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Computer assisted sperm analysis – the relationship to bull field fertility, possible errors and their impact on outputs: A review

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ABSTRACT

Sperm motility is one of the indicators most evaluated before and after cryopreservation, regarding quality and fertilizing ability. The present review provides complex information about the possible negative effects on the results of computer assisted sperm analysis (CASA) and also reflects a possible connection of these results to bull field fertility. Recently, there has been a growing interest in sperm motility assessment by CASA to determine sperm motion more accurately and objectively than by subjective evaluation. CASA systems have been routinely used in most research laboratories and also with increasing tendency in the case of insemination centres. However, objectivity and comparison of CASA results through laboratories can be impacted unfavourably. This is in particular due to the absence of standardization for bull sperm motility evaluation and the presence of drawbacks in the form of human and non-human factors. Investigators have recently turned to the possible association of CASA results with the prediction of bull field fertility. However, the studies suffer from discrepancies, thus a clear relationship has not yet been confirmed. Specific combinations of motility parameters with accurate determination of sperm subpopulations could represent another part in the complex system of providing the ability to predict fertility *in vivo*. The task of future works should be to establish standardization regarding sperm motility evaluation of specific animals, in addition to the settings and algorithms of CASA systems. Furthermore, predictive value CASA outputs to bull field fertility demand more extensive research aimed at a more precise definition of this relationship.

Key words: CASA, Fertility, Insemination dose, Sperm motility

For successful fertilization, both the male and the female gamete of every species is important. In males, this factor is currently represented by the quality of insemination dose (Beran *et al.* 2012, Beran *et al.* 2013) mainly in cattle, where artificial insemination (AI) is the biotechnological method used on a wide scale (Muino *et al.* 2008, Gravance *et al.* 2009, Sundararaman *et al.* 2012).

Quality control of insemination doses before and after thawing is mostly carried out subjectively, mainly on the basis of an estimation of motile spermatozoa ratio, since sperm motility is considered as closely related to fertility (Puglisi *et al.* 2012). Results of this estimation can be affected by bias and inaccuracy (Amann and Waberski 2014); moreover there are discrepancies among results of different studies (Farrell *et al.* 1998, Januskauskas *et al.* 1999, Gillan *et al.* 2008, Kathiravan *et al.* 2008). Methods routinely used for the assessment of spermatozoa do not always give results correlating sufficiently with fertility (Puglisi *et al.* 2012) and also these outputs are laden by subjectivity and variability (Rijsselaere *et al.* 2012).

In comparison to subjective evaluation, during examination of sperm suspension with computer assisted sperm analysis (CASA), a greater number of sperm cells is semi-automatically evaluated and, moreover, in a shorter time (Verstegen *et al.* 2002, Kathiravan *et al.* 2011). CASA represents a practical tool, which was developed at the beginning of the 1980s, aimed at providing a more objective analysis of sperm motility by reconstruction of sperm trajectories and their classification into different categories, to provide further details of sperm motility for quality assessment and fertility estimation. Nevertheless, there are still difficulties which have to be resolved before the routine use of CASA, as well as requirements which have to be fulfilled and factors which can affect CASA results (Mortimer 1997, Feitsma *et al.* 2011). According to Kathiravan *et al.* (2011), this system of sperm motility analysis can be considered as a more effective, precise and trustworthy tool for fertility assessment than subjective

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evaluation across breeds and age categories of breeding males. They also state that the results obtained are more objective, repeatable with a higher predictive ability and can be standardized. However, there are requirements which have to be fulfilled and factors which influence the results.

