

## CASA-Mot technology: how results are affected by the frame rate and counting chamber

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**Abstract.** For over 30 years, CASA-Mot technology has been used for kinematic analysis of sperm motility in different mammalian species, but insufficient attention has been paid to the technical limitations of commercial computer-aided sperm analysis (CASA) systems. Counting chamber type and frame rate are two of the most important aspects to be taken into account. Counting chambers can be disposable or reusable, with different depths. In human semen analysis, reusable chambers with a depth of 10  $\mu\text{m}$  are the most frequently used, whereas for most farm animal species it is more common to use disposable chambers with a depth of 20  $\mu\text{m}$ . The frame rate was previously limited by the hardware, although changes in the number of images collected could lead to significant variations in some kinematic parameters, mainly in curvilinear velocity (VCL). A frame rate of 60 frames  $\text{s}^{-1}$  is widely considered to be the minimum necessary for satisfactory results. However, the frame rate is species specific and must be defined in each experimental condition. In conclusion, we show that the optimal combination of frame rate and counting chamber type and depth should be defined for each species and experimental condition in order to obtain reliable results.

**Additional keywords:** computer-aided sperm analysis (CASA) system, kinematic, motility.

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### Semen analysis

Traditional analysis of semen samples includes the assessment of concentration, motility and morphology, and is the basic tool for determining male fertility (Chong *et al.* 1983; Overstreet 1984; Budworth *et al.* 1988; Hirai *et al.* 2001; McPherson *et al.* 2014). The most common way to evaluate semen quality is based on subjective observations, which require a great deal of time and experience. For this reason, they often lack consistency. It seems that the first trials to define a standard for semen analysis (Falk and Kaufman 1950; MacLeod and Gold 1951; Barták 1971; MacLeod 1971; American Fertility Society 1980; Belsey *et al.* 1980) were commonly ignored, incorrectly followed or not read at all (Chong *et al.* 1983). Unfortunately, this problem still persists, and many different criteria continue to be used even now, leading to considerable confusion and a complete lack of possible comparison between laboratories. The most reputed institutions have endeavoured to define universal

standards for semen analysis (WHO 2010; see also previous version of Kvist and Björndahl 2002; Barratt *et al.* 2011). However, in the end, these standards as still not correctly followed or possibly not even used at all.

Semen evaluation has two main purposes, one related to male fertility (both in humans and other species) and the other for optimising the production of insemination doses for livestock breeding (Hansen 2014). In terms of human male fertility, it has been shown that, in humans, problems conceiving in approximately 50% of couples are related to the male partner (Kumar and Singh 2015). Even if the final causes of infertility are complex and involve the female partner, performing semen analysis continues to be the easiest way to evaluate what is happening with the male part of the problem. Regarding seminal dose production, inaccurate estimates of sperm concentration along with a incorrect interpretation of the spermiogram can lead to faulty insemination doses. All of this can jeopardise

ejaculate optimisation, fertility and fecundity (Jequier and Ukombe 1983; Mortimer *et al.* 1986; Douglas-Hamilton *et al.* 2005a; Kathiravan *et al.* 2011; Amann and Waberski 2014).

Since the outset of CASA-Mot technology, various studies have dealt with the aim of defining optimal set-ups for use of the systems (Katz and Davis 1987). However, these studies only referred to some species. The aim of this paper is to review the effect of frame rate and counting chamber type and depth on the results of CASA-Mot in a variety of mammalian species.

