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Assessment of an open-access CASA software for bovine and buffalo sperm motility analysis

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Del Prete, C., Blanco Prieto, O., Mislei, B., Iacono, E., Mari, G., Cocchia, N., et al. (2022). Assessment of an open-access CASA software for bovine and buffalo sperm motility analysis. *ANIMAL REPRODUCTION SCIENCE*, 247, 1-8 [10.1016/j.anireprosci.2022.107089].

Availability:

This version is available at: <https://hdl.handle.net/11585/904916> since: 2022-11-21

Published:

DOI: <http://doi.org/10.1016/j.anireprosci.2022.107089>

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This is the final peer-reviewed accepted manuscript of:

Del Prete C, Blanco Prieto O, Mislei B, Iacono E, Mari G, Cocchia N, Gasparrini B, Merlo B, Bucci D. Assessment of an open-access CASA software for bovine and buffalo sperm motility analysis. Anim Reprod Sci. 2022 Oct 6;247:107089.

The final published version is available online at:

<https://doi.org/10.1016/j.anireprosci.2022.107089>

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- 30 **Assessment of an open-access CASA software for bovine and buffalo sperm motility analysis**
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31 **Abstract**

32 The aim of this study was to verify the reliability of an open access CASA software (BGM) to evaluate
33 the sperm motility of cattle and buffalo, comparing motility and kinematic parameters to those of a
34 commercial one (HTM).Thirty frozen-thawed samples for each species were analyzed with both
35 HTM and BGM, after 1 hour of incubation at 37 °C. Sperm viability and mitochondrial membrane
36 potential (MMP) were evaluated through flow cytometric analysis. Agreement of all motility
37 variables between the two systems was assessed. Correlation analysis was performed to identify
38 relationships between motion parameters and sperm viability and MMP. Bland Altman analysis
39 showed good agreement between methods for all motility parameters except for curvilinear velocity
40 (VCL) in cattle, and for average path (VAP), VCL and (amplitude of lateral head displacement) ALH
41 in buffalo, that showed a proportional bias ($P>0.05$). In both systems, positive correlation between
42 both viability and high MMP and total and progressive motility of cattle spermatozoa were found;
43 viability and the sperm with high MMP were positive correlated only with VAP, straight-line (VSL),
44 VCL and ALH evaluated with HTM system. Different results were found for buffalo sperm motility
45 parameters, since viability had positive correlations and mitochondrial activity negative ones. Results
46 suggested that motility assessment performed by these two systems are comparable. The discrepancy
47 of VCL, VAP, and ALH could be due to the difference in the algorithms between software. The open-
48 access CASA plug-in is a reliable alternative to the expensive commercial CASA system for sperm
49 motility assessment in cattle and buffalo.

50 **Keywords:** Sperm motility; Computer assisted sperm analysis; Image J; Semen quality.

51

52 **1. Introduction**

53 Semen analysis is the basis of primary male fertility evaluation. The assessment of sperm
54 motility and kinematic parameters, such as average path velocity (VAP), straight line velocity
55 (VSL) and curvilinear velocity (VCL), is considered essential for evaluating sperm quality (Yániz
56 et al., 2018). Sperm motion parameters, along with sperm morphology and acrosome integrity, have
57 been reported to have a high predictive value of sperm fertilizing potential (Irvine et al., 1994;
58 Krause, 1995; Macleod and Irvine, 1995). Poor sperm motility has been correlated with low fertility
59 in both cattle and buffalo (Kjoestad et al., 1993; Farrell et al., 1998; Kathiravan et al., 2008; Puglisi
60 et al., 2012; Kumar et al., 2014).

