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

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

*Published Version:*

Implementing an open-access CASA software for the assessment of stallion sperm motility: Relationship with other sperm quality parameters / Giaretta, Elisa; Munerato, Mauro; Yeste, Marc; Galeati, Giovanna; Spinaci, Marcella; Tamanini, Carlo; Mari, Gaetano; Bucci, Diego. - In: ANIMAL REPRODUCTION SCIENCE. - ISSN 0378-4320. - ELETTRONICO. - 176:(2017), pp. 11-19. [10.1016/j.anireprosci.2016.11.003]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/586261> since: 2020-12-05

*Published:*

DOI: <http://doi.org/10.1016/j.anireprosci.2016.11.003>

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Elisa Giaretta, Mauro Munerato, Marc Yeste, Giovanna Galeati, Marcella Spinaci, Carlo Tamanini, Gaetano Mari, Diego Bucci

The final published version is available online at:

<http://dx.doi.org/10.1016/j.anireprosci.2016.11.003>

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1 **Title**

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

4

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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

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## 53 1. Introduction

54

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

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

70 The availability of an open-access, low cost CASA system could much benefit the  
71 analyses of sperm motility, especially for those that, due to economic constraints, may not  
72 afford the costs of a commercial device. In addition, the relevant information that a CASA  
73 system provides goes beyond a more objective evaluation of the percentages of total and  
74 progressive motile sperm. Indeed, some CASA systems give the individual kinetic  
75 parameters for a single sperm cell and this may be used for evaluating motile sperm  
76 population in differently treated samples (Abaigar et al., 1999; Flores et al., 2009, 2008;  
77 Miró et al., 2005, 2009; Schmidt and Kamp, 2004; Varner, 2008). In 2007, Wilson-Leedy  
78 and Ingermann developed a CASA software package that worked as a plugin to the United  
79 States National Institutes of Health (NIH) Image J software (Wilson-Leedy and Ingermann,  
80 2007). Successively, more than hundred scientific studies used the plugin to assess fish  
81 or invertebrate sperm motility. In particular, Purchase and Earle (2012) implemented the  
82 original plugin, creating a new one that permits the automation of some video processing  
83 steps. Very few studies applied the system to mammalian sperm motility evaluation.  
84 Elsayed et al. (2015) used the plugin to study sperm motility in bull and adapted the  
85 system to their specific experimental conditions. . Boryshpolets et al. (2015) used the

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

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

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## 98 **2. Materials and methods**

99

### 100 *2.1. Collection and preparation of semen*

101

102 Twenty-five ejaculates were collected from four Standardbred stallions of proven  
103 fertility, individually housed at the National Institute of Artificial Insemination (University  
104 of Bologna, Italy), using a Missouri artificial vagina with an inline filter (Nasco, Fort  
105 Atkinson, WI, USA). Semen was diluted in Kenney's extender (Kenney et al., 1975) at a  
106 semen/extender ratio of 1:3 (v:v) and sent to the laboratory within 1 h post-collection at  
107 20-25°C.

108 Upon arrival, an aliquot of 2 mL of extended semen was further diluted to a final  
109 concentration of  $30 \times 10^6$  spermatozoa/mL, and then split into three aliquots. One was  
110 evaluated with the Hamilton-Thorne CASA system Vers.12, another was evaluated with  
111 the Image J software and the new CASA\_bgm, and the last one was used to evaluate the  
112 sperm viability with mitochondrial membrane potential and acrosome integrity.

113

### 114 *2.2. Video microscopy system for motility assessment*

115

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

126 Prior to any observation, spermatozoa ( $30 \times 10^6$  sperm/mL) were loaded onto a fixed  
127 height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos of  
128 separate fields and lasting three seconds each were recorded per sperm sample.

129

### 130 *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  $\mu\text{m}:\text{pixels}$  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*

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164

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

169

- 170 -  $x$  and  $y$  coordinates for each spermatozoon analyzed when the number “1” is entered  
 171 in field “ $n$ ”
- 172 - motility parameters for each spermatozoon analyzed when the number “1” is  
 173 selected in field “ $o$ ” (Fig. 2)
- 174 - the mean and median values for the sperm when the number “1” is selected in field  
 175 “ $p$ ” (Fig. 2)