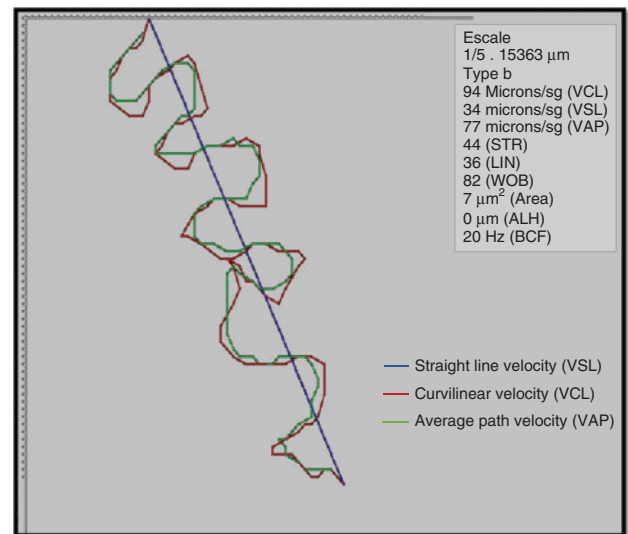
### History of sperm kinematic analysis

During the second part of the last century, several attempts were made to develop an objective method for evaluating sperm motility that were initially based on flagellar motility alone (Taylor 1951, 1952; Hancock 1953; Rothschild 1953; Gray 1955, 1958; Gray and Hancock 1955; Rikmenspoel and van Herpen 1957; Brokaw 1965, 1970, 1972; Fray *et al.* 1972; Blum and Lubliner 1973; Denehy 1975; Yundt *et al.* 1975; Katz and Dott 1975; Cosson 1996). These trials later focused on head position using the following techniques: kinemicrography (van Duijn *et al.* 1971), photography (Glover 1968; Janick and MacLeod 1970; Elliot *et al.* 1973; Revell and Wood 1978), spectrophotometry (Atherton 1975; Cooke and Hallet 1976; Atherton *et al.* 1978; Majumder and Chakrabarti 1984), Laser light scattering (Dubois *et al.* 1975; Shimizu and Matsumoto 1977; Mitsukawa 1979), videomicrography (Tash *et al.* 1986; Katz and Overstreet 1981; O'Connor *et al.* 1981; Samuels and van der Horst 1986), cinematography (Katz *et al.* 1978; David *et al.* 1981), haemocytometry (Ishii *et al.* 1977), photomicrography (Katz and Dott 1975; Makler 1978a; Overstreet *et al.* 1979; Amann and Hammerstedt 1980; Bartoov *et al.* 1981; Aitken *et al.* 1982, 1985) and stroboscopy (Cosson *et al.* 1985). These approaches were time consuming and of little use for diagnostic purposes. Nevertheless, these results offer interesting results and provide a strong background upon which current methods are based.

### Era of CASA technology

With the introduction of computer-aided sperm analysis (CASA) technology at the end of the 1970s, the intention was to overcome these problems and many approaches were developed that were both scientific and clinical (Liu and Warme 1977; Katz and Overstreet 1981; Walker *et al.* 1982; Acott *et al.* 1983; Suarez *et al.* 1983; Schoëvaert-Brossault 1984; Holt *et al.* 1985; Katz *et al.* 1985; Mathur *et al.* 1986; Katz and Davis 1987; Ginsburg *et al.* 1988; Aanesen and Bendvold 1989; Johnson *et al.* 1990, 1996a, 1996b). All these efforts greatly improved the significance of semen analysis in determining fertility (Aitken *et al.* 1994; MacLeod and Irvine 1995) and the seminal doses produced for AI programs (in the case of domestic animals; Hansen 2014). It should be considered that there are different technologies inside the general concept of CASA-Mot systems (Amann and Waberski 2014). However, herein we focus only on the one that is based on head centroid position to define sperm kinematics.

The use of CASA systems provides increasingly accurate and more quantitative information (Fig. 1) than using classical



**Fig. 1.** Description of CASA-Mot kinematic parameters on a 1-s track captured at a rate of 100 frames  $s^{-1}$ . STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

assessment (David *et al.* 1981; Serres *et al.* 1984; Verstegen *et al.* 2002; Chantler *et al.* 2004; Didion 2008) and reduces intertechnician variation in the estimation of sperm concentration, regardless of the type and depth of counting chamber used (Johnson *et al.* 1996a, 1996b; Lu *et al.* 2007).

Unfortunately, the high price of these systems, the lack of homogeneous results between different commercial equipment and the fact that these systems were basically only used to replicate subjective analysis, instead of using the great battery of data obtained, has limited the widespread deployment of this kind of technology.

However, CASA instruments are not 'ready-to-use' robots, and the reliability of their results depends largely on the expertise and training of the user (Holt *et al.* 1994). Unfortunately, this technology is still accepted uncritically by many of its users, reducing the feasibility of the results obtained (Kraemer *et al.* 1998). As a consequence, a fully and well-designed definition of accuracy and limits of each commercial CASA system is needed for its correct use (Vantman *et al.* 1988; Mortimer 1990; Davis and Katz 1992; Kraemer *et al.* 1998; Verstegen *et al.* 2002; Björndahl 2011; Palacios *et al.* 2012; Simonik *et al.* 2015).