61 The percentage of motile sperm have been routinely assessed by visual estimation using a
62 bright field microscope. Manual evaluation has proved to be slow, approximate, and basic (only
63 percentage of total and progressive motility). Moreover, it is highly sensitive to subjective errors
64 and need to train technicians with the goal of increasing repeatability of the estimation (Broekhuijse
65 et al., 2011). Computer-assisted sperm analysis (CASA) systems were developed in 80s for the
66 objective assessment of sperm motility in commercial andrology laboratories (Amann and Katz,
67 2004). Unlike subjective evaluation, CASA systems produce consistent and reliable results by the
68 analysis of more than 500 sperm cells for sample, tracking for each sperm cell the movement pattern
69 (Abaigar et al., 1999; Schmidt and Kamp, 2004). Those systems can extract individual motion
70 kinematic parameters and divide the overall sperm cell population in subpopulations of
71 spermatozoa with similar motility characteristics (usually rapid, medium fast, slow and immotile)
72 (Yániz et al., 2018) . Motility assessment by CASA systems quickly became a gold standard in
73 semen evaluation of many species (Amann and Katz, 2004). The high costs of the commercial
74 CASA systems cannot be afforded by many research groups and motivated Wilson- Leedy and
75 Ingermann to develop an open access plugin of Image J software that works as a CASA system
76 (Wilson-Leedy and Ingermann, 2007). This system analyzes videos that are recorded by a camera-
77 equipped microscope, that is a common laboratory equipment. In the first moment, the development

of this system and following implementation enabled various research studies on sperm motility of fish and marine invertebrate species (Purchase and Earle; 2012; Neumann et al., 2017; Caldeira et al., 2019). Only recently, the plugin was tested in mammalian species (Boryshpolets et al., 2015; Elsayed et al., 2015; Bucci et al., 2017; Giaretta et al., 2017). The only study performed on cattle spermatozoa modified the existing plugin to analyze motion of sperm cells in microfluidic environments (Elsayed et al., 2015). A recent study on stallion sperm motility revealed that the open access plugin can easily adapted to other mammalian species (Giaretta et al., 2017).

The aim of this study was to evaluate a setting of the open-access CASA-BMG plug-in of Image J software (BGM) for bovine and buffalo sperm analysis, comparing motility and kinematic parameters to those of a commercial one. Moreover, the correlations between sperm viability or sperm mitochondria membrane potential and motility parameters obtained from the two CASA systems were investigated.

2. Material and Methods

Thirty frozen-thawed ejaculates from 15 cattles and 30 frozen-thawed ejaculated from 12 buffaloes, coming from a commercial semen collection station were included in this study. Each sample was examined for motility and sperm motion parameters using both commercial Hamilton-Thorne IVOS Vers.12 (HTM) and BGM. Moreover, all samples were examined by flow cytometry analysis to assess sperm cells viability and sperm mitochondria membrane potential.

2.1 Semen processing

For each sample, two straws of 0.25 mL of the same batch were thawed for 30 sec at 37 °C in a water bath and pooled together. Samples were washed with 5 mL of Tris-glucose-citrate buffer (TGC) by centrifugation at 535 xg for 10 min to remove freezing medium. After supernatant removal, the pellet was resuspended with 1 mL of TGC to reach a concentration of 30×10^6 spz/mL. Ejaculates were stored in a portable incubator maintained at 37 °C-for 1 hour before evaluation.

2.2 Motility evaluation

For both commercial and non-commercial systems, after incubation, 5 μ l of well-mixed sample were loaded into a prewarmed chamber of a 4-chamber Leja® slide (Microptic S.L, Spain) kept at 37 °C. The percentage of total (TM; %) and progressive motility (PM; %), VAP (μ m/s), VSL (μ m/s), VCL (μ m/s), amplitude of lateral head displacement (ALH; μ m), straightness (STR; %) and linearity (LIN; %) were assessed with both systems (HTM and BGM).

For the HTM system, analysis settings evaluated sperm with minimum size of 8 pixels and classified sperm with STR 75 % and VAP 25 μ m/s as progressively motile and sperm with VAP below 24.9 μ m/s and VSL below 20 μ m/s as static. Sixty frames per second with minimum contrast of 35 were acquired. At least 500 cells were analyzed in no less than five randomly selected fields.

2.2.1 Motility evaluation with BGM system

The analysis was carried out as described in Giaretta et al. (2017). Briefly, to record video for the analysis a Leitz diaphan microscope (Wild Leitz GmbH, D6330, Wetzlar, Germany) with a 10x plan objective with negative phase-contrast was equipped with a Z31A Ascon technologic heated stage (Ascon technologic, PV – IT) and with a video camera, 3.1 megapixel CMOS USB 2.0 Infinity1-3 Camera (Lumen- era corporation, Ottawa, ON, Canada). Videos were recorded at a resolution of 800 \times 600 pixel and 60 frames/s (fps) and then converted to avi format by Infinity analyzing and capture software 6.4 (Lumenera corporation). For each sample, three seconds videos of five separate fields were recorded.