176

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

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

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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  $30 \times 10^6$  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,  $\mu\text{m}\cdot\text{s}^{-1}$ )  
213 mean velocity (VAP,  $\mu\text{m}\cdot\text{s}^{-1}$ ), straight-line velocity (VSL,  $\mu\text{m}\cdot\text{s}^{-1}$ ), straightness (STR, %),  
214 linearity (LIN, %), beat cross frequency (BCF, Hz), and amplitude of lateral head  
215 displacement (ALH,  $\mu\text{m}$ ). The settings were as follows, frames per second: 60; number of  
216 frames: 45; threshold path velocity:  $15 \mu\text{m}\cdot\text{s}^{-1}$ ; 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  $\mu\text{L}$ ) of semen ( $30 \times 10^6$  spermatozoa/mL) was  
224 incubated with 2  $\mu\text{L}$  of a 300  $\mu\text{M}$  propidium iodide (PI) stock solution, 2  $\mu\text{L}$  of a 10  $\mu\text{M}$   
225 SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit  
226 (Molecular Probes, Inc.) and 2  $\mu\text{L}$  of a 150  $\mu\text{M}$  JC-1 solution for 20 min at  $37^\circ\text{C}$  in the  
227 dark. Ten  $\mu\text{L}$  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, Badhoevedop, The Netherlands). Spermatozoa stained

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230 with SYBR-14 but not with PI were considered as viable (SYBR-14<sup>+</sup>/PI<sup>-</sup>), whereas SYBR-  
231 14<sup>+</sup>/PI<sup>+</sup> and SYBR-14<sup>-</sup>/PI<sup>+</sup> spermatozoa were considered as non-viable. JC-1 monomers  
232 emit green fluorescence in mitochondria with low membrane potential (JC-1<sup>-</sup>) and form  
233 aggregates in mitochondria with high membrane potential (JC-1<sup>+</sup>), then emitting a bright  
234 red-orange fluorescence. Therefore, those viable sperm showing orange-red fluorescence  
235 in the mid piece (JC-1<sup>+</sup>) were considered as viable sperm with high mitochondrial  
236 membrane potential (SYBR-14<sup>+</sup>/PI<sup>-</sup>/JC-1<sup>+</sup>).

237

### 238 *2.7.2. Evaluation of acrosome integrity*

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

248

### 249 *2.8. Statistical analyses*

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

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259 **3. Results**

260

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

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

268 Tables 2 and 3 show the correlation between sperm motility parameters, evaluated  
269 through the two CASA systems (CASA\_bgm and Hamilton CASA respectively), and the  
270 percentages of viable sperm cells with active mitochondria (SYBR-14<sup>+</sup>/PI<sup>-</sup>/JC-1<sup>+</sup>) and  
271 sperm with intact acrosomes (PSA<sup>+</sup>).

272 Results from CASA\_bgm showed a significant positive correlation with the  
273 percentage of motile sperm (total motility) and the percentage of SYBR-14<sup>+</sup>/PI<sup>-</sup>/JC-1<sup>+</sup>  
274 sperm ( $P < 0.001$ ). Moreover, motility variables such as VAP, VSL and WOB evaluated  
275 through CASA\_bgm showed a significant negative correlation with the percentage of sperm  
276 exhibiting an intact acrosome ( $P < 0.05$ ). Finally, a significant positive correlation between  
277 BCF evaluated by CASA\_bgm and sperm with intact acrosomes was observed ( $P < 0.05$ ).

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

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#### 282 4. Discussion

283

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

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

308 We analyzed 25 samples from four stallions with both Hamilton-Thorne IVOS and  
309 CASA\_bgm Image J plugin. To avoid any technical differences, we used the same sperm  
310 concentration for both instruments, as recommended by Holt and colleagues (Holt et al.,  
311 1996, 1994). Leja chambers were also used, as it is well known that different supports  
312 yield different results on the motility parameters (Hoogewijs et al., 2012). Finally, our  
313 settings for determining the sperm motion took into account previous studies. Indeed, and  
314 as reported by (Holt et al., 1996), there have been several approaches to detect properly

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

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

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

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

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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<sup>+</sup>/PI-/JC-1<sup>+</sup> 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 thermotactic  
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

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381 developed a new CASA system for the analysis of bull spermatozoa under very particular  
382 experimental conditions: in fact, those Authors implemented the original plugin to study  
383 bull spermatozoa in different microfluidic environments. These two studies demonstrate  
384 that the plugin is reliable and could also be used properly for mammalian sperm. Anyway,  
385 Boryshpolets et al. (2015) did not improve the original plugin, while Elsayed et al. (2015)  
386 did improve it, with very new peculiar parameters (orientation, proximity to wall,  
387 swimVAP). In the present work, we added progressive motility (PM) and amplitude of lateral  
388 head displacement (ALH), two parameters that are important for all sperm analyses and  
389 also for detecting or defining hyperactivation (Cremades et al., 2005; Schmidt and Kamp,  
390 2004). Furthermore, being this software an open source, any investigator may freely  
391 implement or modify (as we and others already did) the plugin and create a more specific  
392 or versatile system, according to his/her needs; this could then be made available to the  
393 whole scientific community, which would benefit from that tool.

