Implementing an open-access CASA software for the assessment of stallion sperm motility: Relationship with other sperm quality parameters

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Abstract

Setting an open-access computer assisted sperm analysis (CASA) may benefit the evaluation of motility in mammalian sperm, especially when economic constraints do not allow the use of a commercial system. There have been successful attempts to develop such a device in Zebra fish sperm and the system has been used in very few studies on mammalian spermatozoa. Against this background, the present study aimed at developing an open-access CASA system for mammalian sperm using the horse as a model and based upon the Image J software previously established for Zebra fish sperm. Along with determining the sperm progressive motility and other kinetic parameters (such as amplitude of lateral head displacement), the "results" window was adjusted to simplify subsequent statistical analyses. The path window was enriched with colored sperm trajectories on the basis of the subpopulation they belong to and a number that allowed the sperm track to be associated to the sperm motility data shown in the "results" window. Data obtained from the novel plugin (named as CASA_bgm) were compared with those of the commercial CASA Hamilton-Thorn IVOS Vers.12, through Bland Altman's plots. While the percentage of total and progressive motile sperm, VCL, VAP, VSL, LIN and STR and ALH were in agreement with those obtained with the commercial system, BCF significantly differed between the two systems probably due to their settings. Interestingly, a positive and significant correlation between the percentages of total motile sperm evaluated through CASA_bgm and those showing high mitochondrial membrane potential evaluated by JC-1 staining was found. In conclusion, CASA_bgm ImageJ plugin could be useful and reliable for stallion sperm motility analysis and it is our aim to apply this system to other mammalian species.

Keywords: CASA; stallion; sperm motility; sperm quality

1. **Introduction**

At present, more than 12 different computer-assisted sperm analysis (CASA) systems are available for sperm motion detection in spermatology labs and in commercial semen production units (Amann and Waberski, 2014). The development of a powerful CASA software has made possible kinetic studies of spermatozoa and objective measurements of sperm movements (Verstegen et al., 2002).

The evaluation of sperm motility and other kinetic parameters such as curvilinear, straight line, and average path velocities, is an essential part of sperm quality examination in many mammalian species. Despite the immediacy and accuracy of these softwares, several investigators rely on non-automated analysis due to the high cost of commercial options. CASA systems historically evolved for commercial purposes and were initially sold to clinical laboratories to assess human sperm fertility (Amann and Katz, 2004). Later on, CASA systems were produced for stallion sperm analysis by Hamilton-Thorne in 1986, and soon after they were adapted to many species. In spite of being much widespread across research laboratories, “teaching the instrument” is still needed, as reported by Amann and Katz (2004).

The availability of an open-access, low cost CASA system could much benefit the analyses of sperm motility, especially for those that, due to economic constraints, may not afford the costs of a commercial device. In addition, the relevant information that a CASA system provides goes beyond a more objective evaluation of the percentages of total and progressive motile sperm. Indeed, some CASA systems give the individual kinetic parameters for a single sperm cell and this may be used for evaluating motile sperm population in differently treated samples (Abaigar et al., 1999; Flores et al., 2009, 2008; Miró et al., 2005, 2009; Schmidt and Kamp, 2004; Varner, 2008). In 2007, Wilson-Leedy and Ingermann developed a CASA software package that worked as a plugin to the United States National Institutes of Health (NIH) Image J software (Wilson-Leedy and Ingermann, 2007). Successively, more than hundred scientific studies used the plugin to assess fish or invertebrate sperm motility. In particular, Purchase and Earle (2012) implemented the original plugin, creating a new one that permits the automation of some video processing steps. Very few studies applied the system to mammalian sperm motility evaluation. Elsayed et al. (2015) used the plugin to study sperm motility in bull and adapted the system to their specific experimental conditions. . Boryshpolets et al. (2015) used the

original plugin to study human sperm motility in response to thermotaxis. Since this plugin deposited is an open source, this allows any research laboratory to have access to CASA software and to perform the motility sperm analysis.

The first aim of our study was to set up an Image J CASA system for mammalian sperm analysis, using the horse as a model, and also including progressive motility and amplitude of lateral head displacement measurements; second, we compared the results obtained with our system to those of a commercial one and to the data reported in the literature. Finally, we checked the correlations between motion values obtained from the two CASA systems and other parameters of semen quality such as mitochondrial activity and acrosome integrity.

2. Materials and methods

2.1. Collection and preparation of semen

Twenty-five ejaculates were collected from four Standardbred stallions of proven fertility, individually housed at the National Institute of Artificial Insemination (University of Bologna, Italy), using a Missouri artificial vagina with an inline filter (Nasco, Fort Atkinson, WI, USA). Semen was diluted in Kenney's extender (Kenney et al., 1975) at a semen/extender ratio of 1:3 (v:v) and sent to the laboratory within 1 h post-collection at 20-25°C.

Upon arrival, an aliquot of 2 mL of extended semen was further diluted to a final concentration of $30x10^6$ spermatozoa/mL, and then split into three aliquots. One was evaluated with the Hamilton-Thorne CASA system Vers.12, another was evaluated with the Image J software and the new CASA_bgm, and the last one was used to evaluate the sperm viability with mitochondrial membrane potential and acrosome integrity.

2.2. Video microscopy system for motility assessment

Settings for video camera and microscope were established according to the indications of Wilson-Leedy and Ingermann (2007) and a Leitz diaplan microscope (Wild Leitz GmbH, D6330, Wetzlar, Germany) with a 10x plan objective with negative phase-contrast was used. The microscope was equipped with a Z31A Ascon technologic heated stage (Ascon technologic, PV – IT). The video camera, 3.1 megapixel CMOS USB 2.0 Infinity1-3 Camera (Lumenera corporation, Ottawa, ON, Canada), was coupled to the microscope by a c-mount adapter and videos were registered for three seconds at a resolution of 800x600 pixel and 60 frames/sec (fps). Images were recorded on a hard drive using the Infinity analyzing and capture software 6.4 (Lumenera corporation) and converted to avi format.

Prior to any observation, spermatozoa ($30x10^6$ sperm/mL) were loaded onto a fixed height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos of separate fields and lasting three seconds each were recorded per sperm sample.

2.3. Installation of plugin and video adjustment and analysis

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132 The indications of Wilson-Leedy and Ingermann (2007) were followed to install the
133 plugin and to import the central second of each video. After importing, each video was
134 converted into greyscale 8-bit image and the threshold was adjusted to highlight the sperm
135 heads over the background.
136 Launching the plugin results in the initiation of a dialog box, where parameters for
137 analysis need to be indicated. In order to adapt the existing plugin to the analysis of
138 mammalian spermatozoa, the input parameters related to the bulk flow were eliminated.
139 Therefore, the dialog box generated for CASA_bgm is more simple than that of CASA and
140 the implemented input settings consisted of two VAP cut-off values that divided the sperm
141 population into slow, medium and rapid spermatozoa. The input parameters used to
142 identify and characterize the sperm motion are reported in Figure 1.
143 As shown in Figure 1a, the first two (*a* and *b*) parameters regard the minimum and
144 maximum pixel areas that the program takes into account for the analysis. The particles
145 over or below the selected area are not analyzed. The minimum track length (*c*) indicates
146 the minimum number of frames in which a particle must appear in the video in order to
147 be considered in the analysis. The maximum sperm velocity between frames (*d*) regards
148 the maximum distance a spermatozoon would be expected to travel within the time period,
149 so it is related to the sperm cells velocity.
150 The cut-off values for VSL, VAP and VCL for a spermatozoon to be considered as
151 motile are given in rows *e*, *f* and *g*. In addition, VAP cut-off values for slow and medium
152 velocity spermatozoa, which are then used to describe the sperm subpopulations based
153 upon the VAP, can be set in rows *h* and *i*. In row *l*, the user inserts the number of frames
154 acquired per second, whereas the ratio μm:pixels of the particles analyzed should be
155 indicated in the following field (row *m*). Rows *n*, *o* and *p* are binary variables: 0 will not
156 produce the specific output, while 1 is the positive choice and will produce an output.
157 As far as the two "advanced parameters" are concerned, they were included in order
158 for the progressive motility to be determined; therefore, field *q* is the VAP cut-off and field
159 *r* is the straightness cut-off for progressive motile cells. Each analysis needs the
160 parameters to be entered correctly by the user. To automatize this procedure, Image J
161 function called Macro recorder could be used.
162
163 *2.4. Output and successive analysis*