The basic principle behind microscopy-based CASA-Mot systems is that a series of successive images of motile spermatozoa is acquired and analysed (within a field of view; Elliot *et al.* 1973; Jecht and Russo 1973; Katz and Dott 1975; Jouannet *et al.* 1977; Liu and Warme 1977; Overstreet *et al.* 1979; Amann and Hammerstedt 1980; Stephens *et al.* 1988; Holt and Palomo 1996). As noted previously, the CASA-Mot image analysis technique is based on the assessment of centroid-based values that are well correlated with the flagellar movement patterns. Therefore, although centroid-derived kinematic measurements are used for the definition of sperm movement, it is understood

that these values are secondary indicators of aspects of flagellar movement (Mortimer *et al.* 1997).

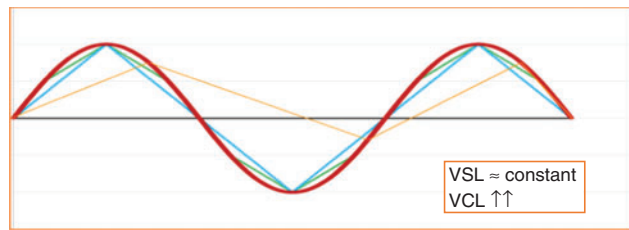
Nevertheless, CASA-Mot systems do not follow one common standard and the motility reports generated with one commercial system cannot be compared directly with those obtained using other systems (Gill *et al.* 1988; Knuth and Nieschlag 1988; Vantman *et al.* 1988; Jasko *et al.* 1990; Olds-Clarke *et al.* 1990; Hoogewijs *et al.* 2012). The effects of technical settings (Smith and England 2001; Rijsselaere *et al.* 2003), such as frame rate, the number of frames analysed and counting chamber, are some of the factors affecting the final results. Sperm concentration and the semen diluents used are also important (Rijsselaere *et al.* 2003; Contri *et al.* 2010).

Following on from this, the final results could be affected by two different sources: (1) hardware and computational variations between systems; and (2) non-computational issues, such as specimen preparation and microscopic technique (Holt *et al.* 1994). One of the non-computational variables is related to sampling. Regarding this, and given that spermatozoa are not uniformly distributed in seminal plasma or other diluting fluids, the number of randomly selected fields needed to achieve a good level of precision must be defined (Vantman *et al.* 1988).

Two recent papers have reviewed not only the history of the CASA-Mot systems, but also their future perspectives (Amann and Waberski 2014; Mortimer *et al.* 2015). The review by Amann and Waberski (2014) is devoted to animal production, whereas that of Mortimer *et al.* (2015) is more focussed on human samples. The literature reporting semen analysis describes a wide variety of methodologies both in terms of CASA-Mot settings (Loomis and Graham 2008; Waite *et al.* 2008; Ortega-Ferrusola *et al.* 2009) and in the use of different counting chambers in combination with CASA systems (Ortega-Ferrusola *et al.* 2009).

### Effects of frame rate on CASA-Mot results

Many systems use standard video image acquisition rates (expressed in frames  $s^{-1}$ ; Hz) and are limited by hardware and software capabilities, with the most common previously being 16, 25, 30, 50 or 60 frames  $s^{-1}$  (Holt *et al.* 2007; Contri *et al.* 2010). The effect of frame rate on cell track recognition was pointed out even when manual image analysis was first introduced for semen evaluation (Mack *et al.* 1988). The increased quality of both hardware and software enables us to currently work with higher frame rates. However, conflicting results can arise when studies use different frame rates, even when using the same device (Morris *et al.* 1996; Rijsselaere *et al.* 2003). Results in different species confirm the observation of increasing velocity parameters as the frame rate increases (Mortimer *et al.* 1988; Mortimer and Swan 1995; Castellini *et al.* 2011; Gallego *et al.* 2013), and this is particularly important when hyperactivation needs to be evaluated (Morales *et al.* 1988; Mortimer and Swan 1995; Mortimer *et al.* 1997, 1988). The most sensitive parameter is the curvilinear velocity (VCL), whereas the least sensitive one must be the straight line velocity (VSL). The former is calculated using centroid positions in each image, whereas the latter indicates only the link between the first and the last points of the track (Fig. 2). In previous papers, it was



**Fig. 2.** Theoretical example of changes in kinematic parameters using CASA-Mot system technology according to frame rate. The red line shows the real track in 1 s; the black line shows straight line velocity (VSL). The yellow, blue and green lines show VCL at rates of 5, 7 and 13 frames  $s^{-1}$  respectively.

observed that straightness (the relationship between both velocities (VSL/VCL)) also increased with increasing frame rate (Morris *et al.* 1996). The amplitude of lateral head displacement (ALH) is reduced at higher frame rates (Morris *et al.* 1996; Rijsselaere *et al.* 2003), but even this parameter is greatly affected by the algorithm used for its characterisation. In contrast, the beat cross frequency (BCF) increases greatly at higher frame rates (Rijsselaere *et al.* 2003).