Each video was imported into Image J software, where CASA_bgm plugin had been previously installed. Only the central second of each video (60 to 120 frames) was imported and converted into greyscale 8-bit image. Subsequently, the threshold of each video was adjusted manually in order to highlight only the sperm heads and remove the background.

The system was set as follows: minimum of 25 and a maximum of 250 area pixels, minimum track length 20 frames; maximum sperm velocity 60 pixels/s. The cut-off of motion

parameters, VSL, VAP and VCL were set at, respectively, 10, 15 and 25 $\mu\text{m/s}$. The maximum VAP threshold values for slow and medium spermatozoa subpopulations were 30 and 50 $\mu\text{m/s}$. The frame rate was set at 60 fps and the conversion between pixels and microns was set as 500 microns per 1000 pixels. Finally, VAP and VSL were adjusted at 25 and 75 $\mu\text{m/s}$ to define progressive motility.

2.3 Sperm viability and mitochondrial membrane potential

Two different flow cytometry protocols were conducted to evaluate sperm viability and mitochondrial activity. The FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488 nm argon-ion laser and a 635 nm red diode laser was used in this study with the following filters: 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2), >670 long pass (far-red/FL-3) and 661/16 band-pass (orange far red laser/FL-4). Side scatter and forward scatter in logarithmic mode were used to back-gate and identify sperm population. A minimum of 10,000 events (sperm cells) was evaluated per sample. The BD CellQuest Pro software (Becton Dickinson) was used for instrument control and data acquisition. Settings were adjusted to each staining method.

Assessment of sperm viability was performed by determining the membrane integrity with a double fluorochrome staining with SYBR-14 and propidium iodide (PI) (LIVE/ DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy). Sperm samples diluted in 500 μL of Tyrode's medium (concentration 1×10^6 spz/mL) were stained with 5 μL SYBR-14 working solution (final concentration: 100 nM for cattle spermatozoa and 1 nM for buffalo) and with 3 μL of PI (final concentration: 12 mM) and incubated for 10 min at 37°C in the dark.

Flow cytometry analysis with 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbo- cyanine iodide (JC-1; Thermo Fisher Scientific, Waltham, MA, USA) was also performed to evaluate mitochondrial membrane potential (Ψm). JC-1 selectively enters into mitochondria, forming multimers when membrane potential is high (staining mitochondria with high membrane potential; HMMP) and emits orange fluorescence at 590 nm, detected by the FL-2

photomultiplier. In mitochondria with low membrane potential (LMMP), JC-1 maintains its monomeric form and emits green fluorescence at 530 nm, detected by FL-1 photomultiplier. For JC-1 staining, an aliquot of semen was diluted with Tyrode's medium to reach a concentration of 1×10^6 spz/mL (500 μ L) and incubated with 2.5 μ L of JC-1 (in DMSO; 1 mg/mL final concentration) at 37 °C for 30 min in the darkness. The percentage of cells with HMMP on the entire sperm population were calculated.

2.4 Statistical Analysis

All data were tabulated in an Excel sheet and were analyzed using the SPSS version 22.0 (IBM Corp., Chicago, IL, USA). Normal distribution was checked by the Shapiro Wilk test. As data were found to be not always normally distributed, non-parametric tests were applied. Intra-system variation of each parameter was calculated to determine the reproducibility of all variables, using the percentage coefficient of variation ($CV \% = SD/mean \times 100$). The CV was calculated for each sample analyzed and then an average CV was calculated for each system (BGM and HTM) in both species.

Bland-Altman analysis were used to assess the agreement between two CASA systems (Bland and Altman, 1999). The mean difference between values obtained by the two different systems and 95% confidence interval ($\pm 1.96 \times SD$ of the mean difference between the two methods) were calculated and a Bland-Altman plot (plot of individual differences against the mean) was produced. Moreover, presence of proportional bias (a significant association between difference and mean) was tested with linear regression analysis between difference and mean values of the two methods (Giavarina, 2015).