A primary disadvantage of CASA is the fairly high price of these systems which relates to its availability for laboratories. An overwhelming majority of managers and laboratory leaders have extensive information from CASA suppliers, reassuring them of accurate and reliable results. However, independent accurate comparison among different CASA systems has been lacking. Moreover, there is no standard among different CASA types (Kathiravan *et al.* 2011, Lu *et al.* 2014). Despite the indicated results by suppliers, it is appropriate to carry out comparison among different CASA systems and performance tests before purchasing (Feitsma *et al.* 2011, Amann and Waberski 2014). According to Feitsma *et al.* (2011), it is recommended that tests are performed on at least 100 ejaculates in duplicates, with the intention of evaluating accuracy and repeatability. The repeatability of the CASA results can be determined by regression of CASA measurement and WHO standards. The coefficient of determination resulting from this analysis represents repeatability. For a more detailed description of other possible coefficients and their relationships, see the study of Feitsma *et al.* (2011). Reliability of CASA is positively related to repeatability, which is increased with a higher number of successfully analysed spermatozoa, thus with decreasing coefficient of variation. Subsequently, in respect of this costly investment, there is a need for economic evaluation together with judging the level of lab technicians' skills in order to achieve the expected trustworthy outputs (Feitsma *et al.* 2011).

Introduction of CASA requires a higher level of economic and technical evaluation for determination of repeatability, calculation of economic profit, as well as training courses for laboratory technicians. Moreover, there is the consideration whether one CASA system would be sufficient for the analysis of a huge number of samples (Feitsma *et al.* 2011). Owing to these facts, a decision to purchase CASA has to be based on technical and economic analyses. Furthermore, managerial staff has to be confident of having skilled employees with the natural undertaking to be further trained, so that the required reliability of results is ensured. Recently, CASA systems have more often been introduced into boar and stallion insemination centres. Nevertheless, there has also been the same tendency in bull insemination centres (Amann and Waberski 2014).

The CASA system was first introduced in 1980 (Mortimer 2000), and over the years it has been developed from the analysis of microphotographs exposition, through analysis of records from each video record, to the latest semi–automatic methods (Rijsselaere *et al.* 2012). The first available systems were the CellSoft Automated Semen Analyser and Hamilton Thorn Motility Analyser (HTM), after which other types of this system were also presented (Katila 2001). The arrangements of CASA systems differ from each other mainly in hardware, optics (microscope, lens, camera) and software accessories (Verstegen *et al.* 2002). The CASA system is based on acquiring consecutive frames from a microscope by means of a simple chip camera (Quintero-Moreno *et al.* 2003). The image is consequently converted to a black/white resolution, exported to the computer and evaluated by specific software with the CASA module, which is able to analyse these images (Mortimer 2000). The obtained data are subsequently mathematically processed and trajectories are defined in numerical form. The results from this operation are reflected as a series of parameters which accurately define the motion of each spermatic cell (Quintero-Moreno *et al.* 2003). The process of motility analysis by CASA is partially automated, but its reliability depends on several factors.

The objective of this review is to summarise and provide complex information about possible errors and difficulties during the analysis of samples and subsequent interpretation of the obtained results. In addition, it reflects a possible relationship between the sperm motility parameters assessed by CASA and their value for estimation or prediction of bull field fertility.

Evaluation of sperm motility by CASA

In general, sperm motility has been considered as one of the most important characteristics in connection with fertilizing ability (Rodriguez-Martinez 2006, Awad 2011). With respect to the fact that cryopreserved semen is used entirely in bovine reproduction, estimation of the motility before cryopreservation closely relates to the quality of spermatozoa post-thaw (Fitzpatrick *et al.* 2002). Subjective motility assessment is considerably affected by the technicians' experience, thus there is a higher probability of the incidence of systematic mistakes (Januskauskas *et al.* 1999, Amann and Waberski 2014). Evaluation of motion characteristics by the CASA system provides a wide scale of kinematic parameters described by Mortimer (2000), which can be seen in Table 1 and are graphically illustrated in Fig. 1. These indicators have a high informative value, characterising the physiological state of spermatozoa and their possible fertilizing ability; thus they provide more accurate information with regard to the estimation of bull fertility (Farrell *et al.* 1998, Kathiravan *et al.* 2011). Because