Outputs obtained from CASA_bgm additional parameters were implemented to CASA plugin to match the needs of analyzing mammalian spermatozoa. The output, as the pre-existing, shows two windows: Results and Path. On the Results window, and depending on the choice made in fields *n*, *o* and *p*, it is possible to obtain:

- *x* and *y* coordinates for each spermatozoon analyzed when the number "1" is entered in field "*n*"
- motility parameters for each spermatozoon analyzed when the number "1" is selected in field "*o*" (Fig. 2)
- the mean and median values for the sperm when the number "1" is selected in field "*p*" (Fig. 2)

When typing 1 in row "*o*", the Results window shows a list of individual sperm motion parameters, where the following values related to each spermatozoa were analyzed: VCL, VAP, VSL, LIN, STR, WOB, Beat Cross Frequency (BCF) and ALH appear in each row. Excluding ALH, algorithms for other parameter calculations are the same used in the pre-existing CASA plugin (Wilson-Leedy and Ingermann, 2007). Instead, ALH values were worked out following Mortimer (2000). Finally, the last row summarizes the total number of sperm analyzed in the selected frames.

By selecting the third set of parameters (row "*p*"), the output of Results shows average (Avg) and median (Median) values for the populations of sperm examined, with the respective standard deviations (Sigma). Additionally, the same output shows the numbers of total (Total Sperm), total motile (TM), progressive motile (PM), slow, medium and rapid spermatozoa analyzed. If multiple analyses are performed, the results of each analysis are added to new rows below and are depicted in the Results window. Moreover, the plugin also produces an output window called *Path* (Fig. 3), that is an image showing the paths of each single spermatozoon. In CASA_bgm, black marks identify non-motile sperm, whilst paths of motile sperm are traced with different colors indicating different velocities, so that red, yellow and green colors are utilized for rapid, medium and slow sperm, respectively. This classification is made on the basis of VAP. In addition, each path of motile sperm is tagged with an ordinal number that corresponds to the order through which spermatozoa are listed in the Results windows.

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198
199 *2.5. Image J settings (Macro Horse)*
200
201 We set the parameters for performing the motility analysis with horse spermatozoa
202 in accordance to the Standard Operating Procedure of Italian Experimental Institute
203 "Lazzaro Spallanzani" (Law D.M. 403/2000). The same values of VAP and STR for PM
204 evaluation were used for the analyses with Hamilton CASA system.
205 The parameters chosen for this purpose are shown in Figure 1.
206
207 *2.6. Motility evaluation using Hamilton CASA system*
208
209 Motility was measured using a Hamilton Thorne, IVOS Ver. 12. Sperm cells were
210 evaluated for each sample diluted to 30x10^6 sperm/mL using a fixed-height Leja Chamber
211 SC 20-01-04-B (Leja, The Netherlands). The assessed sperm motility were: proportions of
212 total motile (TM) and progressive motile (PM) spermatozoa, curvilinear velocity (VCL, μm·s-
213 ^{1}) mean velocity (VAP, μm·s^{-1}), straight-line velocity (VSL, μm·s^{-1}), straightness (STR, %),
214 linearity (LIN, %), beat cross frequency (BCF, Hz), and amplitude of lateral head
215 displacement (ALH, μm). The settings were as follows, frames per second: 60; number of
216 frames: 45; threshold path velocity: 15 μm·s^{-1}; and straightness threshold: 75%.
217
218 *2.7. Sperm mitochondrial activity and viability and acrosome integrity*
219 Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan,
220 Italy).
221
222 *2.7.1. Evaluation of mitochondrial activity and viability*
223 For each sample, an aliquot (25 μL) of semen (30 x 10^6 spermatozoa/mL) was
224 incubated with 2 μL of a 300 μM propidium iodide (PI) stock solution, 2 μL of a 10 μM
225 SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit
226 (Molecular Probes, Inc.) and 2 μL of a 150 μM JC-1 solution for 20 min at 37°C in the
227 dark. Ten μL of the sperm suspension were then placed on a slide and at least 200
228 spermatozoa per sample were scored using a Nikon Eclipse E 600 epifluorescence
229 microscope (Nikon Europe BV, Badhoeverdop, The Netherlands). Spermatozoa stained

with SYBR-14 but not with PI were considered as viable (SYBR-14$^+$/PI$^-$), whereas SYBR-14$^+$/PI$^+$ and SYBR-14$^-$/PI$^+$ spermatozoa were considered as non-viable. JC-1 monomers emit green fluorescence in mitochondria with low membrane potential (JC-1$^-$) and form aggregates in mitochondria with high membrane potential (JC-1$^+$), then emitting a bright red-orange fluorescence. Therefore, those viable sperm showing orange-red fluorescence in the mid piece (JC-1$^+$) were considered as viable sperm with high mitochondrial membrane potential (SYBR-14$^+$/PI$^-$/JC-1$^+$).

2.7.2. Evaluation of acrosome integrity

The integrity of the acrosome was evaluated using FITC-conjugated agglutinin derived from *Pisum sativum* (FITC-PSA) that specifically binds to α-mannoside sugar residues found within the acrosomal contents. Briefly, spermatozoa were washed twice with PBS and subsequently fixed and permeabilized with 95% ethanol at -20°C for 30 min. Sperm were placed onto microscope slides, air-dried, and incubated with FITC-PSA (0.1 mg/mL PSA-FITC) for 15 min at room temperature in the dark. Samples were finally examined under the aforesaid microscope, and 200 cells were counted. Acrosomes were considered as intact when stained with FITC-PSA, and damaged when presenting total or partial loss of FITC-PSA- fluorescence.

2.8. Statistical analyses

Data were analyzed with a statistical package (IBM SPSS for Windows Ver 21.0; IBM Corp., Chicago, IL, USA) and are expressed as mean ± standard deviation. After determining the normality and homogeneity of variances through Shapiro-Wilk and Levene tests, a t-test comparing the two CASA devices was conducted. Data were transformed through √x or arcsin √x when required. The agreement between the two systems' results was studied by Bland Altman's plots. Correlations between kinetic parameters and sperm viability, mitochondrial activity and acrosome integrity were determined through Pearson correlation. In all cases, the level of significance was at least at $P \leq 0.05$.

3. Results

Motility parameters obtained from CASA_bgm plugin and Hamilton-Thorne IVOS are shown in Table 1. Total and progressive motility values were similar, with no significant differences between paired samples.

The agreement between the two systems' measurements was assessed by Bland Altman's Plot (see Supplementary file 1). The different parameters showed a good agreement, in particular TM, PM, VAP, VSL , STR, LIN. VCL, BCF and ALH showed good agreement with some data outside the ± 2 SD.

Tables 2 and 3 show the correlation between sperm motility parameters, evaluated through the two CASA systems (CASA_bgm and Hamilton CASA respectively), and the percentages of viable sperm cells with active mitochondria ($SYBR\text{-}14^+/PI^-/JC\text{-}1^+$) and sperm with intact acrosomes (PSA^+).

Results from CASA_bgm showed a significant positive correlation with the percentage of motile sperm (total motility) and the percentage of $SYBR\text{-}14^+/PI^-/JC\text{-}1^+$ sperm ($P<0.001$). Moreover, motility variables such as VAP, VSL and WOB evaluated through CASA_bgm showed a significant negative correlation with the percentage of sperm exhibiting an intact acrosome ($P<0.05$). Finally, a significant positive correlation between BCF evaluated by CASA_bgm and sperm with intact acrosomes was observed ($P<0.05$).