Spearman correlation was performed to analyze the relationship between motility parameters of each system (BGM and HTM) and viability (% of sperm with SYBR-14 +/- PI -) or percentage of spermatozoa with HMMP. In addition, Wilcoxon test was used to check differences between viability and percentage of spermatozoa with HMMP. In all cases, the level of significance was set at $P < 0.05$.

182 3. Results

183 Motility parameters of cattle and buffalo semen obtained with HTM and BGM are shown
184 in Table 1A and 1B, respectively.

185

186 **Table 1.** Sperm motility parameters and coefficient of variations (CV) of cattle (A) and buffalo
187 (B) frozen-thawed semen obtained by HTM and BGM systems expressed as median and
188 interquartile range (IQR).

<i>A</i>		<i>Cattle semen</i>		
Median (IQR)	HTM parameters	CV (%)	BGM parameters	CV (%)
TM (%)	30.5 (21.5-37.5)		32 (13.2-40.3)	
PM (%)	9.5 (6.7-15.2)		17.2 (7.5-22.5)	
VAP ($\mu\text{m/s}$)	98.9 (90.5-114.2)	40.3	72.8 (57.5-89.5)	65.5
VSL ($\mu\text{m/s}$)	76 (70.0-90.0)	47	60.5 (45.0-74.5)	72.4
VCL ($\mu\text{m/s}$)	191.7 (155.4-220.6)	42.4	145.4 (118.9-188.3)	63.0
ALH (mm)	8 (7-8.7)	44.3	5.5 (4.5-7.7)	69.1
BCF (Hz)	30.9 (29.8-33.1)	41	24.5 (23.6-26.0)	43.8
STR (%)	77 (74.0-81.0)	22.5	0.76 (74.0-79.0)	41.3
LIN (%)	43 (41.0-47.0)	38.1	40 (37.0-48.0)	56.1

189

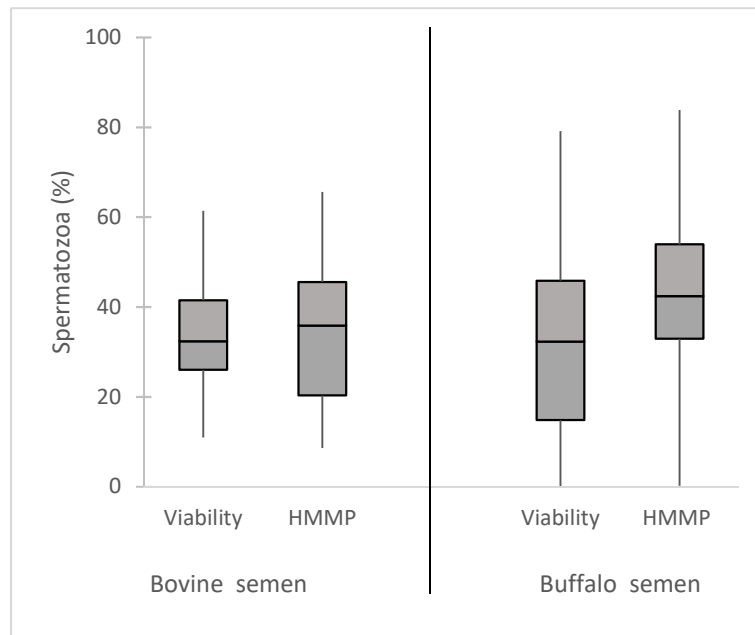
<i>B</i>		<i>Buffalo semen</i>		
Median (IQR)	HTM parameters	CV (%)	BGM parameters	CV (%)
TM (%)	45.0 (25.3-55.5)		36.3 (23.7-52.0)	

PM (%)	21.0 (9.0-27.0)		20.1 (20.1-30)	
VAP (µm/s)	96.3 (84.9-102.1)	44.8	67.3 (48.4-92.9)	48.4
VSL (µm/s)	79.5 (66.9-82.7)	50.3	52.8 (36.3-79.8)	53.4
VCL (µm/s)	161.0 (144.5-173.5)	47.5	125.4 (104.1-186.3)	51.4
ALH (mm)	6.0 (5.1-6.6)	50.3	5.1 (4.2-7.1)	60.6
BCF (Hz)	35.3 (33.7-38.9)	34.9	25.3 (24.2-27.9)	27.4
STR (%)	83.0 (77.0-86.5)	22.3	78.0 (74.0-80.5)	20.0
LIN (%)	50.0 (47.5-57)	35.5	48.0 (40.0- 51.0)	40.9

TM= Total motility; PM= Progressive motility; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; ALH = amplitude of lateral head movement; BCF = beat cross frequency; STR = straightness; LIN = linearity. Results are reported as median and Interquartile range (IQR).