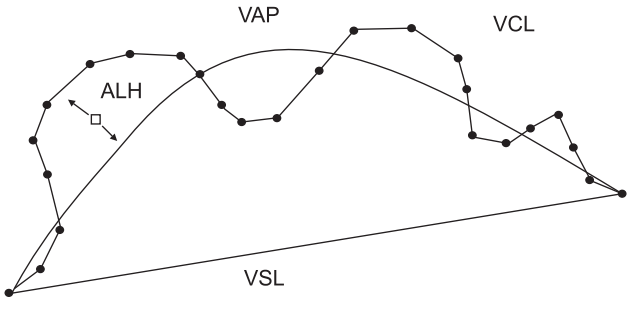

Fig. 1. Expression of selected movement characteristics.

Table 1. Abbreviations used

VCL	curvilinear velocity
VAP	average path velocity
VSL	straight line velocity
STR	straightness (VSL/VAP)
LIN	linearity (VSL/VCL)
WOB	wobble (VAP/VCL)
TMOT	total motility
PMOT	progressive motility
ALH	amplitude of lateral head displacement
BCF	beat cross frequency
LVV	low VAP cut-off
MVV	medium VAP cut-off

there is no standard for animal spermatozoa assessment (Amann and Katz 2004, Feitsma *et al.* 2011), it could be divided according to the World Health Organization (WHO) classification for human spermatozoa into 4 categories – fast, medium fast, slow and immotile. Categorization according to WHO is performed, depending on the lowest and middle value of VAP (LVV and MVV) and the minimal value of STR, in which fast sperms are considered sperms with VAP > MVV, middle fast LVV < VAP < MVV, slow VAP < LVV and immotile without any movement during the analysis. The percentage of sperm with progressive motility (PMOT) is consequently established by VAP which exceeds MVV and the minimal value of STR (WHO 2010). In bull sperm, the accurate values for categorization have not yet been established. This fact is evident in studies by Farrell *et al.* (1998);, Gillan *et al.* (2008), Contri *et al.* (2010), Shojaei *et al.* (2012) and Oliveira *et al.* (2013), where, despite the fact that the authors used the CASA system from the same manufacturer, the values for categorization were set at different levels.

Another method for distinction of the different sperm groups is determination of subpopulations, which are differentiated by cluster analysis. Identification of subpopulations in sperm sample is recently accepted by a wide range of scientists, due to the more meaningful approach to establish a ratio of specific subpopulations in sperm sample in contrast to values of means or medians (Amann and Waberski 2014). In addition to other mammalian species (Abaigar *et al.* 1999, Abaigar *et al.* 2001, Quintero-Moreno *et al.* 2003, Quintero-Moreno *et al.* 2004, Quintero-Moreno *et al.* 2007,), the presence of these subpopulations has also been ascertained in bulls (Muino *et al.* 2008, Muino *et al.* 2009). More importantly, results of studies (Muino *et al.* 2008, Muino *et al.* 2009) showed possible similarities in the distribution of subpopulations among bull sires, thus that subpopulation structure could be constant for bovine sperm samples (Muino *et al.* 2009). The diversity of these subpopulations is a reflection of the differences in morphology, motility, longevity and finally also in their fertilizing ability (Muino *et al.* 2008, Amann and Waberski 2014, Martinez-Pastor *et al.* 2011). Muino *et al.* (2009) reported the presence of the following four sperm subpopulations: the first subpopulation with a relatively low velocity but high progressivity (high LIN, STR, WOB, BCF and low ALH); the second subpopulation which was characterized by high activity but without PMOT, with high values of VCL and ALH simultaneously with low LIN, and STR so-called hyperactivated motility; the third included sperms with very low motility and without progressive movement, which is characterized by the lowest values of VCL, VSL, VAP, ALH, BCF, LIN, STR, WOB; and the fourth subpopulation represented spermatozoa with fast and PMOT with the highest values of both VCL, VSL, VAP, BCF and LIN, STR, WOB, ALH.