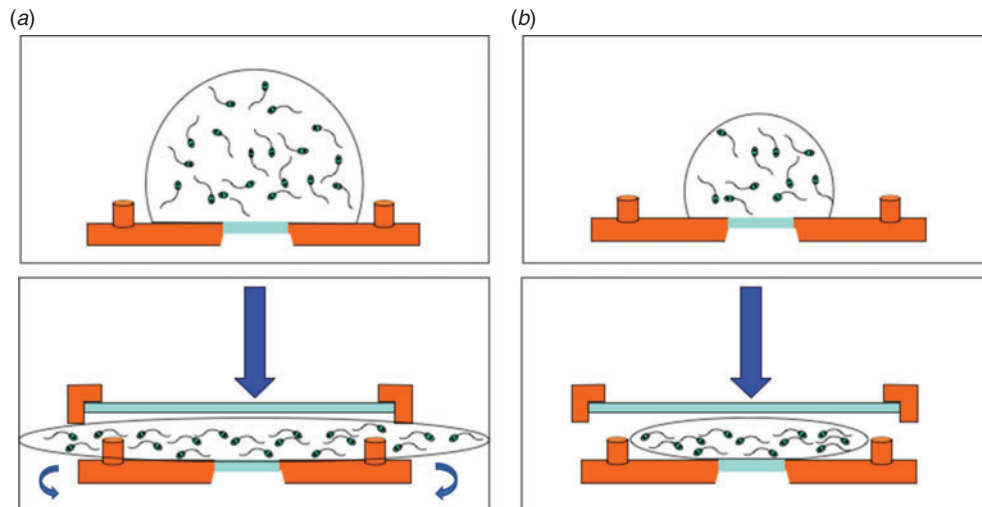
### Effect of counting chamber type on CASA-Mot results

CASA-Mot technology requires the use of specific counting chambers and it is necessary to understand the proprieties associated with each type of chamber (Le Lannou *et al.* 1992; Massányi *et al.* 2008; Hoogewijs *et al.* 2012). There are two general physical principles for charging the chambers: (1) by capillary action in most disposable chambers; and (2) by droplet displacement in reusable chambers (Coetzee and Menkveld 2001; Del Gallego *et al.* 2017). In the case of some reusable chambers based on drop displacement (e.g. Makler chamber (Sefi Medical Instruments); Matson *et al.* 1999), the time involved in placing the cover affects the results by increasing the apparent concentration the longer it takes to place the cover; this does not happen with chambers (e.g. Spermtrack (Proiser R+D); Soler *et al.* 2012). This could be related to the glass composition of the chambers and the number of ions exposed at the surface of the glass, or to the swim-up process of cells moving to the centre of the drop.

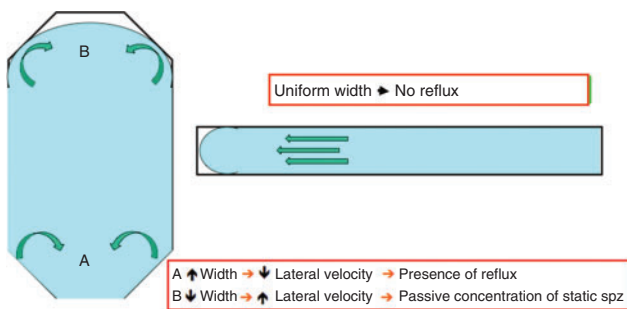
Another factor that may introduce errors is the volume used. Because the reusable chambers are usually circular, the volume required to fill them is defined by the area ( $\pi r^2$ ) multiplied by depth. In common practice, this is not calculated, producing an excessive volume that is displaced outside the plateau and leading to a false distribution of the cells. This makes it necessary to define the exact volume to be used in each chamber (Fig. 3).

In the case of disposable chambers, most of the commonly used disposable chambers include a cover slide that is attached using different kinds of glue. At the manufacturing level, the glue is used not only to design the shape of the counting chamber, but also to define its depth, depending on the height of the glue (Fig. 4).

Thus, samples inside counting chambers are charged by capillary progress between the two layers (slide and cover),



**Fig. 3.** Effect of volume used in reusable counting chambers. (a) When the placed volume is higher than needed the excess of fluid outcome the plateau on the chamber causing variations in sample distribution.; (b) adequate volume, well distributed.



