some relationships between motility variables and fertility (either positive or negative) would no longer be valid with more sensitive techniques. The enhanced techniques used in the present study may allow for a more subtle identification of sperm subpopulations and allow for assessment of relationships between motility descriptors and fertility.

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Declaration of Competing Interest

The authors declare no conflict of interest. Some of the authors are working for the company that manufacture the CASA-Mot system and the counting chambers used but the results are not related with the possible interest and no publicity is contained in the manuscript.

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