

# Technical Note: The use of iSperm technology for on-farm measurement of equine sperm motility and concentration

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**ABSTRACT:** The iSperm is a newly released semen analysis tool from Aidmics Biotechnology Co. LTD, which allows an iPad Mini to be transformed into a handheld microscope with objective semen analysis software for equine available through the Apple Store (version 4.5.2). The aim of this study was to compare iSperm values for sperm motility and sperm concentration to current acceptable methods for semen analysis and to determine the agreement with these methods using statistical methods. Two ejaculates from each of five Standardbred stallions were used to compare sperm motility (computer-assisted semen analysis [CASA] vs. iSperm) and concentration (NucleoCounter SP-100 [NC] vs. hemocytometer vs. iSperm). Data were analyzed by first testing for the differences between the means of each method using a linear mixed-effects model. The agreement between the two continuous measurements for each method was then investigated by computing Lin's concordance correlation coefficient (CCC), with a value of 1 indicating perfect agreement between methods. Results are reported as the CCC with the associated 95% confidence interval in parentheses. Means for both total motility (TM) and progressive motility (PM) were equal

between CASA and iSperm values ( $P = 0.0741$  and  $P = 0.725$ , respectively). However, means for all velocity measurements were significantly different between CASA and iSperm readings ( $P < 0.001$ ). For concentration, means were equal between NC and iSperm values ( $P = 0.748$ ) and for hemocytometer and iSperm values ( $P = 0.953$ ). The CCC for TM was 0.871 (0.788, 0.923) and for PM was 0.916 (0.847, 0.955) indicating good agreement between methods. Low levels of agreement were observed for all velocity measurements. Finally, the CCC for concentration compared by iSperm and NC was 0.970 (0.949, 0.982) and for iSperm and hemocytometer it was 0.962 (0.934, 0.978), both close to the line of perfect concordance. Although more work is needed to improve the iSperm software for velocity measurements to be acceptable by research standards, in its present form the iSperm will introduce a low-cost and affordable method for on-farm semen analysis (TM, PM, concentration) for breeders and veterinarians. As a result, more farms will have access to accurate sperm analysis tools which will help to standardize semen processing procedures leading to better overall quality of semen used for artificial insemination.

**Key words:** equine, iSperm, motility, sperm

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## INTRODUCTION

Current on-farm semen evaluation techniques can vary depending on the type and scale of the

equine breeding operation. On smaller farms and private veterinary practices, evaluation often includes a subjective measurement of motility with a microscope, slide and coverslip, and determination of concentration with either a spectrophotometer or a manual count with a hemocytometer. One major drawback is that subjective motility often relies on the experience of the semen evaluator and may vary from person to person as well as sample to sample (Amann and Waberski, 2014). Although considered the gold standard for humans, hemocytometer counts are time consuming and require adequate training in order to avoid variable results, which have been demonstrated by various studies (WHO, 2010; Brito et al., 2016). Spectrophotometers, while precise, fast and relatively inexpensive, also have drawbacks of not being as accurate due to the inability to discriminate between spermatozoa and other cells, particles, or debris that may be in the suspension (Anzar et al., 2009; Camus et al., 2011; Brito et al., 2016).

On larger breeding farms and at Universities, veterinarians and researchers often have access to equipment that allows for objective and more accurate determination of motility and concentration parameters, including computer-assisted semen analysis (CASA) and NucleoCounter (NC) concentration determination. CASA systems have been used more frequently over the past 40 yr, especially in animal andrology laboratories, with marked improvements in the algorithms that detect sperm motion increasing accuracy and eliminating the problem of subjective measurements by technicians (Verstegen et al., 2002; Amann and Waberski, 2014). NC concentration estimates are also now considered the gold standard for equine andrology and have gained popularity since their introduction in 2010. The NC uses a DNA-specific dye, propidium iodide, to label the sperm heads after permeabilization with a detergent (SP-100 reagent, 0.2% pluronic acid) and can discern between sperm and non-sperm debris reporting a concentration reading in about 30 s (Anzar et al., 2009; Love, 2016). The higher costs associated with purchasing this advanced equipment often makes purchase impractical for smaller operations, including breeding farms and private veterinary practices. In addition, consumables for the CASA and NC systems can also be expensive introducing added costs that smaller farms and practices are not able to absorb.

The need for affordable, portable, and objective semen analysis equipment and software for on-farm analysis has led to the development of systems that rely on the camera of a cell phone or tablet in order to perform analysis. Early technology using the camera of a cell phone was able to demonstrate lens-free

microscopy in order to visualize red blood cells, white blood cells, platelets, and some waterborne parasites (Tseng et al., 2010). Recently, a smartphone-based semen analyzer was successfully validated in comparison to CASA values for semen analysis in humans (Kanakasabapathy et al., 2017). The iSperm system created by Aidmics Biotechnology Co., LTD (Taiwan) is another recently developed method of portable semen analysis designed for use in a number of different domestic animal species.