With regard to the motility parameters obtained through Hamilton CASA analysis, only a significant negative relationship between ALH and sperm with intact acrosome was observed ($P<0.01$).

4. Discussion

The present study aimed at setting an open-access CASA system for evaluating the motility of mammalian sperm from adapting the system developed by Wilson-Leedy and Ingermann , (2007) for fish sperm. In effect, the original ImageJ-plugin created by these Authors was intended to fish sperm and, consequently, requires some adaptation and implementation to fit the request of a standard mammalian sperm analysis. Therefore, the first objective was to modify the previously mentioned plugin, adding the progressive motility and amplitude of lateral head displacement, that were absent in the original version. For this purpose, two new fields were added to the input window and an algorithm was created *ex novo*. Moreover, we included three additional fields in the input window in order to classify as "slow", "medium" and "rapid" spermatozoa, as other systems, like the Hamilton-Thorne IVOS, do.

After adding or completing the input setup, we also introduced some modifications in the output window. Briefly, we included the progressively motile sperm count (PM) and amplitude of lateral head displacement measure (ALH) in the outputs, as well as the number of total motile sperm and the total count of sperm analyzed. This allows a better and faster use of these data for further statistical analyses. Regarding the "Paths" window, our output allows to distinguish through the track color between static (black paths), slow (green paths), medium (yellow paths) and fast (red paths) sperm. This classification originates from the different cut-off VAP values, which are set in the input window and may be modified and adapted to the peculiarities of other mammalian species. In addition, each sperm track is associated with a number, which allows the user to identify quickly (if sperm concentration is not too high) the path of a given spermatozoon and to associate it with the single data obtained in the results window. Thus, each number corresponds to a specific line in the results window (e.g. number 1 correspond to the first row, etc.).

We analyzed 25 samples from four stallions with both Hamilton-Thorne IVOS and CASA_bgm Image J plugin. To avoid any technical differences, we used the same sperm concentration for both instruments, as recommended by Holt and colleagues (Holt et al., 1996, 1994). Leja chambers were also used, as it is well known that different supports yield different results on the motility parameters (Hoogewijs et al., 2012). Finally, our settings for determining the sperm motion took into account previous studies. Indeed, and as reported by (Holt et al., 1996), there have been several approaches to detect properly

the true movements of sperm cells, and to distinguish motile from non-motile spermatozoa. In this regard, it has been reported that VAP values around 20 $\mu m \cdot s^{-1}$ or less could be due to spurious movements of non-viable sperm.

It is worth noting that our results on TM, PM, VCL, VAP, VSL, STR and LIN obtained with CASA_bgm are consistent with those obtained by the Hamilton-Thorne CASA. It should be pointed out that data from our instrument and the commercial one are numerically different, but the excursion of measurements between the two systems is not significant. Our coefficients of variation are also in agreement with data reported by other Authors (Holt et al., 1994), who found values exceeding 20%. In that work, CV for total motility, VCL, VAP, LIN and ALH were 24%, 19%, 44%, 22.5% and 39%, respectively. The higher degree of variation of that work is possibly due to the comparison between numerous systems (five CASA). In the present study, although Hamilton Thorne CASA showed lower CV than CASA_bgm, the extent of these differences was not as high as that reported by other Authors (Holt et al., 1994). Moreover, BCF, was quite different between the two systems. This could be due to the difference in the algorithms between software. Indeed, various CASA systems, which generally utilize proprietary software, use different algorithms to determine the same measures. Therefore, in the absence of a "gold standard" with reference value, internal validation is required, which is setting-, system-, and species- specific (Amann and Waberski, 2014).

Besides, a new parameter ALH, not originally present in the Image J CASA, was set on the basis of the algorithm proposed by Mortimer (2000). In this context, it is worth noting that Holt et al. (1994) reported that caution is needed when comparing results between CASA systems, particularly with regard to ALH and VAP. Not only do our data support this idea, but also indicate that such assertion could be extended to the other kinetic parameters. In addition, sperm preparation, previous incubation periods, qualities of optics, camera and imaging are amongst the factors responsible for the differences between CASA devices. Therefore, a direct comparison between such devices is not possible (Holt et al., 1996). Apart from this, one should note that other researchers have also pointed out additional critical steps in semen motility analysis, ranging from sample preparation (Contri et al., 2010) to the support used (Hoogewijs et al., 2012).

The original plugin was already tested with fish sperm in comparison with two other systems (Boryshpolets et al., 2013). In that study, Image J plugin was shown to be in accordance with the other two examined systems, but for determined conditions (different

348 frame rate) and fish species, VCL from Image J and CRISMAS CASA systems were in
349 accordance, while the Hobson Sperm Tracker was not.
350 Since a reliable method to evaluate sperm motility should give a global picture of
351 sperm quality and should be in agreement with other sperm parameters, such membrane
352 integrity, the second part of the work was intended to correlate the motility values obtained
353 from the two CASA systems with sperm mitochondrial activity and acrosome integrity.
354 Interestingly, we observed that the motion values obtained by CASA_bgm were better
355 correlated with the other sperm quality parameters than those obtained through the
356 commercial CASA. Indeed, a significant positive correlation between TM and the
357 percentage of SYBR-14^{+}/PI^{-}/JC-1^{+} positive sperm was observed, kinetic parameters such
358 as VAP, VSL and WOB showed a significant negative correlation with the percentage of
359 sperm exhibiting an intact acrosome. The correlation between TM and viability and
360 mitochondrial activity has been previously demonstrated (Bucci et al., 2016; Plaza Dávila
361 et al., 2015). The significant and positive correlation between all these parameters
362 corroborates the reliability of CASA_bgm. Moreover the relationship between the single
363 kinetic sperm characteristics and its functional status, in terms of viability, mitochondrial
364 activity and acrosome integrity, could be interesting for further investigations, since they
365 could improve the predictive accuracy of the true sperm's fertilizing potential ((Jung et al.,
366 2015; Nagy et al., 2015; Oliveira et al., 2013; Santolaria et al., 2015). In addition, the
367 motion values of individual spermatozoon analyzed, which can be recorded by CASA_bgm,
368 could be useful to study sperm subpopulations, after various techniques for processing
369 semen, such as cryopreservation, capacitation or hyperactivation (Henning et al., 2014;
370 Martínez-Pastor et al., 2011). In fact, mounting evidence indicates that the distribution of
371 spermatozoa in different subpopulations based on motion features may have functional
372 relevance and can be useful to study sperm biology (Amann and Waberski, 2014).
373 Finally, and as for the possibility of using CASA_bgm for other mammalian species,
374 it is possible to establish specific settings for each species analyzed and to easily modify
375 these parameters in the input. Two studies applied so far the original CASA plugin to
376 mammalian species (Boryshpolets et al., 2015; Elsayed et al., 2015): the first one analyzed
377 human sperm motility parameters (only VCL, VAP, VSL, LIN and WOB) by the enhanced
378 CASA version (Purchase and Earle, 2012). Those Authors studied the thermotactive
379 behavior of spermatozoa subjected to different temperature (31°C then 37°C and again
380 31°C) and obtained interesting results on sperm parameters changes. The second study

developed a new CASA system for the analysis of bull spermatozoa under very particular experimental conditions: in fact, those Authors implemented the original plugin to study bull spermatozoa in different microfluidic environments. These two studies demonstrate that the plugin is reliable and could also be used properly for mammalian sperm. Anyway, Boryshpolets et al. (2015) did not improve the original plugin, while Elsayed et al. (2015) did improve it, with very new peculiar parameters (orientation, proximity to wall, swimVAP). In the present work, we added progressive motility (PM) and amplitude of lateral head displacement (ALH), two parameters that are important for all sperm analyses and also for detecting or defining hyperactivation (Cremades et al., 2005; Schmidt and Kamp, 2004). Furthermore, being this software an open source, any investigator may freely implement or modify (as we and others already did) the plugin and create a more specific or versatile system, according to his/her needs; this could then be made available to the whole scientific community, which would benefit from that tool.