**Fig. 4.** Effect of the design of disposable counting chambers on cell distribution. spz, spermatozoa.

under a force derived from the surface tension between the fluid and the glass, with a meniscus forming at the leading edge. This is known as the laminar Poiseuille flow (Douglas-Hamilton *et al.* 2005b). This leads to a transverse lifting force, causing the particles to migrate perpendicular to the direction of the flow, which results in an uneven distribution of suspended particles throughout the sample (Vasseur and Cox 1976). This was described by Segré and Silberberg (1962a, 1962b) and is known as the SS effect (Douglas Hamilton *et al.* 2005a). The SS effect can affect the final result depending on sample viscosity, with the final result being that particles exhibit a tendency to become concentrated at the meniscus (Douglas-Hamilton *et al.* 2005a, 2005b).

The design of most chambers is such that there are changes in the width and shape of the space where the sample is distributed, which implies variations in the capillary forces when the semen is moving on, causing non-uniform distribution of spermatozoa (Douglas-Hamilton *et al.* 2005a, 2005b; Ibănescu *et al.* 2016). In contrast, when the chamber design is based on a thin parallel space, this problem is solved and the distribution of spermatozoa

is uniform along the length of drop displacement (Soler *et al.* 2012, 2014; Del Gallego *et al.* 2017; Fig. 4).

Most capillary-filled chambers produce an underestimation of concentration due to the fact that the cells tend to accumulate at the edges, with a lower density in the centre where the concentration is evaluated (particularly with some CASA systems; Amann and Waberski 2014).

That different chamber designs affect both the final distribution of spermatozoa inside the chamber and their motility characteristics has been demonstrated for the boar (Christensen *et al.* 2005), cattle (Prathalingam *et al.* 2006; Contri *et al.* 2010; Lenz *et al.* 2011; Gloria *et al.* 2013), goats (Del Gallego *et al.* 2017), humans (Tomlinson *et al.* 2001; Soler *et al.* 2012; Peng *et al.* 2015), rabbits (Massányi *et al.* 2008), sheep (Palacín *et al.* 2013) and stallion (Len *et al.* 2010; Spizziri *et al.* 2010; Hoogewijs *et al.* 2012). This must be taken into account when comparing different studies.

The traditional gold standard for sperm concentration was defined by the use of haemocytometers, such as Bürker and Neubauer, despite there being discrepancies between them (Johnson *et al.* 1996a; Mahmoud *et al.* 1997). In general, there are significant differences in the results obtained after using these classical haemocytometers and the counting chambers used for CASA systems (Rijsselaere *et al.* 2003). This discrepancy could be attributed to many factors. First, in statistical terms, calculating the concentration by taking an aliquot from the original sample is only an estimate of the true concentration (Coetzee and Menkveld 2001). The volume analysed with both types of chambers is very different. In haemocytometers, the height of the space is 100 µm, whereas the height of sperm counting chambers is 10 or 20 µm. This means that the volume in sperm counting chambers, which is one-fifth to one-tenth that in the reference standard (haemocytometers), is also less representative of the entire sample. This can produce an underestimation of the number of cells present in the sample (Rijsselaere *et al.* 2003).



Conversely, others have observed an increase in concentration values using CASA systems that could be related to the inclusion of fragmented tracks as a consequence of cell crossing (Knuth and Nieschlag 1988; Vantman *et al.* 1988; Chan *et al.* 1989; Neuwinger *et al.* 1990) or the incorrect recognition of some non-sperm structures (e.g. lipid or protein globules, other cells) identified as sperm cells.

Finally, it is not possible to know whether one chamber is better than another. However, it is necessary to rationally analyse differences between both the chambers themselves and the results obtained given that differences do, indeed, exist (Kuster 2005).

To this end, it was shown that chambers based on capillary filling (e.g. MicroCell Chamber (Vitrolife)) resulted in similar sperm concentrations in human samples when samples were analysed both manually and with a CASA-Mot system to those obtained using a haemocytometer, whereas the concentration obtained using a drop-displacement chamber (Makler; both manual and CASA-Mot analysis) was significantly greater (Johnson *et al.* 1996a; Bailey *et al.* 2007).