From the viewpoint of bovine insemination doses production, it was shown that the percentage of sperm with fast and progressive motility before cryopreservation correlates with the longevity of spermatozoa after thawing (Nunez-Martinez *et al.* 2006). This result indicates that a higher resistance of the ejaculate to the cryopreservation process is linked to a higher incidence of spermatozoa from this subpopulation before freezing. Nunez-Martinez *et al.* (2006), who evaluated Holstein bulls, concluded that the ratio of each subpopulation was similar in both native ejaculate and insemination dose after thawing. Only the case of the percentage of fast sperms with progressive motility was found by these authors as significantly different. Similar results were published in a study where the authors assessed post-thaw motility in a group of Asturiana de los Valles beef cattle (Muino *et al.* 2009).

Factors affecting analysis outputs

Processing of sample: A very important factor for the standardization of the CASA system results is the pre–analytical part, which includes the preparation of a sample for analysis (Amann and Waberski 2014). Thus the concentration, sample volume, counting chamber type, temperature and type of extender are crucial for correct interpretation of CASA results.

The samples have to be diluted before analysis, since the concentration of the native ejaculate is too high for a successful analysis of the pathway of each sperm (Verstegen *et al.* 2002). These authors also indicate that the optimum concentration of sperm for CASA analysis is within the range of 30-50 × 10^6/ml. Additionally, Contri *et al.* (2010) reported that the optimum concentration as 20 × 10^6/ml, when in their experiments the concentration of 50 × 10^6/ml caused a large amount of erroneously generated trajectories and inferior sperm detection. The sample volume of semen after dilution used for CASA analysis varies between 4 – 10 µl, depending on the counting chamber used. For Microcell chambers, 7 µl is used; in Makler chambers it is 4 µl (Januskauskas *et al.* 1999), as in the Leja chambers (Shojaei *et al.* 2012).

In the same way that the use of different types of chambers can affect sperm motility parameters (Lenz *et al.* 2011, Gloria *et al.* 2013, Amann and Waberski 2014), the

duration of the analysis can affect the results of the analysis, both the motion parameters and the total percentage of progressive motile spermatozoa. The duration of the analysis should not exceed 5 min (Contri *et al.* 2010). Due to the small volume which is used for the analysis, the samples have to be maximally homogenized and processed gently. For the safety of the objectivity of the results, higher demands are required on the preparation of samples in comparison with subjective analysis (Feitsma *et al.* 2011, Michos *et al.* 2013).

For analysis of insemination dose, the thawing procedure must be taken into account, since a possible effect on some analysed parameters has been shown (Iguer-ouada and Verstegen 2001, Contri *et al.* 2010). Particularly the temperature of thawing and further incubation (37°C) is crucial (Iguer-ouada and Verstegen 2001).

In addition, the fact that the sample already contains an extender plays a role, because no medium has the same properties as the fluids in the female reproductive tract (Amann and Hammerstedt 2002). Therefore it is necessary to know which type of extender was used before the interpretation of the results. In egg yolk extenders, there are objects with a similar size as the sperm cells yolk granules – which can adversely affect the accuracy of results' objectivity (Wall and Foote 1999, Amirat *et al.* 2005) and the percentage of motile spermatozoa as well (Moussa *et al.* 2002). In contrast, the addition of only effective compound of egg yolk can improve post-thaw motility of spermatozoa (Simonik *et al.* 2013) and can lead to better assessment, due to no presence of yolk granules. The higher viscosity in comparison with non-egg yolk types of media is also related to this influence. This fact has been demonstrated by the reduction of speed parameters (VAP, VSL and VCL) and the percentage of fast-moving sperm cells. Conversely, using HEPES or TALP for sample dilution caused increase of the percentage of motile and rapidly moving spermatozoa. In the case of a saline solution, its usage can avoid the compromise of speed parameters, however, the percentage of progressive and moderately fast moving sperm is also lower (Rijsselaere *et al.* 2003). With regard to the comparison of other diluting media and their relation to the analyzed parameters, there were no significant differences between saline solution, physiological buffer saline and Bioxcell®, with the exception of VCL and ALH, which were significantly higher, and LIN, which was lower when using Bioxcell® compared with saline solutions (Contri *et al.* 2010).