In conclusion, the present study demonstrates the possibility of implementing an open-access CASA for evaluating stallion sperm motility and to easily adapt this background to other mammalian species. Whilst no "gold standard" is available to assert which system is the most suitable, the high and significant correlation of those sperm kinetic parameters evaluated through CASA_bgm with other sperm quality parameters, including membrane integrity and mitochondrial activity, demonstrates the reliability of this tool. This open source system could benefit those researchers that cannot afford the purchase of a commercial CASA system.

The plugin is provided as an e-component of the present article (see e-component. BGM_java)

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506 Table 1. Motility parameters from CASA_bgm and Hamilton-Thorne IVOS

507

	CASA_bgm	% CV	IVOS	% CV	Paired *t*-test p value	% Total CV
% TM	75.27±10.80	18.22	78.64±11.43	14.23	=0.05	16.64
% PM	37.04±11.21	30.36	37.96±9.33	24.97	>0.05	27.62
VCL	203.24±33.67	16.50	190.78±19.43	9.61	>0.05	13.69
VAP	110.98±26.81	22.88	101.20±13.54	13.30	>0.05	19.29
VSL	75.02±15.20	18.89	71.49±11.52	17.35	>0.05	18.06
LIN	38.20±5.70	14.49	38.12±3.94	11.78	>0.05	13.12
STR	69.25±7.13	9.51	69.44±5.12	7.95	>0.05	8.69
WOB	54.21±6.94	12.32	NA	NA	NA	12.32
BCF	28.19±2.39	9.11	40.78±2.40	6.18	<0.01	20.69
ALH	8.10±1.49	17.78	7.64±0.51	7.45	>0.05	14.01

508

509 Abbreviations: TM – total sperm motility; PM – Progressive sperm motility; VAP – average
510 path velocity; VSL – straight line velocity; VCL – curvilinear velocity; ALH – amplitude of
511 lateral head displacement; BCF– beat cross frequency; STR – straightness of track; LIN –
512 linearity of track; WOB – wobble. CV – coefficient of variation. Data are reported as mean
513 ± SD.
514

515

Table 2. Correlation between CASA_bgm parameters and percentage of sperm with intact acrosome and percentage of viable sperm with active mitochondria. *P<0.05 **P<0.01

	% Sperm with an intact acrosome	% SYBR-14^{+}/PI^{-}/JC-1^{+}-sperm
TM	0.32	0.47**
PM	0.33	0.17
VCL	-0.24	0.28
VAP	-0.39*	0.21
VSL	-0.45*	0.17
LIN	-0.25	-0.17
STR	0.08	-0.10
WOB	-0.41*	-0.05
BCF	0.38*	0.11
ALH	-0.31	0.25

Abbreviations: TM – total sperm motility; PM – Progressive sperm motility; VAP – average path velocity; VSL– straight line velocity; VCL – curvilinear velocity; ALH – amplitude of lateral head displacement; BCF – beat cross frequency; STR – straightness of track; LIN – linearity of track; WOB - wobble.

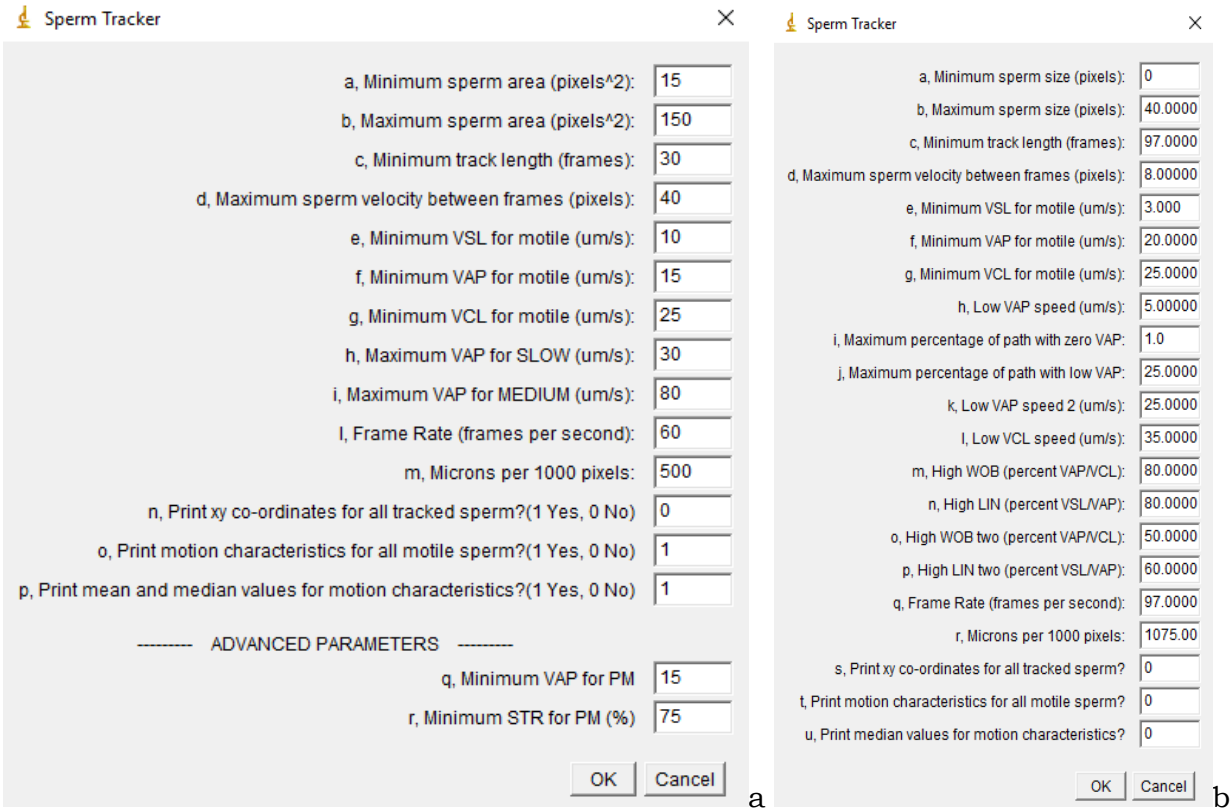
526 Table 3. Correlation between IVOS Hamilton-Thorne CASA parameters and percentage
527 of cells with intact acrosome and percentage of viable sperm with active mitochondria.
528 *P<0.05.
529

	% Sperm with an intact acrosome	% SYBR-14^{+}/PI^{-}/JC-1^{+} -sperm
TM	0.13	0.26
PM	-0.07	0.33
VCL	-0.04	-0.07
VAP	-0.10	-0.13
VSL	-0.25	-0.04
LIN	-0.16	0.02
STR	-0.22	0.17
WOB	-0.14	-0.19
BCF	0.32	-0.23
ALH	-0.36*	0.20

530
531 Abbreviations: TM – total sperm motility; PM – Progressive sperm motility; VAP – average
532 path velocity; VSL– straight line velocity; VCL – curvilinear velocity; ALH – amplitude of
533 lateral head displacement; BCF – beat cross frequency; STR – straightness of track; LIN –
534 linearity of track; WOB - wobble.
535

536 Figure 1.

537



538

539 *Figure 1.* CASA_bgm (a) and CASA_ (b) sperm tracker input dialog box. Some fields from
540 the original input dialog box have been removed as not necessary for mammalian sperm
541 analysis.