Good examples of the analysis of these kinds of differences between drop-displacement and capillary chambers have been performed in the ram (Palacín *et al.* 2013) and goat (Del Gallego *et al.* 2017). In these papers, the effect of chamber charging on motility and kinematics was investigated, with the results showing that the drop-displacement chambers resulted in higher parameter values than obtained with capillary chambers. However, it is necessary to point out that 'higher' does not necessarily mean 'better', merely 'different' (Contri *et al.* 2010; Palacín *et al.* 2013; Del Gallego *et al.* 2017). Other studies have not shown differences between different counting chambers, in either mammals (boar; Gączarzewicz 2015) or fish (eel; Gallego *et al.* 2013).

It was proposed that one of the possible explanations for the lower sperm motility could be the toxicity of chemical substances present in the capillary-filled chambers (Gloria *et al.* 2013), which could be species specific.

### Effect of counting chamber depth effect on CASA-Mot results

The natural movement of spermatozoa is helical in nature and, depending on the species and physiological status, considerable space is needed to develop this kind of motility correctly (Kraemer *et al.* 1998; Soler *et al.* 2018).

Despite this, in the case of human spermatozoa, the reduced amplitude of movement, the relatively short flagellum and the high viscosity of seminal plasma enabled us to use chambers with a depth of 10 µm without involving extensive modifications, even if the resultant movement is only two-dimensional instead of three-dimensional (Makler 1978b). However, when the reusable Spermtrack counting chamber was used, there were differences in human samples in sperm concentration and total motility comparing chambers with depths of 10 and 20 µm (Makler 1978b). This difference was not observed when ISASD4C (Proiser R+D) chambers (depths 10, 16 and 20 µm) were used, which also demonstrates the importance of the charging method on the final results (Soler *et al.* 2012). The

effects of the capacitation medium on human samples also differ considerably between chambers with depths of 10 and 20 µm, indicating that the latter is much more suitable (Le Lannou *et al.* 1992).

In the veterinary literature, the depth of the sperm suspension for motility analysis is variable and frequently left unmentioned (Hoogewijs *et al.* 2012). In species such as the goat, progressive motility and cell velocities were higher with capillary-loading chambers with depths of 20 than 10 µm (Del Gallego *et al.* 2017). However, these results were not confirmed in other species, including human (Le Lannou *et al.* 1992; Soler *et al.* 2012) and hamster (Shivaji *et al.* 1995), which could be related to the fact that the spermatozoa of different species exhibit different motility patterns.

The SS effect mentioned above is also dependent on the depth of the chambers, because the filling velocity is proportional to depth and decreases as the meniscus penetrates the chamber. Thus, shallow chambers fill more slowly than deeper chambers, providing a possible explanation for differences between chambers of different depths (Kuster 2005) in the sense that the deeper the chamber, the better the uniform distribution.

### Sampling in the counting chamber

The final question regarding counting chamber sampling refers to the variability between field analysis with drop- and capillary-filled chambers. Sperm motility was similar in all fields analysed in drop-filled Spermtrack chambers (Soler *et al.* 2012; Del Gallego *et al.* 2017). When capillary-filled chambers were used, variations in the distribution of spermatozoa along the chamber were different for different species. No changes were observed for human (Soler *et al.* 2012) and bull (Nöthling and dos Santos 2012) spermatozoa, but in other species, such as the fox, sperm motility was reduced in some individuals in the outermost region (from the deposition line) while remaining constant in others (Soler *et al.* 2014). In contrast, in goats, motility increased with the distance to the deposition point (Del Gallego *et al.* 2017).

The differences observed between counting chambers (with results varying with depth and charging method) imply that correlation studies are needed to translate results from one type of chamber to another to overcome the problem of different results being generated. This work must be based on good protocols, defining suitable sampling methods and the total number of cells analysed. This is particularly important when results of semen analysis are used for the calculation of commercial semen doses because, if it is not taken into account, the number of doses can differ markedly depending on the chamber used (Hoogewijs *et al.* 2012).

The new generation of CASA-Mot systems based on laser microscopy allows the use of counting chambers with a depth >20 µm (i.e. the maximum depth could be 100 µm). It has been shown that kinematic values obtained in chambers with a depth of 100 µm are significantly higher than those obtained using chambers with a depth of 10 or 20 µm, compared to the optical microscope (Soler *et al.* 2018).

In conclusion, the use of standardised procedures is not sufficient to guarantee reliable kinematic parameters with the

use of CASA-Mot systems (in general with all CASA technology). Optimal analytical conditions need to be defined, including proper training for the technicians who must understand why one procedure is more appropriate than another. Subsequently, a well-defined quality control analysis is needed in order to obtain good scientific and good clinical results.

### Conflicts of interest

The authors declare no conflicts of interest.

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