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

402

403 The plugin is provided as an e-component of the present article (see e-component.  
404 BGM\_java)

405

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506 Table 1. Motility parameters from CASA\_bgm and Hamilton-Thorne IVOS

507

	CASA_bgm	% CV	IVOS	% CV	Paired <i>t</i> -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

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516 Table 2. Correlation between CASA\_bgm parameters and percentage of sperm with  
 517 intact acrosome and percentage of viable sperm with active mitochondria. \*P<0.05  
 518 \*\*P<0.01

519

	% Sperm with an intact acrosome	% SYBR-14 <sup>+</sup> /PI <sup>-</sup> /JC-1 <sup>+</sup> -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

520

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

525

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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.

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536 Figure 1.

537

Figure 1 shows two versions of the Sperm Tracker input dialog box. The left version (a) is the original dialog box, and the right version (b) is the modified version for mammalian sperm analysis. The parameters and their values are as follows:

Parameter	Value
a, Minimum sperm area (pixels <sup>2</sup> ):	15
b, Maximum sperm area (pixels <sup>2</sup> ):	150
c, Minimum track length (frames):	30
d, Maximum sperm velocity between frames (pixels):	40
e, Minimum VSL for motile (um/s):	10
f, Minimum VAP for motile (um/s):	15
g, Minimum VCL for motile (um/s):	25
h, Maximum VAP for SLOW (um/s):	30
i, Maximum VAP for MEDIUM (um/s):	80
l, Frame Rate (frames per second):	60
m, Microns per 1000 pixels:	500
n, Print xy co-ordinates for all tracked sperm?(1 Yes, 0 No)	0
o, Print motion characteristics for all motile sperm?(1 Yes, 0 No)	1
p, Print mean and median values for motion characteristics?(1 Yes, 0 No)	1
----- ADVANCED PARAMETERS -----	
q, Minimum VAP for PM	15
r, Minimum STR for PM (%)	75

Parameter	Value
a, Minimum sperm size (pixels):	0
b, Maximum sperm size (pixels):	40.0000
c, Minimum track length (frames):	97.0000
d, Maximum sperm velocity between frames (pixels):	8.00000
e, Minimum VSL for motile (um/s):	3.000
f, Minimum VAP for motile (um/s):	20.0000
g, Minimum VCL for motile (um/s):	25.0000
h, Low VAP speed (um/s):	5.00000
i, Maximum percentage of path with zero VAP:	1.0
j, Maximum percentage of path with low VAP:	25.0000
k, Low VAP speed 2 (um/s):	25.0000
l, Low VCL speed (um/s):	35.0000
m, High WOB (percent VAP/VCL):	80.0000
n, High LIN (percent VSL/VAP):	80.0000
o, High WOB two (percent VAP/VCL):	50.0000
p, High LIN two (percent VSL/VAP):	60.0000
q, Frame Rate (frames per second):	97.0000
r, Microns per 1000 pixels:	1075.00
s, Print xy co-ordinates for all tracked sperm?	0
t, Print motion characteristics for all motile sperm?	0
u, Print median values for motion characteristics?	0

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

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544 Figure 2

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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	MedianWC	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									

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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