542

543

544 Figure 2

Parameters for motile sperm									
VCL	VAP	VSL	LIN	STR	WOB	BeatCross	ALH		
208.4422	117.5997	92.12508	0.441969	0.783379	0.564184	22.69231	6.781614		
57.10527	33.98052	33.06013	0.578933	0.972914	0.595051	33.12281	2.076376		
129.6436	50.9638	40.01078	0.308621	0.785082	0.393107	31.05263	5.116851		
144.441	70.79696	41.66586	0.288463	0.588526	0.490145	32.18182	5.977822		
244.0964	100.948	21.53316	0.088216	0.213309	0.413558	31.05263	9.370712		
180.4653	73.34014	68.32744	0.378618	0.931651	0.406395	30.51724	5.03472		
204.4206	92.29341	71.11768	0.347899	0.770561	0.451488	31.95833	8.313763		
187.8413	92.70441	72.69722	0.387014	0.784183	0.493525	21.73684	6.442191		
102.7384	68.11353	59.32798	0.577467	0.871016	0.662981	33.39623	3.969582		
199.5729	90.0397	33.60023	0.168361	0.373171	0.451162	36.22807	7.658349		
241.8574	106.7313	41.69513	0.172396	0.390655	0.441299	33.71429	9.16523		
208.7	86.18127	49.3397	0.236414	0.572511	0.412943	37.84906	7.227401		
76.0532	19.15179	15.73174	0.206852	0.821424	0.251821	25.87719	1.602928		
140.6687	66.61184	61.41458	0.43659	0.921977	0.473537	33.1875	4.324525		
121.0462	65.35769	62.766	0.518529	0.960346	0.53994	28.9434	4.557494		
54.04993	18.49949	17.39703	0.32187	0.940406	0.342267	35.19298	2.233654		
115.7715	46.49909	40.86501	0.35298	0.878835	0.401645	26.71698	3.260101		
108.198	43.05379	33.07	0.305644	0.768109	0.397917	31.60714	4.58126		
180.2396	77.94228	72.54892	0.402514	0.930803	0.432437	28.48276	6.111791		
100.9077	29.959	24.90571	0.246817	0.831327	0.296895	28.98246	2.371706		
132.0983	73.93782	68.84734	0.521182	0.931152	0.559718	26.22222	4.748324		
203.0996	90.36935	37.69428	0.185595	0.417113	0.444951	32.08772	6.62122		
91.42439	66.02258	60.7103	0.664049	0.919538	0.722155	30.01754	4.684298		
81.10976	30.64762	23.28496	0.28708	0.759764	0.377854	30.55357	3.196392		
171.3814	67.98292	20.4941	0.119582	0.30146	0.396676	23.17857	5.500337		
194.5852	89.10887	80.53256	0.413868	0.903755	0.457943	28.60606	7.859629		
92.89756	63.31046	58.71605	0.632052	0.92743	0.681508	28.98246	3.358674		
79.45986	32.02757	29.26935	0.368354	0.91388	0.403066	34.15789	3.14254		
98.73236	64.23033	36.09186	0.365553	0.561913	0.65055	23.80702	4.135894		
192.1678	104.9203	74.44733	0.387408	0.709561	0.545983	25.87719	7.032392		
109.3685	52.77604	39.45234	0.360729	0.747543	0.482552	32.08772	4.843184		
72.93187	35.76814	33.78304	0.463214	0.944501	0.490432	28.98246	3.288517		
88.70194	42.0184	18.73911	0.211259	0.445974	0.473703	30.01754	3.674224		
71.0462	35.08382	29.83453	0.419931	0.850379	0.493817	38.29824	2.85478		
190.4572	96.78941	85.78745	0.450429	0.886331	0.508195	31.23529	7.854505		
195.9439	109.1582	96.40215	0.491988	0.883142	0.557089	31.46667	9.582038		
200.0686	100.7108	54.26265	0.27122	0.538797	0.503381	34.22	7.64082		
223.7871	82.24772	23.39241	0.10453	0.284414	0.367527	22.87755	8.010928		
140.1676	84.55843	78.46845	0.559819	0.927979	0.603267	30.15556	5.648967		
174.6872	68.16888	56.45772	0.323193	0.828204	0.390234	27.53333	5.39762		
151.3767	39.70294	29.18845	0.19282	0.735171	0.262279	28.48276	2.023471		
78.01864	61.79254	58.24905	0.746604	0.942655	0.792023	29.5	2.284161		
55.20258	22.50139	20.29848	0.367709	0.902099	0.407615	17.7	1.631619		
120.6371	60.33001	56.47726	0.468158	0.936139	0.500095	22.37931	5.266281		
145.6646	97.53716	88.55895	0.607965	0.907951	0.669601	21.07143	6.907838		
128.6104	49.32986	40.31792	0.313489	0.817313	0.38356	26.22222	4.369081		
TotalSperm	68								
TM	PM	AvgVCL	AvgVAP	AvgVSL	AvgLIN	AvgSTR	AvgWOB	AvgBeats	AvgALH
46	32	141.0845	66.77824	48.97734	0.370955	0.761181	0.473321	29.35246	5.16817
SLOW	MEDIUM	RAPID							
4	26	16							
MedianVCL	MedianVAP	MedianVSL	MedianLIN	MedianSTR	MedianWO	MedianBea	MedianALH		
136.133	67.29738	41.6805	0.366631	0.829765	0.46574	30.01754	4.938952		
SigmaVCL	SigmaVAP	SigmaVSL	SigmaLIN	SigmaSTR	SigmaWOB	SigmaBeats	SigmaALH		
54.43197	26.79796	22.59057	0.153401	0.209589	0.116159	4.522693	2.198211		
##########	---	Color	LEGEND	---	##########				
RED:	RAPID								
ORANGE:	MEDIUM								
GREEN:	SLOW								
BLACK:	NOT	CLASSIFIED							

546

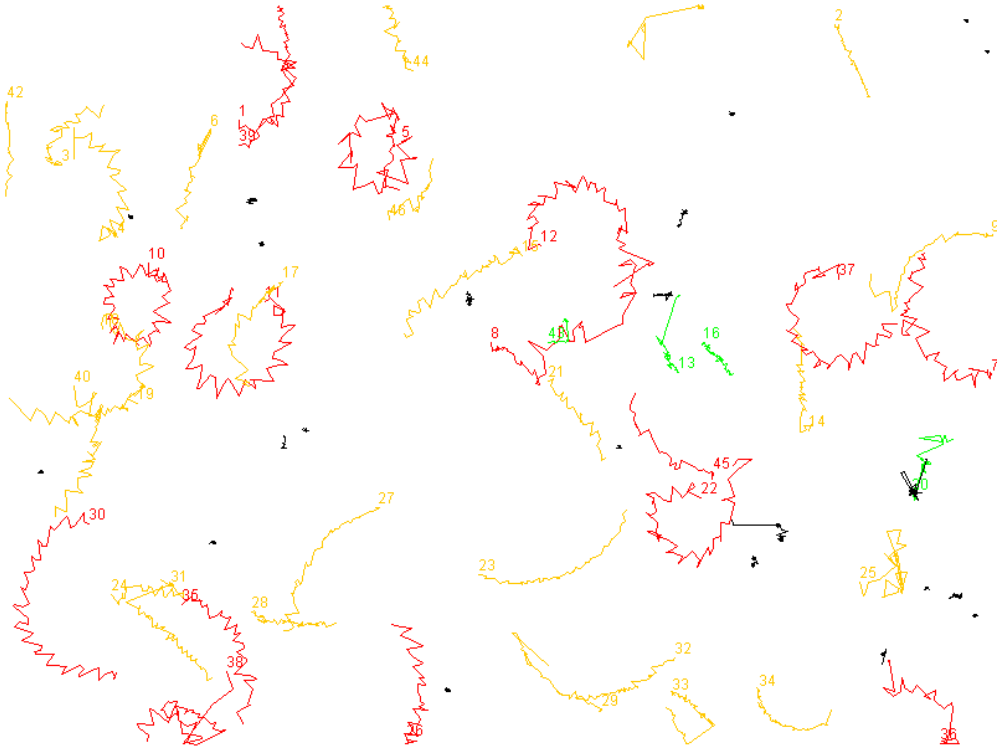
547 *Figure 2.* Output window produced by the plugin when the choice "1" is set at "o" and "p"
548 fields. In case "o" field is set to "0", motility characteristics from single cells are not
549 displayed; when "p" field is set to "0", median, mean values and standard deviations of the
550 analyzed video are not displayed.