Other factors

When the possible effect of camera properties were assessed, a significant influence of lower imaging speed on total motility (TMOT), progressive motility (PMOT), all the kinematic parameters and categories of fast, middle fast, slow and immotile spermatozoa were found upon a comparison among cameras with 30, 45 and 65 fps (Contri *et al.* 2010). The most common sources of errors in CASA systems are inaccurate object recognition, collisions (Rijsselaere *et al.* 2003, Rijsselaere *et al.* 2012), loss of trajectories due to the high sperm concentration, errors during the reconstruction of the conflicting trajectories and their interpolation. However, there are ongoing innovations of the CASA systems, such as the endeavour to determine new algorithms for a more reliable assessment of static objects and collisions. One of the most basic methods of prevention of incorrect results caused by sperm collisions is, as mentioned previously, appropriate sperm concentration (Verstegen *et al.* 2002). Different types of CASA software represent another 'non-human' factor which plays a role in the reliability of results, especially during comparison among laboratories. Software functions on the same principles, but there are differences in the settings and algorithms for generating trajectories and calculating velocities of each spermatozoon (Amann and Waberski 2014). Moreover, variances among CASA in the capability of distinguishing spermatozoa from debris and egg yolk particles are known (Vincent *et al.* 2012). These factors also present possible influences related to the introduction of CASA in scientific laboratories and insemination centres, as well.

As was previously stated, also the human factor can affect results of analysis in different ways, thus specific requirements have to be fulfilled. The lab technician plays a crucial role in these complex conditions of assessment and can influence almost all of the aforementioned factors, in order to keep obtained results reliable and repeatable (Michos *et al.* 2013, Amann and Waberski 2014). Hence, CASA as a sophisticated system places high demands on skilled operators. Moreover, since there is no defined golden standard for methods of animal spermatozoa motility analysis, so establishment of a standard operating procedure for a specific laboratory is recommended. This should be accompanied by determination of repeatability of assessment, when repeatability higher than 95% is feasible (Feitsma *et al.* 2011). In other words, one of the difficulties in the usage of CASA is the human factor. The purchase and establishment of CASA not only entail maintaining the facilities, but operators need to know the principles of CASA and have to be periodically trained (Michos *et al.* 2013).

Relationship of sperm motility to bull fertility

Motility is accepted as the most important parameter in the set of evaluated characteristics in terms of the fertilization ability of sperm, according to e.g. Fitzpatrick *et al.*(2002) and Kathiravan *et al.* (2011). A number of scientific laboratories deal with the subject of determining one particular parameter, evaluated by the CASA system, and its close correlation with fertility *in vivo*. However, this parameter or laboratory test, which could accurately report information on the fertilization ability of insemination dose, has not yet been established (Oliveira *et al.* 2013). To a certain extent, this cannot be expected due to the fact that the reproductive process is very complex (Feitsma *et al.* 2011, Vincent *et al.* 2012, Amann and Waberski 2014, Ferraz

et al. 2014) and can be affected by different physiological conditions (Rehak *et al.* 2012).