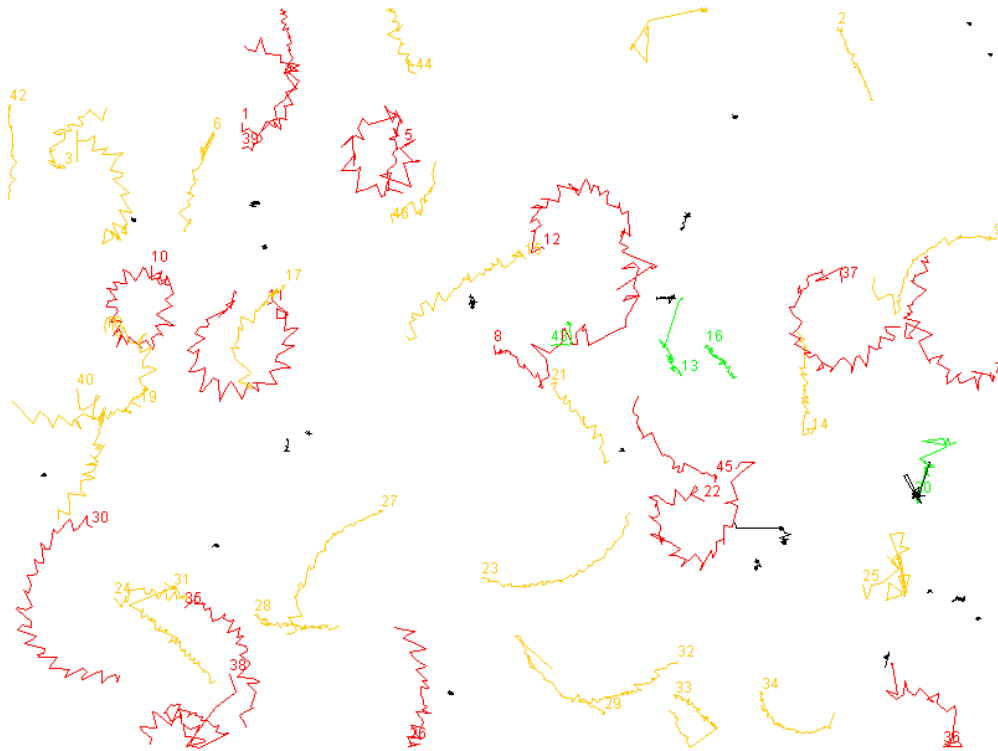
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553

554 Figure 3.



555

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

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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  $\pm 2$  SD.

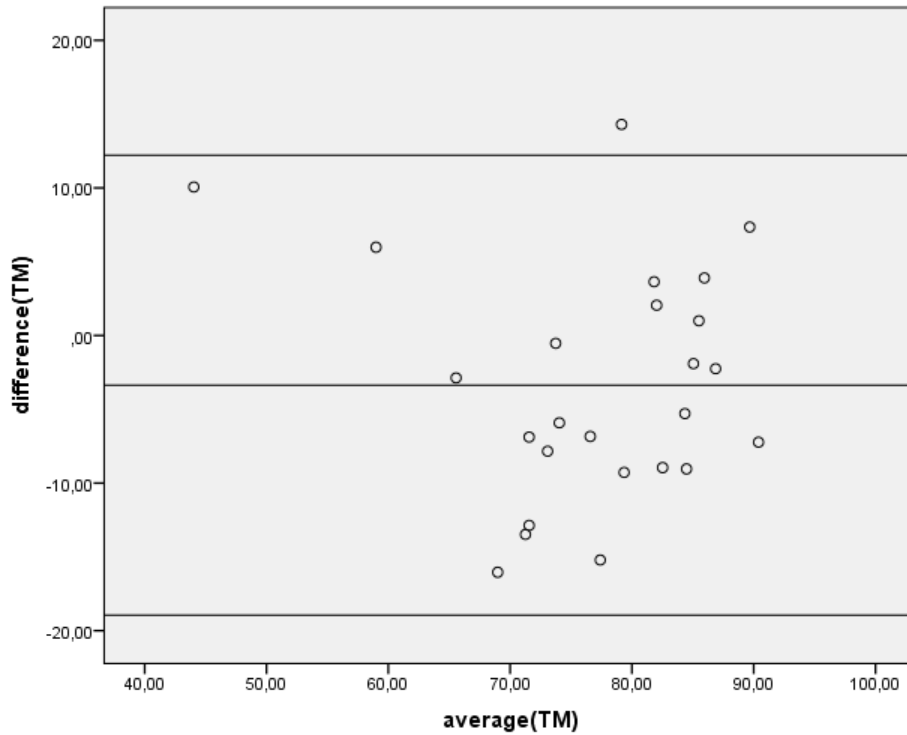
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571 Total motility(TM) Bland Altman's plot