551

552

553

554 Figure 3.



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556

557 *Figure 3.* "Paths" window. Each spermatozoon analyzed by the plugin is assigned a
558 number, following the order of the "results" window and a color, depending on the VAP.
559 Red, rapid cells with VAP higher than the value inserted in the input field "i"; yellow,
560 medium cells with VAP between the value in the input field "i" and "h"; green, slow cells,
561 with VAP lower that the value inserted in the input field "h". Black tracks are those of non
562 motile cells (velocities beneath).

563

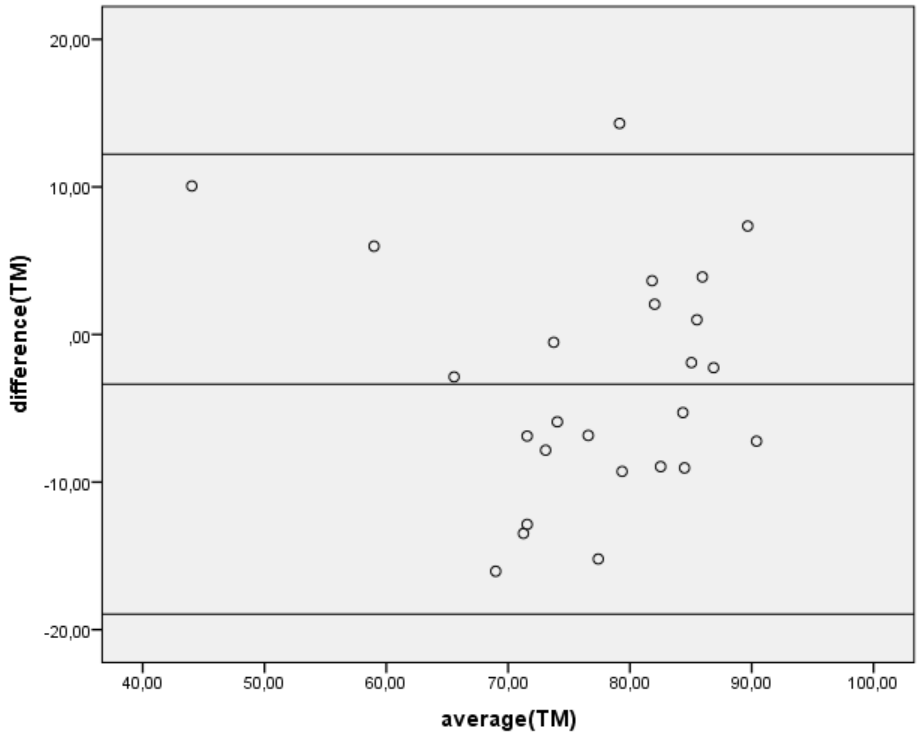
564

565 Supplementary file 1.

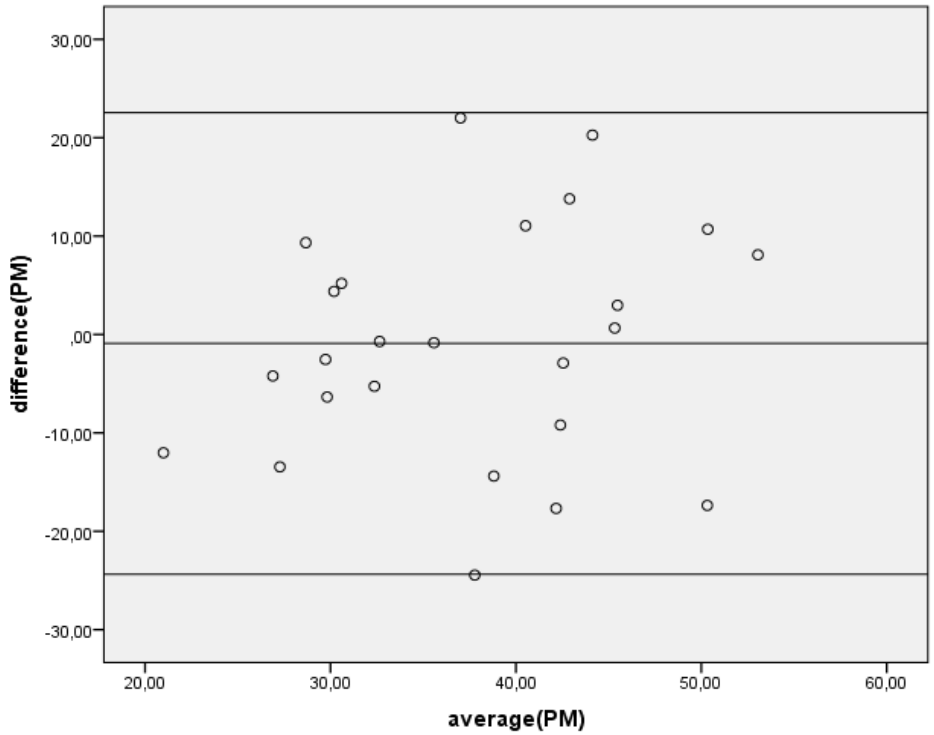
566 Bland Altman plots representing agreement between parameters measured by IVOS Hamilton-
567 Thorne CASA and CASA_bgm plugin. Each point represent a sample, measured with both
568 instruments. Y axis expresses difference in means, while X axis expresses the average between the
569 two measurements. The external lines represents ± 2 SD.