Bland-Altman plots demonstrated good agreement between evaluation systems (HTM vs BGM) for the most of both cattle and buffalo sperm parameters, with at most two measurement pairs outside the limits of agreement (Supplementary files 1 and 2). A proportional bias was found for VCL of cattle spermatozoa ($P=0.035$) and for VAP, VSL, VCL and ALH ($P < 0.001$) of buffalo spermatozoa.

As showed in Fig. 1, results of sperm viability (intactness of plasma membrane) of frozen-thawed cattle and buffalo semen has a median (IQR) of 32,4 (26-41,5)% and of 12,8 (1,9-33,4) %, respectively. Meanwhile, the median (IQR) percentages of cattle and buffalo spermatozoa with HMMP (on the entire sperm population) were 35,9 (20,3-45,6)% and 40,5 (27,5-46,5) %, respectively (Fig. 1). No differences were found between viability and HMMP within each species ($P > 0.05$).



207

208 Fig. 1. Viability (percentage of sperm with SYBR14+/PI-) and high mitochondrial membrane
 209 potential (HMMP) of frozen-thawed cattle (n=30) and buffalo (n=30) spermatozoa as assessed by
 210 using cytofluorimetric determination of SYBR-14/PI and JC-1, respectively. For each box, the central
 211 line represents the median, the edges of the boxes represent the IQR (25th and 75th percentiles), the
 212 whiskers represent the extreme points.

213

214 Correlations between viability or percentage of HMMP and HTM or BGM parameters
 215 of cattle and buffalo semen are shown in Fig. 2 A and B. Significant positive correlations were
 216 found between viability or % of spermatozoa with HMMP of cattle spermatozoa and TM and
 217 PM of both systems (HTM and BGM; $P < 0.001$). Moreover, VAP ($P < 0.001$ for both), VSL
 218 ($P < 0.001$ for viability and $P = 0.009$ for HMMP), VCL ($P = 0.002$ for viability and $P = 0.01$ for
 219 HMMP) and ALH ($P < 0.001$) only obtained by HTM system were positively correlated with
 220 cattle sperm viability or % of spermatozoa with HMMP.

221 Motility variables of buffalo semen such as TM and PM evaluated through both HTM
 222 and BGM systems were found positively correlated with viability ($P < 0.001$ and $P < 0.05$, for
 223 HTM and BGM respectively). Moreover, VAP measured by HTM system showed a significant
 224 positive correlation with viability ($P = 0.029$) and VAP, VSL, VCL and ALH evaluated by BGM

system showed a significant positive correlation with viability ($P = 0.002$, $P = 0.002$, $P = 0.001$, $P = 0.001$, respectively).

HTM parameters PM, STR and LIN showed a significant negative correlation with HMMP spermatozoa ($P = 0.032$, $P = 0.033$ and $P = 0.013$, respectively). Finally, the % of spermatozoa with HMMP was negatively correlated with VAP ($P < 0.001$), VSL ($P < 0.001$), VCL ($P = 0.001$) and ALH ($P < 0.001$) evaluated by BGM method.

Fig. 2 Spearman correlation coefficient between HTM or BGM parameters and viability, and percentage of spermatozoa with HMMP evaluated through flow-cytometry in both cattle and buffalo semen. Color coding represents the correlation coefficient: large positive values (≥ 0.5) are green, intermediate values (0.5 to -0.5) are yellow, and small values (≤ -0.5) are red.

A.