572



573

574

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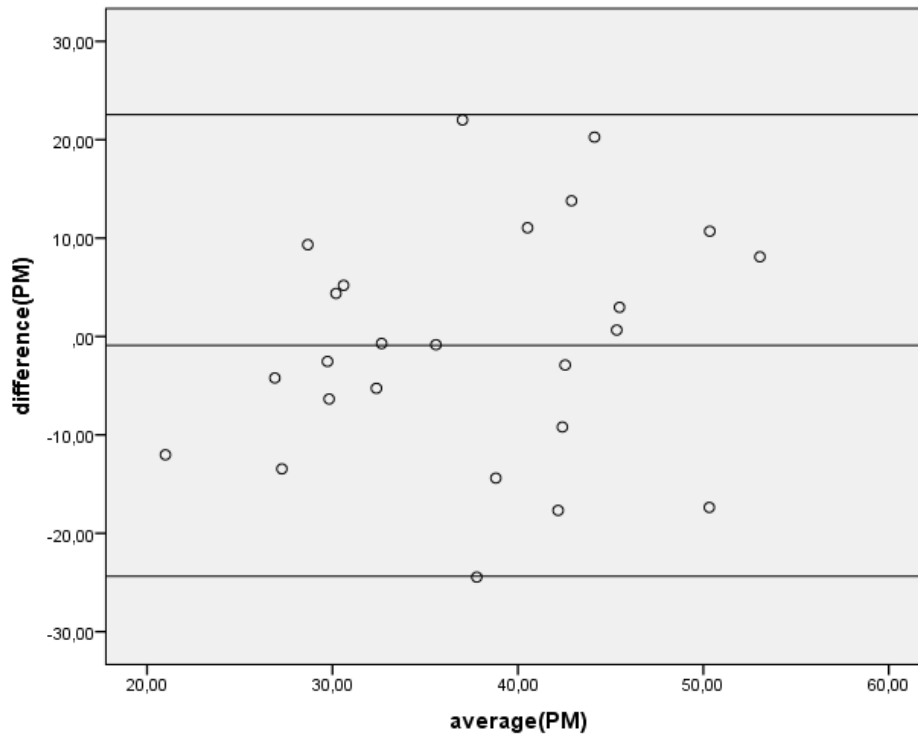
576