570

Total motility(TM) Bland Altman's plot



Progressive motility(PM) Bland Altman's plot

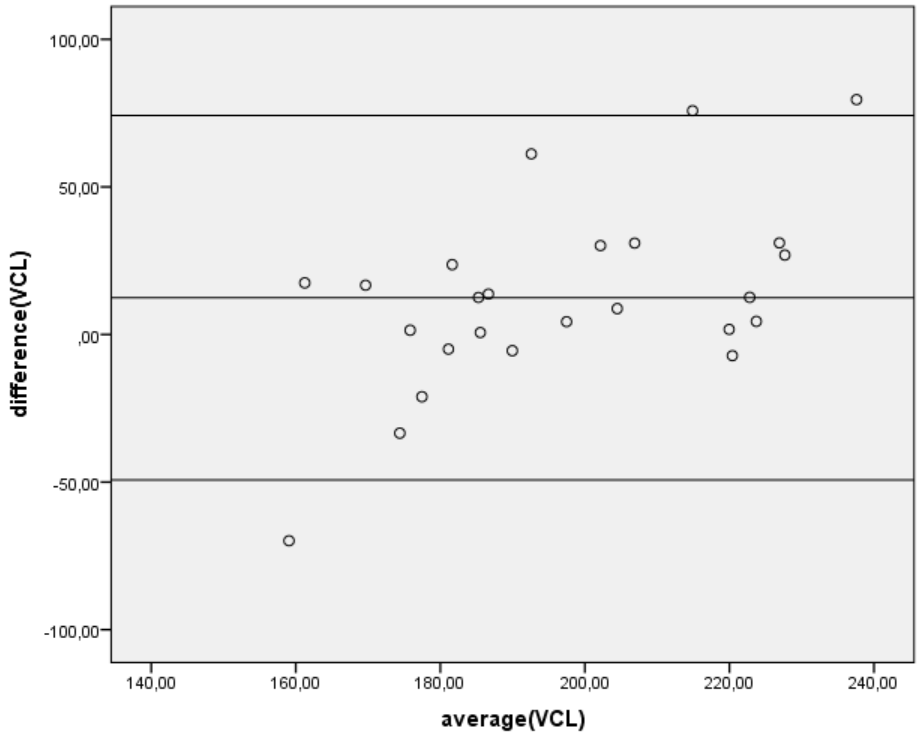


579

580

581 Linear velocity (VCL) Bland Altman's plot

582



583

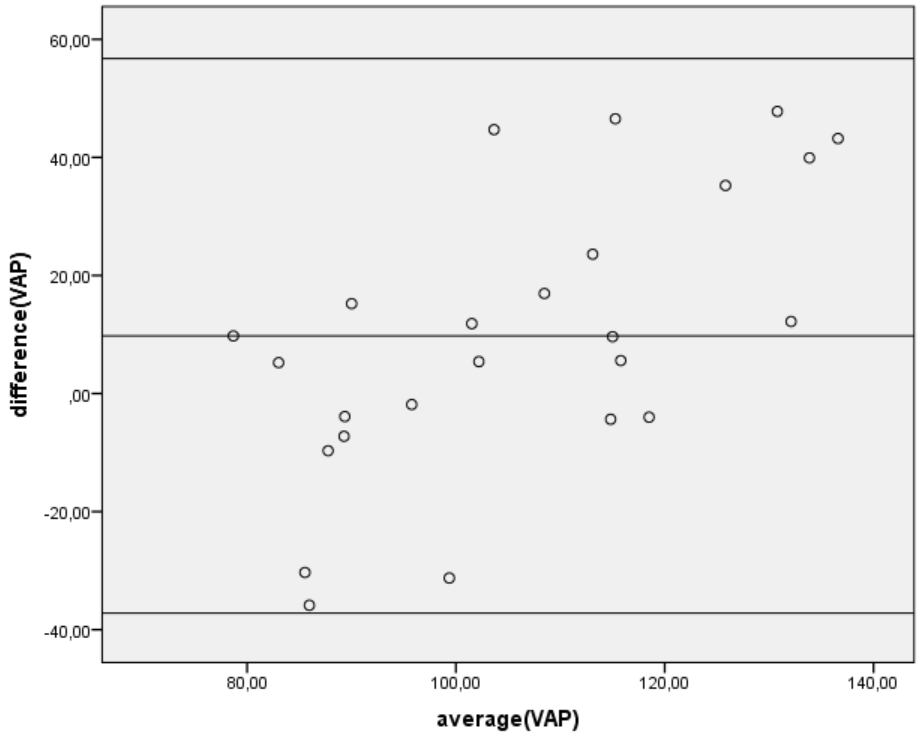
584

585

586

587 Mean velocity (VAP) Bland Altman's plot

588

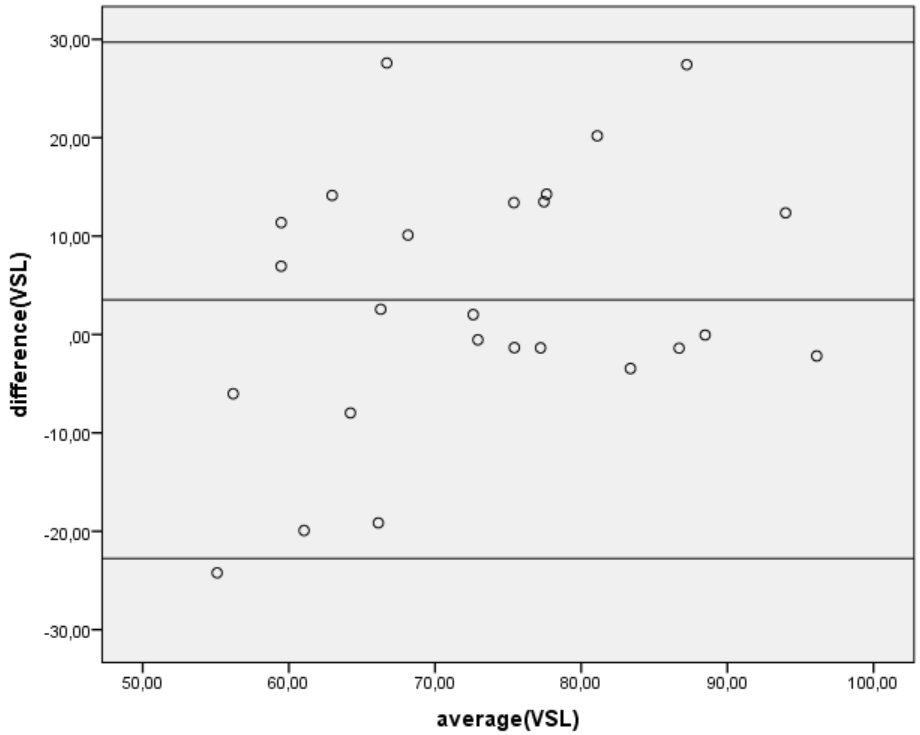


589

590

591 Linear velocity (VSL) Bland Altman's plot

592



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597 Linearity (LIN) Bland Altman's plot

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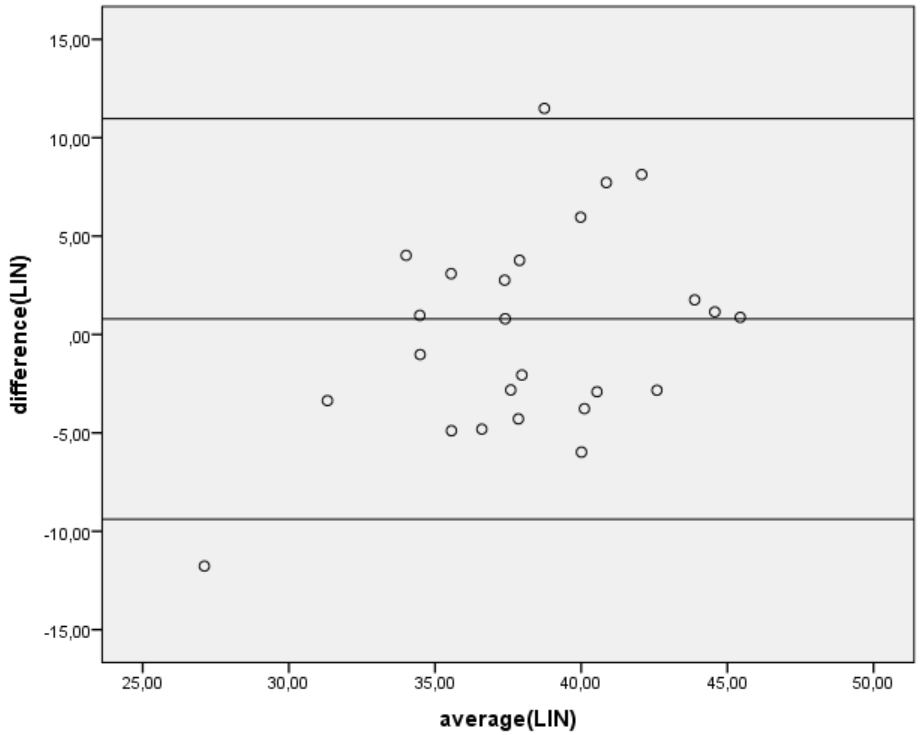
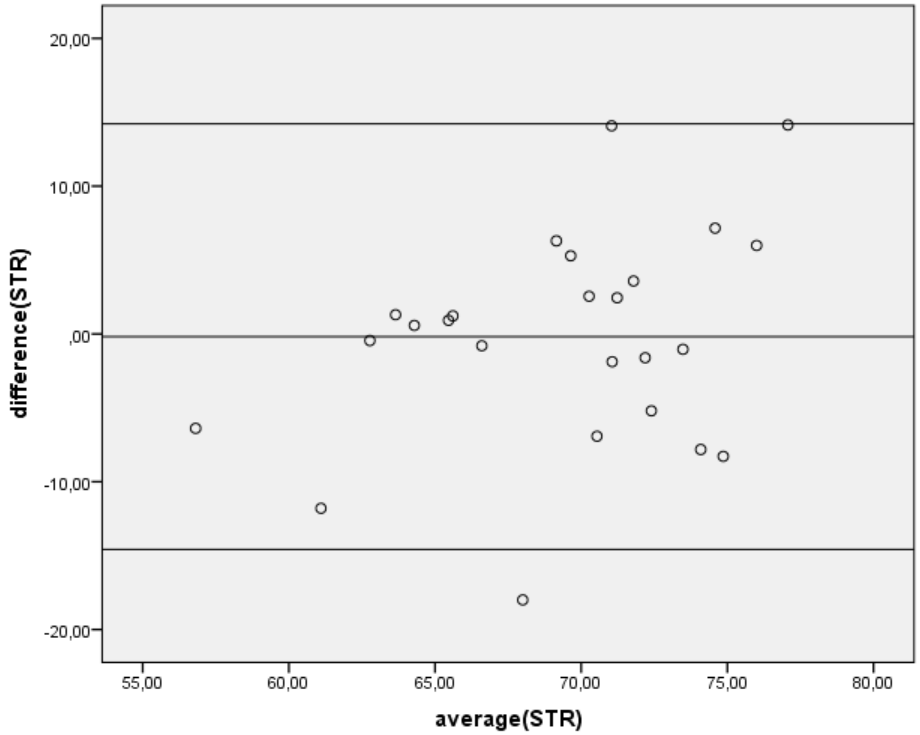