The iSperm uses the camera of an iPad Mini 4 and analysis software in the form of a species-specific application (app) from the Apple Store to provide a method of objective and highly portable semen analysis. The hardware includes a specialized case with a heated chamber to allow for sample analysis at 37 °C. To sample the semen, the sample collector is fitted with a sampling chip that is dipped into the semen sample and subsequently covered with a base chip to create a sampling chamber depth of 20 µm. The sampling collector is then inserted into the back of the case and heated chamber, the light source is turned on and the iPad camera is then used to visualize the sperm at a magnification of 200×. Analysis takes approximately 10 s and the results are then reported on the screen. In addition, the video used for analysis can be saved and played back later. There is also the option to use the Semen Packaging tool in order to determine the number of insemination doses that can be prepared from the ejaculate specific for equine semen.

It appears that with this affordable and objective technology, the standardization of semen processing and packaging for shipped equine semen could be greatly improved. The objectives of this study were to compare iSperm values for sperm motility and sperm concentration to current acceptable methods of equine semen analysis. The agreement between these different methods was then determined using statistical methods to assess the validity of using iSperm for assessment of equine sperm motility and concentration.

## MATERIALS AND METHODS

### *Semen Collection and Processing*

Stallions were housed at a commercial Standardbred stud farm in accordance with *ADSA-ASAS-PSA Guide for Care and Use of Agricultural Animals in Research and Teaching* (2010). Ejaculates were collected during the breeding season (2018) using an artificial vagina and two ejaculates

collected 2 d apart from each of five sexually active Standardbred stallions were used in the study. After collection, semen was filtered to remove the gel fraction. Initial sperm concentration was estimated on-farm using an equine densimeter (Animal Reproduction Systems, Chino, CA). Samples were subsequently diluted 1:1 in pre-warmed INRA 96 (IMV, Maple Grove, MN) and stored in an insulated container for transport back to the laboratory (~50 min). At the laboratory, concentration was estimated with a NucleoCounter SP-100 (ChemoMetec A/S, Allerød, Denmark) and samples were prepared accordingly for the motility and concentration experiments.

### Motility

For motility analysis, samples were diluted to approximately 30 million/ml in pre-warmed INRA 96. Aliquots of dead sperm were prepared by snap freezing a sample in liquid nitrogen then thawing it in a 37 °C water bath. This process was repeated twice to ensure membrane rupture of all spermatozoa. Samples were then mixed with flash-frozen dead sperm at the ratios of 100:0, 75:25, 50:50, and 25:75, live:dead in order to achieve a range of varying motilities. Motility was evaluated for each sample with both CASA (Ceros II, Hamilton Thorne, Inc.) and iSperm (version 4.5.2; Aidmics Biotechnology Co., LTD, Taiwan) methods. Endpoints included total motility (TM, %), progressive motility (PM, %), average path velocity (VAP,  $\mu\text{m/s}$ ), curvilinear velocity (VCL,  $\mu\text{m/s}$ ), straight-line velocity (VSL,  $\mu\text{m/s}$ ), straightness (STR), and linearity (LIN) for both methods.

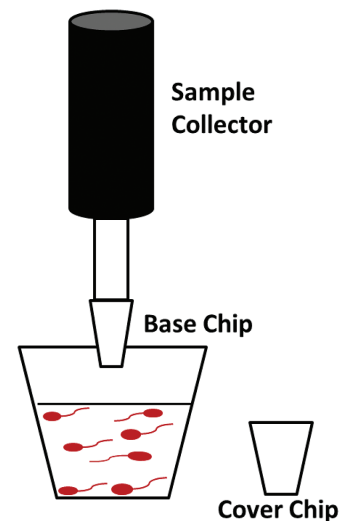
For CASA, semen (3  $\mu\text{l}$ ) was loaded into a pre-warmed counting chamber (Leja, 20  $\mu\text{m}$ , four-chamber slide) and a minimum of seven fields and 500 sperm were analyzed per sample. This was repeated in triplicate with a new chamber of the slide for each sample. The preset values for the Ceros II system were as follows: frames acquired, 45; frame rate, 60 Hz; minimum contrast, 70; minimum cell size, 4 pixels; minimum static contrast, 30; STR threshold for PM, 75; VAP threshold for PM, 50; VAP threshold for static cells, 0; and static elongation, 1 to 95.

Samples for iSperm motility were measured concurrently with the CASA samples. At least 1 ml of each sample was placed in the iSperm sampling cup and aliquots were maintained at 37 °C on a heated stage between measurements. The sample was mixed thoroughly prior to dipping the base chip into the sample, then applying the cover chip

(Fig. 1). The sample collector was then inserted into the case with a heated 37 °C chamber for sample analysis. New samples were prepared if bubbles or drift were observed on the iPad screen. This process was repeated in triplicate with a new base and cover chip for each replicate. The preset values for the iSperm system were as follows: chamber depth: 20  $\mu\text{m}$ ; progressive cutoff values: STR  $\geq$  75%, VAP  $\geq$  50  $\mu\text{m/s}$ ; TM cutoff values: VAP  $\geq$  20  $\mu\text{m/s}$ , VSL  $\geq$  3  $\mu\text{m/s}$ .