577 Progressive motility(PM) Bland Altman's plot

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579

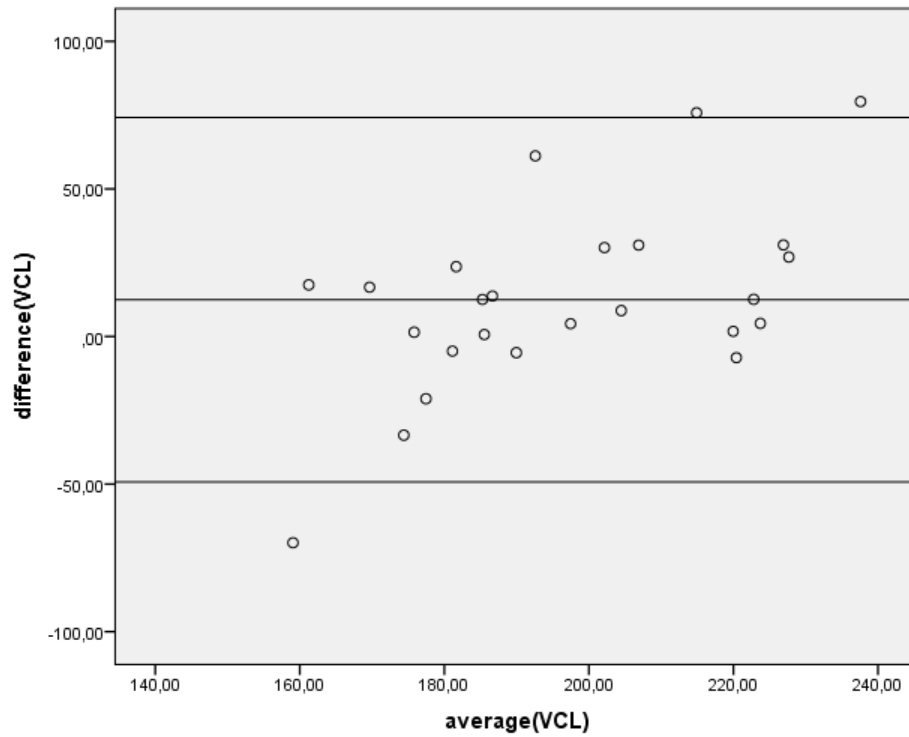
580

581 Linear velocity (VCL) Bland Altman's plot

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583

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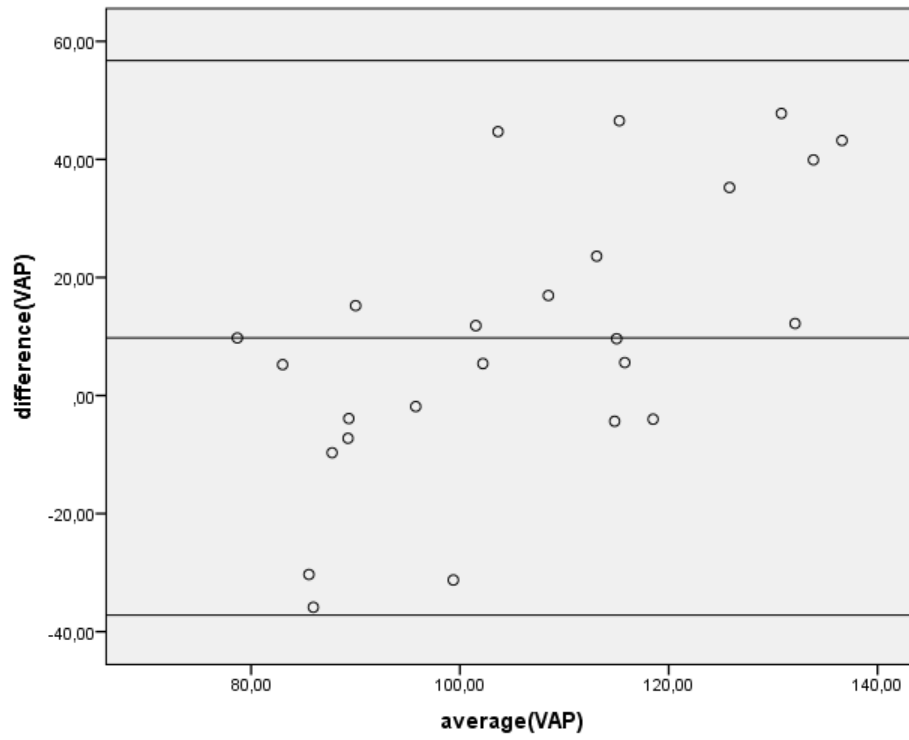
587 Mean velocity (VAP) Bland Altman's plot

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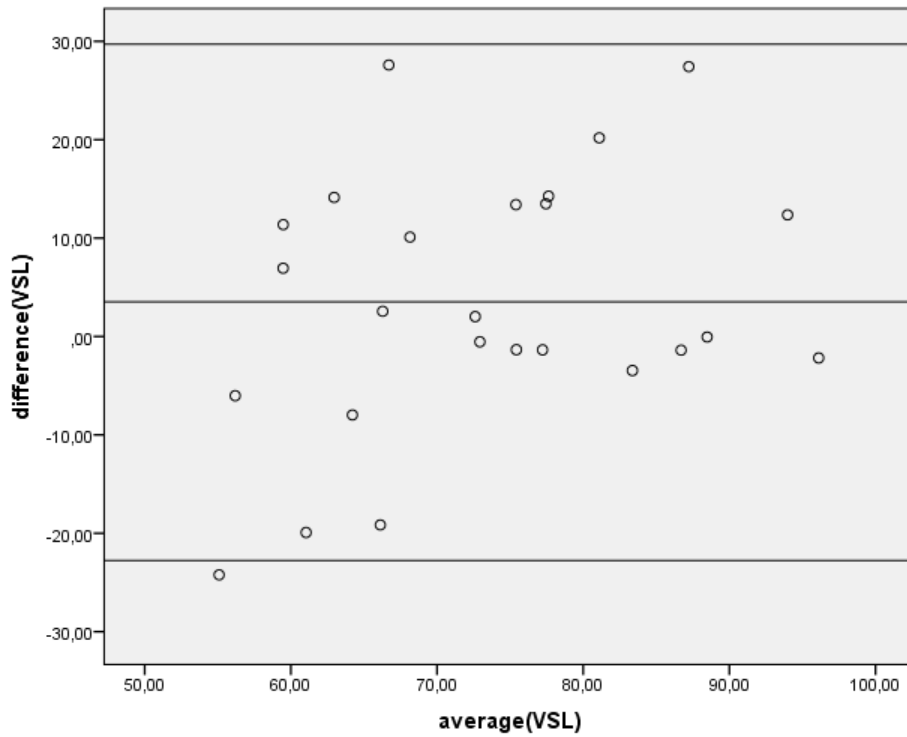
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591 Linear velocity (VSL) Bland Altman's plot