599

600 Straightness (STR) Bland Altman's plot

601



602

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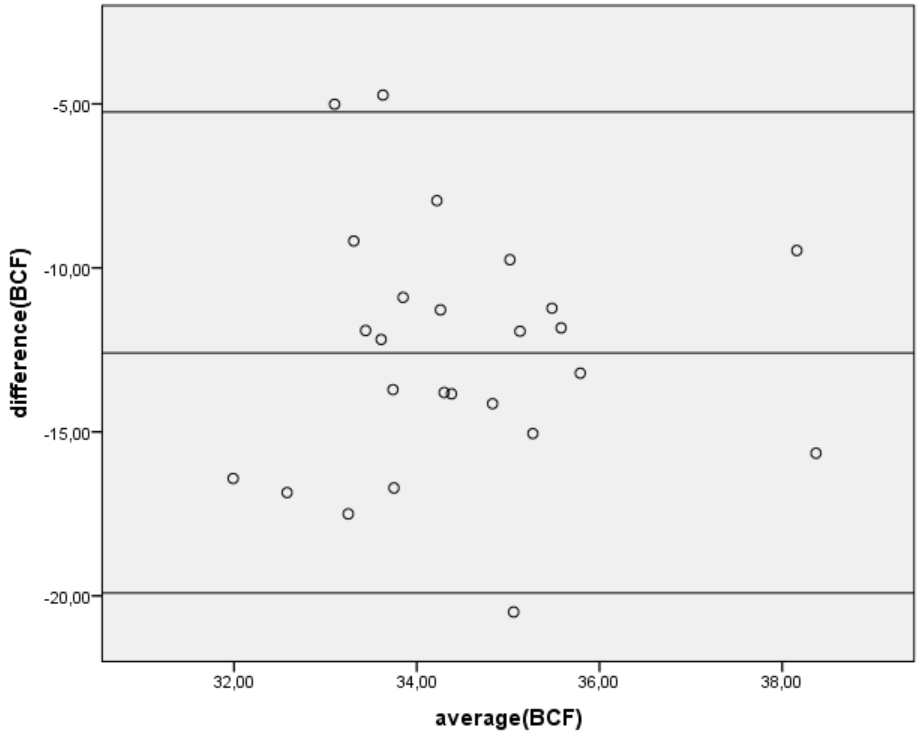
604

605

606 Beat Cross Frequency (BCF) Bland Altman's plot

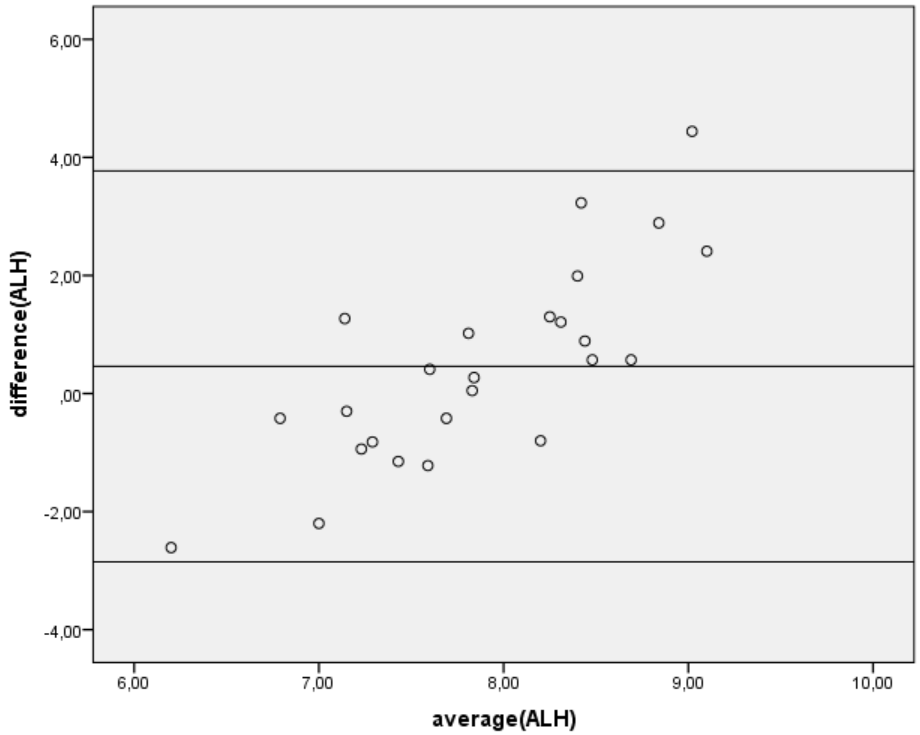
607



608

609 Amplitude of lateral head displacement (ALH) Bland Altman's plot

610



611

612

613

614

615 Declaration of intent.
616
617 The CASA_BGM plugin was obtained by modifying and renewing the “casa” plugin Computer Assisted
618 Sperm Analyzer designed by JG Wilson-Leedy JG and RL Ingermann and owned by the Regents of the
619 University of California and the Howard Hughes Medical Institute.
620
621 The plugin is subjected to the following conditions and terms of use:
622
623 Wilson-Leedy JG, Ingermann RL, Development of a novel CASA system based on open source software for
624 characterization of zebrafish sperm motility parameters, Theriogenology (2006),
625 doi:10.1016/j.theriogenology.2006.10.003.
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648 MODIFICATIONS.
649
650
651 The purpose of our work is to implement an open source tool and share with the international reseach
652 community our findings and, possibly, start collaborations for further implementations. We neglect and reject
653 any commercial use of our plugin.
654
655

Brief instructions for analysis with ImageJ CASA_BGM plugin

Plugin installation

We report the original instruction from Wilson Leedy and Ingermann

"The plugin must first be downloaded to the computer's plugins folder and compiled (Plugins->Compile and Run, locate the file and open, see http://rsb.info.nih.gov/ij/docs/menus/plugins.html for more detailed instruction). Running the plugin will result in initiation of a dialog box where parameters for analysis may be entered (and must be entered for each analysis performed - we recommend use of a macro to input these values, macros can then be saved with particular settings for a specific species). Macros can be recorded by opening the macro recorder (Plugins->Macros->Record), and performing the analysis as normal.Clicking Create will open a new window with the text of the macro, running this macro will open the plugin, input the previously used values, and run the plugin. See http://rsb.info.nih.gov/ij/docs/menus/plugins.html#macros and http://rsb.info.nih.gov/ij/developer/macro/macros.html#recorder for more detailed instructions regarding macro recording."

Import a video in Image J and analysis

After saving videos of sperm samples, these should be convertedin .avi format.

To import the video into Image J after launching the software, choose *File>Import>Avi…* and chose the file from the correct folder.

A dialog box opens and it should be indicated to import fames from 60 to 120 and to convert the image to greyscale.

The video opens and the threshold should then be adjusted. Use Image->Adjust->Threshold file menu. Adjust the sliders to select spermatozoa 8red) and avoid selecting any background image (the background should be white). By clicking "Apply" the image is converted into black and white (black spermatozoa and white background).

Launch the plugin and fill each field with the appropriate values (see the main article for stallion) or alternatively create and run a specific Macro.