		<i>Cattle semen</i>			
		HTM	BGM		
	1	Viability		1	
1	0,795**	<i>Sperm with HMMP</i>		0,795**	1
0,806**	0,848**	TM		0,571**	0,655**
0,701**	0,840**	PM		0,596**	0,619**
0,476*	0,631**	VAP		0,227	0,235
0,471*	0,632**	VSL		0,242	0,227
0,848*	0,554*	VCL		0,097	0,083
0,607*	0,623**	ALH		0,206	0,223
0,137	0,335	BCF		-0,040	-0,113

0,111	0,240	STR	0,091	-0,035
-0,087	0,099	LIN	0,350	0,311
<i>Sperm with HMMP</i>	<i>Viability</i>		<i>Viability</i>	<i>Sperm with HMMP</i>

236

237

B.

<i>Buffalo semen</i>				
HTM BGM				
	1	Viability	1	
1	-0,643**	<i>Sperm with HMMP</i>	-0,643**	1
-0,265	0,478**	TM	0,393*	-0,126
-0,400*	0,586**	PM	0,431*	-0,191
-0,210	0,406**	VAP	0,560**	-0,649**
-0,249	0,325	VSL	0,541**	-0,670**
-0,032	0,269	VCL	0,598**	-0,580**
-0,292	-0,097	ALH	0,586**	-0,618**
-0,224	0,309	BCF	0,310	-0,292
-0,398*	0,142	STR	-0,046	-0,133

-0,457*	0,296	LIN	0,133	-0,135
<i>Sperm with HMMP</i>	<i>Viability</i>		<i>Viability</i>	<i>Sperm with HMMP</i>

238

239 **P* <0.05; ** *P* <0.001; HMMP= *High mitochondrial membrane potential*; TM= *Total motility*; PM=
240 *Progressive motility*; VAP = *average path velocity*; VCL = *curvilinear velocity*; VSL = *straight line velocity*;
241 ALH = *amplitude of lateral head movement*; BCF = *beat cross frequency*; STR = *straightness*; LIN = *linearity*.
242

243

244

2454. Discussion

246 In this study the possibility to use CASA-BGM plug-in to assess both cattle and buffalo
247 sperm motility was explored for the first time.

248 Our results demonstrated that sperm motility assessments performed by HTM and BGM
249 are comparable. Although during the years the original open access CASA plug-in or its
250 variations were used to evaluate mammalian sperm motility (Boryshpolets et al., 2015; Elsayed
251 et al., 2015; Giaretta et al., 2017; Bucci et al., 2019; Nesci et al., 2020; Ortiz-Rodriguez et al.,
252 2021), only one study conducted on stallion semen aimed at demonstrating the agreement
253 between results obtained by commercial CASA system (HTM) and BGM (Giaretta et al., 2017).
254 The reliability of BGM results for cattle and buffalo spermatozoa was corroborated not only by
255 the agreement with HTM result but also by the similar coefficient of variation and by the
256 correlation with other sperm quality characteristics. In the only previous study conducted on
257 cattle semen, the accuracy of CASA plug-in was verified only by tracking sperm cells manually
258 (Elsayed et al., 2015). Our results provide a detailed description of a specific setting that can be
259 used to have reliable and accurate sperm motility results.

The major discrepancies between tested systems were found for kinetic parameters, VCL in cattle spermatozoa and for VAP, VSL, VCL and ALH for buffalo spermatozoa. In the stallion the only parameter that significantly differed between the two systems was BCF (Giaretta et al., 2017). A previous study that compared motility evaluation between CASA systems, showed differences especially for VAP, VCL and ALH (Holt et al., 1994). The cause could be attributed to the difference in the algorithms between software. It is important to note that differences in motion parameters were reported not only between commercial and open access devices but also between different commercial CASA systems (Holt et al., 1994; Boryshpolets et al., 2015). Each CASA uses a specific algorithm to identify all sperm and evaluate their motion features (Tomlinson et al., 2010), thus suggesting the researcher to use considerable caution in the direct comparison between different devices (Holt et al., 1996; Tomlinson et al., 2010). Moreover, the use of a different equipment (microscope, optics, camera), type of counting chamber or sample preparation (concentration, volume, medium) can influence motility parameters (Contri et al., 2010; Hoogewijs et al., 2012). In this study, to minimize the bias, a single semen sample at the concentration recommended for CASA analysis with the use of the same disposable slide (Leja chambers) were used with both systems.