Nevertheless, different sets of specific parameters of motility and their relationship between *in vitro* and *in vivo* fertility were described earlier (Farrell *et al.* 1998, Gillan *et al.* 2008, Oliveira *et al.* 2013). One of the first studies to demonstrate the relationship among kinematic parameters of spermatozoa assessed by CASA and bull fertility was published by Farrell *et al.* (1998). This study was performed on the basis of motility parameters of native ejaculates from 11 bulls and their lifetime fertility. Results indicated that, for the prediction of bull fertility, the following combinations of parameters had the highest correlation values - ALH, BCF, LIN, VAP, VSL ($r^2 = 0.98$); BCF, LIN, VSL, VCL, STR ($r^2 = 0.97$); BCF, LIN, VAP, VSL, STR ($r^2 = 0.97$) and BCF, LIN, VAP, VSL, VCL ($r^2 = 0.97$). Conversely, for single parameters r^2 values were much lower, for example for total motility, the coefficient of determination was 0.34. Similarly, the fact that a combination of several parameters correlated more with *in vivo* fertility was confirmed by Oliveira *et al.* (2013). However, this study was carried out on the basis of evaluation of sperm parameters of frozen-thawed insemination doses and a field artificial insemination programme. Although these authors used the same type of CASA system, the settings were different and, moreover, samples were stained with Hoechst 342 for better visualisation and differentiation of spermatozoa in milk-based extender. In contrast to the study of Farrel *et al.* (1998), partial least squares analysis was applied for the first time for this purpose. Oliveira *et al.* (2013) revealed that motility parameters, which could be used as predictors of field fertility immediately after thawing, were total and progressive motility. After two hours of thermal resistance test, important predictors of fertility were total motility, progressive motility, BCF and percentage of rapidly moving cells. On the basis of these two studies, the fact that more parameters have higher predictive value was confirmed. Moreover, it was found that after 2 hr incubation of frozen-thawed insemination doses, a higher number of evaluated sperm motion characteristics had greater predicative capability.

Zhang *et al.* (1998) also assessed sperm qualitative parameters post-thaw and their possible correlations to bull field fertility. They presented a significant positive correlation of sperm with linear movement pattern. On the other hand, different CASA software was used and, moreover, with a different setting. Another study by Cseh *et al.* (2004), conducted with a different CASA system, showed a correlation with strong significance for VAP and bulls with higher *in vivo* fertility. Cseh *et al.* (2004) proved that lower values of VCL (22.51 – 79.5 μm/s); VSL (11.35 – 35.71 μm/s) and VAP (12.67 – 34.45 μm/s) were linked to bulls with low field fertility.

Contrary to the results of Farrel *et al.* (1998), Cseh *et al.* (2004) and Oliveira *et al.* 2013), the results of Gillan *et al.* (2008) were different. They worked with the same CASA system as Oliveira *et al.* (2013) with almost the same settings, however with a higher number of bulls. Fertility of bulls was assessed with FERTSOL expressing fertility by 56-day non-return rate adjusted for season of insemination, inseminator, and number of inseminations, etc. (Vincent *et al.* 2012). Different groups of bulls were involved to experiment according to these statistically derived fertility values. Gillan *et al.* (2008) showed no correlation of almost all the parameters evaluated by CASA and *in vivo* fertility. Interestingly, results also showed that merely VSL, VCL, ALH, BCF parameters were involved in certain combinations with other specific *in vitro* assessed parameters which had high correlation values > r^2 0.986 which is in contrast to other studies (Farrell *et al.* 1998, Cseh *et al.* 2004, Oliveira *et al.* 2013). But like that of Oliveira *et al.* (2013), this study proved that an increased number of tests resulted in greater correlation values with fertility *in vivo.* Even more relationships among motility parameters and other *in vitro* tests were determined. Oliveira *et al.* (2012) also showed discrepancies in relation to the motility parameters assessed by CASA, when bulls characterized with higher field fertility displayed lower VSL, STR and LIN. On the other hand, bulls with lower fertility had lower TMOT. This fact was additionally supplemented with lower values of parameters TMOT, PMOT, VAP, VSL, VCL, ALH, BCF, STR, LIN after 4 hours of thermo resistance test. Nonetheless, it must be taken account that the study included only three bulls and inter-bull differences were not significant.

Factors which can also play a role in determining a set of indicators for predicting fertility of insemination doses are individual differences in sensitivity of ejaculates to cryopreservation (Muino *et al.* 2008) and interbreed differences (Hoflack *et al.* 2007, Beran *et al.* 2011). Hoflack *et al.* (2007) are some of the few researchers to have investigated a comparison of parameters in native semen of different bull sires – Holstein and Belgian Blue-White (*Bos taurus)*. Differences were determined in speed as well as in other physical characteristics. In Belgian Blue-White breed, VCL, VSL and VAP were significantly lower, together with the differences in the kinetic characteristics of ALH and BCF, showing lower movement efficiency. Those differences may also be related to the fertilization ability of sperm after cryopreservation, which was demonstrated by Farrell *et al.* (1998). In the view of the categories, slow, immotile spermatozoa were found in a higher proportion. Moreover, overall and progressively motile sperms were lower as well, in the case of the Belgian Blue-White breed.