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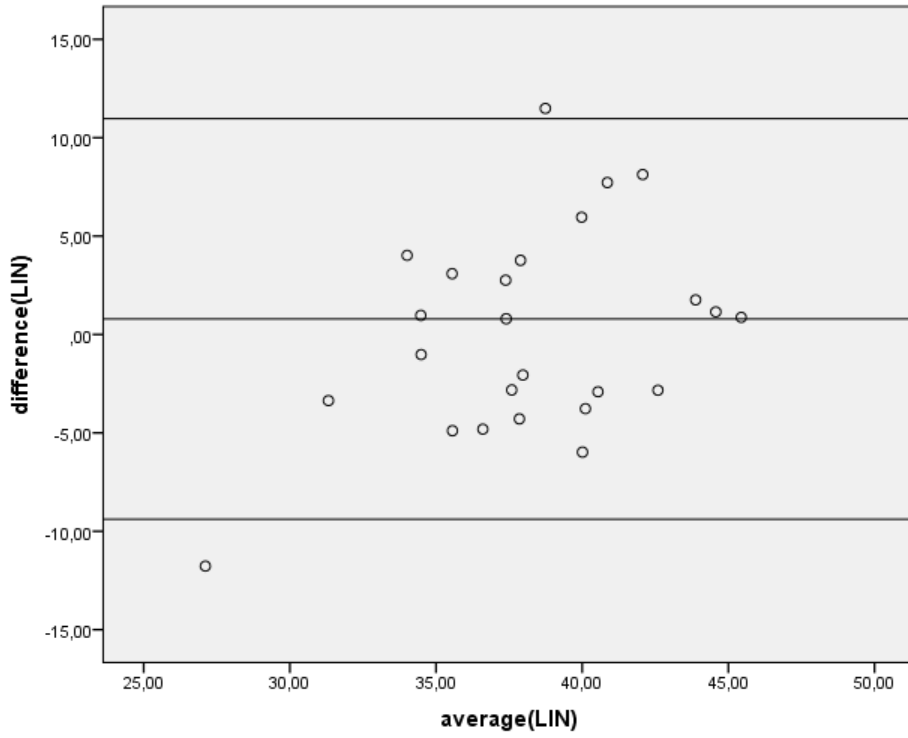
596

597 Linearity (LIN) Bland Altman's plot

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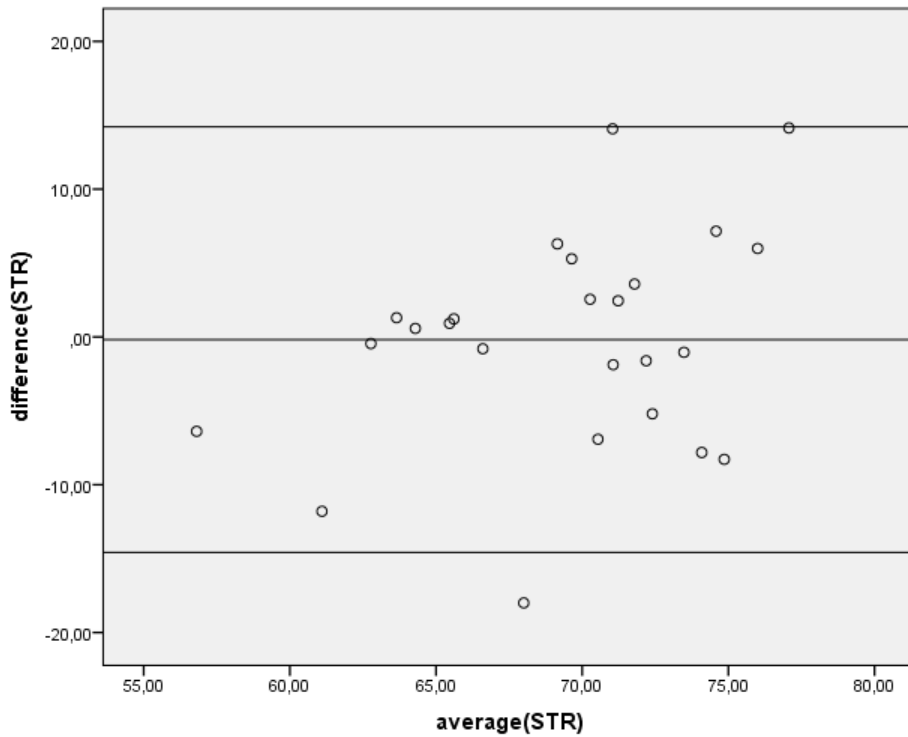
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599