To evaluate the repeatability of results, a coefficient of variation was calculated for all motion parameters of each method. The variation of all parameters of both devices tested in this study was higher than all the other studies comparing CASA systems (Holt et al., 1994; Giaretta et al., 2017). Those authors analyzed samples of fresh semen, whereas in our study frozen-thawed samples were used. Although in this study we used commercial samples, total and progressive motility resulted low. We can suppose that the presence of more dead spermatozoa or more particles of the frozen extender could produce more errors in the tracking evaluation. A previous study already suggested to perform additional replicates in samples with low motility in order to obtain correct results (Boryshpolets et al., 2015). Further study to comparing CASA systems in

the evaluation of different motility classes, including semen with low quality, could be carried out to increase the knowledge on CASA systems and to standardize CASA protocols.

A higher degree of variation for most of the parameters was registered in open access device than in commercial one, in agreement with data reported in stallion semen evaluation by Giaretta et al. (2017).

Evidence of positive correlations between motion parameters and sperm viability or mitochondrial activity consolidate the validation of BGM system. The associations between motility parameters and membrane integrity or mitochondrial activity of mammalian spermatozoa has been indeed proved numerous times (O'connell et al., 2002; Plaza Davila et al., 2015; Bucci et al., 2017). Similar positive correlations between both sperm viability or percentage of spermatozoa with HMMP and cattle TM and PM were found with HTM and BGM systems. This result corroborates the reliability of the evaluation of TM and PM with BGM. On the other hand, viability and the percentage of JC-1 positive sperm were positively correlated with velocities (VAP, VSL and VCL) and ALH of cattle spermatozoa evaluated only with HTM system. A decrease in the correlation between some motility parameters and mitochondrial activity have been, however, observed after freezing and thawing of semen (O'connell et al., 2002).

Different results were found for buffalo sperm motility, since viability had positive correlations and mitochondrial activity negative ones. Those correlations between various motility HTM and BGM parameters and HMMP are unexpected and not consistent with previous studies, as a higher percentage of HMMP is expected to be concomitant with an elevated sperm motility (Aitken, 1995; Kadirvel et al., 2009). It has been reported that the energy required for sperm motility is mainly produced by mitochondria respiration and MMP is the best indicator of mitochondrial energy status (Storey, 2004). While the cytofluorimetric analysis of MMP of spermatozoa with the use of JC-1 dye is considered a reliable method (Martínez-Pastor et al., 2010; Minervini et al., 2013), a combination of fluorescent dyes (e.g. PI/SYBR-14/ JC-1)

becomes particularly important especially for studies in which semen is cryopreserved (Garner and Thomas, 1999; Guthrie and Welch, 2006). Those protocols allow to exclude from the analysis nonviable cells, that may take to overestimate the percentage of sperm with HMMP (Guthrie and Welch, 2006). The results of current study were broadly consistent with previous results in the same race (Italian Mediterranean) of buffalo, that found values ranging between 33.4–43.6 % of viability and 45.2–64.6 % of sperm having HMMP (Minervini et al., 2013). A possible explanation of this paradoxical situation could be found in Storey (2008). As the author reports, in fact, many studies demonstrated, at least in cattle spermatozoa, that oxidative phosphorylation and glycolysis could sustain motility in a coordinated manner (mitochondrial activity coupled with glycolysis) or one in alternative to the other.

5. Conclusions

In conclusion, the use of BGM system requires the same training for new users as the commercial systems, but to obtain results, post-processing analysis is longer than the commercial CASA systems. On the other side, it is easier to set up for the analysis of different species and provides most of the useful sperm motion parameters, as obtained by commercial CASA systems. Our results confirm that this open-access plug-in is reliable and can be used by researchers as an alternative to the expensive commercial CASA systems to evaluate motility of cattle and buffalo semen.

Declarations of interest: none

CRediT authorship contribution statement

Chiara Del Prete: Conceptualization, Investigation, Data curation, Writing – original draft. **Olga Blanco Prieto:** Resources, Analysis. **Beatrice Mislei:** Resources, Analysis. **Eleonora Iacono:** Writing – review & editing; **Gaetano Mari:** Writing – review & editing. **Natascia Cocchia:** Writing – review & editing. **Bianca Gasparrini:** Writing – review & editing. **Barbara Merlo:** Conceptualization, Methodology, Supervision, Writing – review & editing; **Diego Bucci:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration.

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