Studies to investigate the possible connection of sperm motility assessment before cryopreservation with the quality of insemination doses post-thaw, which is obviously connected with fertilisation ability (Holt 2000), were performed by Muino *et al.* (2008) and Muino *et al.* (2009). Muino *et al.* (2008) presented data of the Holstein breed, which suggested a possible relationship of sperm subpopulations before freezing and their quality post-thaw.

Occurrence of these different sperm subpopulations in the native ejaculate can be used to predict resistance to cryopreservation, in other words, fertilizing ability of the insemination dose. The specific prevalent category of rapidly and progressively moving spermatozoa before cryopreservation is directly proportional to this subpopulation after thawing (Muino *et al*. 2008). In addition, evaluation of the cryopreservation effect on sperm subpopulations of the autochthonous breed of Asturiana de los Valles bulls by Muino *et al.* (2009) proved virtually the same results. The lack of studies concerning differences among other breeds raises the question: what will the situation be when comparing more similar breeds? Are the number and characteristics of sperm subpopulations constant for bovine ejaculates?

Another indicator of bull fertility *in vivo* could be the ability of spermatozoa to transform to the hyperactivation state, thus the ratio of sperm cells with motility pattern which classifies them into so called 'transition phase' (Shojaei *et al*. 2012). This movement pattern was characterized by higher VCL, ALH and simultaneously lower LIN, WOB (Mortimer 2000). The full physiological significance of the hyperactivation phenomenon has not been completely understood. However, there is a presumption that is important for the release of the sperms attached to epithelial cells in the isthmus of the oviduct (Verstegen *et al.* 2002, Marquez and Suarez 2007, Suarez 2008a, b). An essential requirement from the point of view of more precise identification of this sperm movement pattern is to use a camera with more than 60 fps. Cut-offs for recognition of the transition phase were: ALH > 7 μm, LIN < 0.65 and VCL > 80 μm/s (Marquez and Suarez 2007). The ability to use the ratio of spermatozoa in the transition phase determined by CASA to predict field bull fertility was proven by Shojaei *et al.* (2012). These authors showed that the aforementioned values were characteristic for the ejaculate of bulls with higher FERTSOL index, suggesting positive correlation of these motility parameters with increased fertility. As a consequence, the same authors subsequently confirmed in an environment simulating the female genital tract *in vitro*, that the sperms of these bulls with higher fertility easily cross into a state of hyperactivation. This hyperactivated status probably increases the passage of sperm cells through the female reproductive tract to the point of fertilization (Suarez 2008), thus the ability to undergo a hyperactive state could be an indicator of the spermatozoa performance characteristics associated with fertility *in vivo* suggested by Shojaei *et al.* (2012).