600 Straightness (STR) Bland Altman's plot

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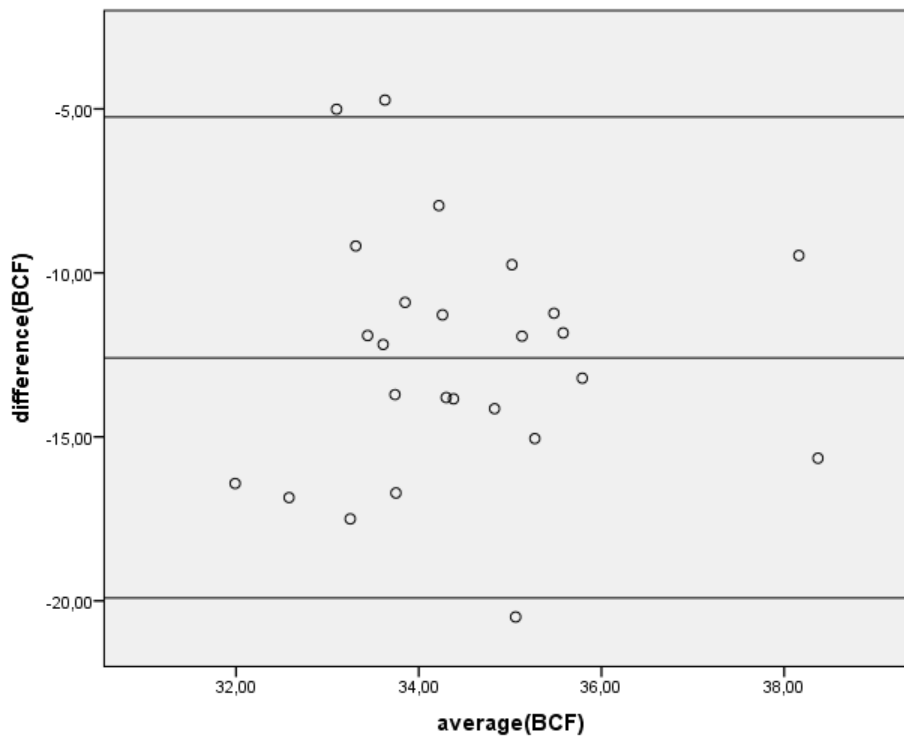
603

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605

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

607



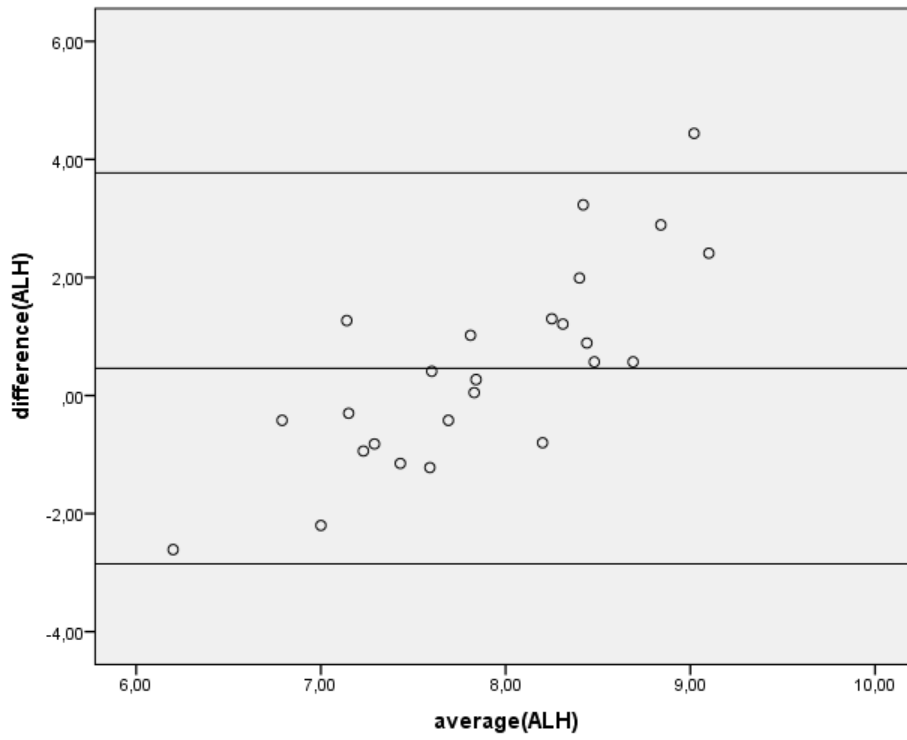
608

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

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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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649

650

651 The purpose of our work is to implement an open source tool and share with the international research  
652 community our findings and, possibly, start collaborations for further implementations. We neglect and reject  
653 any commercial use of our plugin.

654

655

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656 Brief instructions for analysis with ImageJ CASA\_BGM plugin

657

658 Plugin installation

659 We report the original instruction from Wilson Leedy and Ingermann

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

673

674 Import a video in Image J and analysis

675

676 After saving videos of sperm samples, these should be converted in .avi format.

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

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

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

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

687

688

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