Other utilization of CASA

In addition to motility assessment, most CASA systems can be used for an evaluation of sperm concentration, sperm morphology and sperm viability. However, Verstegen *et al.* (2002) reported that, where this parameter was investigated, an accurate assessment of sperm concentration using CASA is a problem in most species. The main problems are underestimation of the number of sperms (Maes *et al.* 2010) and overestimation of the sperm numbers due to collisions and subsequent re-counting of already analyzed objects (Coetzee *et al.* 2001). A major factor which can cause non-uniformity of sperm distribution in a sample, thus the estimation of sperm number, is the counting chamber. After filling, the Segre-Silberberg effect causes excessive sperm movement towards the walls of the chamber, dependent on the Z axis and viscosity of the suspension. It can be reduced by analysing the sample along the central axis of the chamber and also by the use of a correction coefficient (Kuster 2005 , Douglas-Hamilton *et al.* 2005).This physical phenomenon also affects the number of sperms in relation to the time when motile sperms relocate from the walls to the centre of the chamber and immotile sperms cannot be redistributed (Amann and Waberski 2014). In haemocytometer count, which is generally accepted as a gold standard for the determination of sperm concentration, the Segre-Silberberg effect is eliminated due to its depth (100μm). However, the fact that the Bürker chamber has a five times greater depth does not allow its use during CASA concentration evaluation (Hansen *et al*. 2006). These authors presented that the highest agreement for diluted semen was between sperm vision and haemetocytometer. Nonetheless, huge discrepancies can also be found among CASA systems with regard to SpermVision and UltiMate (Douglas and Hamilton 2005). Alternatively, during the comparison of CASA and the subjective evaluation, there has been an overestimation prior to cryopreservation, with the opposite error after thawing. The reason could be the tendency of sperm cells to agglutinate after the cryopreservation process. Another influencing factor may be the system setup for evaluation of native and cryopreserved semen in relation to the used extender (Verstegen *et al.* 2002, Hansen *et al.* 2006). Finally, in bull artificial insemination centres, sperm concentration determination by CASA has been considered as too inaccurate in comparison with standard and routinely used methods. So, the use of the CASA tool is more common in centres where stallion or boar semen is processed (Amann and Waberski 2014). In the case of morphology analysis, Björndahl *et al.* (2010) reported that the current generation of CASA instruments are not able to analyze sperm morphology to a degree that would be suitable for routine use. The problem is the lack of the ability to determine the central part of a sperm flagellum. In subjective evaluation of human sperm morphology, the guidelines are set by WHO, and despite this fact, there can be considerable variability among laboratory technicians and also among all laboratories (Rijsselaere *et al*. 2012, Amann and Waberski 2014). Standardization is not established in morphology evaluation of livestock sperms (Verstegen *et al.* 2002), therefore it is possible to expect even greater variability. Morphology analysis using CASA is also influenced by factors which can distort results, but these can be eliminated by following the standard procedures (Gravance *et al.* 1996, Verstegen *et al.* 2002). Samples for

analysis have to be prepared by washing, staining and fixing before image recording and processing, but this is associated with a greater time consumption and varies according to the CASA system used. Therefore, for maximum image quality, the outputs of quality, type of staining, magnification and the optical device are crucial. These factors, together with different user settings of software may again be a source of variability (Verstegen *et al*. 2002).

Conclusion

In summary, CASA is known and used in laboratories, however certain problems persist. Sperm motility is one of the parameters evaluated routinely. CASA represents a tool for more objective and accurate analysis of sperm motion than subjective evaluation. It must be borne in mind that various human and non-human factors can influence the advisability and objectivity of outputs obtained by CASA. Although CASA provides many parameters for every recognized sperm cell, only a specific approach ensures the meaningful value of a vast number of data for semen evaluation and prediction of bull fertility. Nevertheless, accurate significance of motility parameters to prediction of field fertility has not yet been elucidated.

In all honesty, the use of CASA currently does not represent a tool for precise prediction of bull field fertility. However, a debatable question is: Does it make it possible to discover one or more *in vitro* parameters serving to foretell bull fertility? Outputs of CASA are unlikely going to be individually representative indicators for bull fertility *in vivo*. Nevertheless, specific combinations of motility parameters and defined subpopulations of spermatozoa assessed by CASA should be used together with outputs of other laboratory tests. In this way, they can serve as parameters for improving and increasing the value of *in vitro* assessments for the prediction bull field fertility.

Differences among CASA systems represent obstacles in view of the objective comparison of results across laboratories and also studies investigating prediction values of CASA outputs to bull field fertility. Creation of standards for animal sperm motility assessment by CASA regarding algorithms, parameters, precise object recognition and threshold values for subpopulations can overcome the aforementioned loophole. Moreover, the possible contribution of CASA results to the prediction of bull *in vivo* fertility demands further extensive investigation.

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