### Concentration

For concentration analysis, samples were centrifuged at  $500 \times g$  for 10 min and resuspended in INRA 96 to approximately 480 million/ml. A serial dilution was then prepared to achieve concentrations of approximately 240, 120, 60, and 30 million/ml, which span the reported accuracy interval for iSperm (20 to 500 million/ml). Samples for NC and iSperm evaluation were run concurrently in triplicate (three separate NC cassettes and three separate sampling and base chips for iSperm). Samples for hemocytometer counts were diluted 1:10 in 4% glutaraldehyde for later analysis. iSperm concentration analysis was performed in the same way as previously described for motility. For the NC, samples were prepared by diluting in the SP-100 reagent according to the manufacturer's instructions using the proper dilution factor for each expected concentration. For the hemocytometer counts, samples were further diluted 1:50 for 480 million/ml samples, 1:20 for 240 million/ml samples, and remained at 1:10 for



**Figure 1.** Sample collection with iSperm. The sample collector is fitted with the base chip and dipped into the extended semen sample in the sampling cup after it has been thoroughly mixed. The base chip is then inserted into the cover chip and screwed into the 37 °C sampling chamber on the specialized iSperm case.

120, 60, and 30 million/ml samples. The improved Neubauer Hemocytometer (Fisher Scientific, Waltham, MA) was used with 10  $\mu$ l of sample loaded into each chamber. Slides were placed in a humid chamber for 10 min prior to counting. Counting was performed with a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany) at 400 $\times$ . At least 200 sperm were counted per chamber and the average between the two chambers was calculated for each of the three replicates (six chambers from three slides). If the two chambers were not within 10% of each other, a new slide was prepared and the analysis was repeated.

### Statistical Analysis

The differences between the means of each method were compared using a linear mixed-effects model in R statistical software (R Core Team, 2013). The model accounted for the effect of stallion, ejaculate within stallion and the method of measurement. The “lme4” and “lmerTest” R packages were used for model fitting and hypothesis testing (Bates et al., 2015; Kuznetsova et al., 2017). The minimum significance level was  $P < 0.05$ . As suggested by Watson and Petrie (2010), the agreement between the two continuous measurements for each method was then investigated by computing Lin’s concordance correlation coefficient (CCC) in the “epiR” R package (Stevenson et al., 2019). This measurement combines measures of both precision and accuracy to determine how close the data are to the line of perfect concordance with values ranging from 0 (no agreement) to 1 (perfect agreement). Results are reported as the Lin’s coefficient with the associated 95% confidence interval in parentheses.

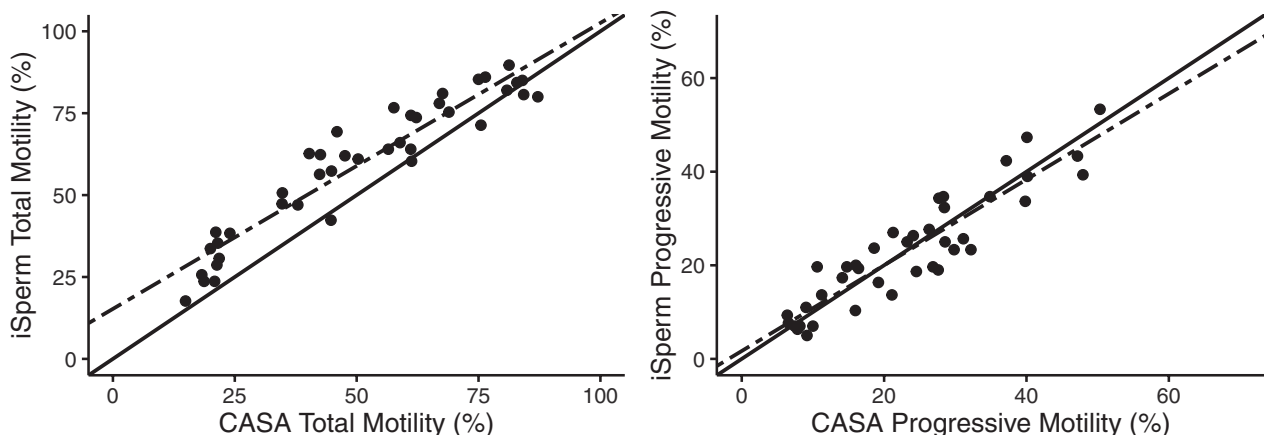
## RESULTS

### Comparison of Motility With CASA and iSperm Methods

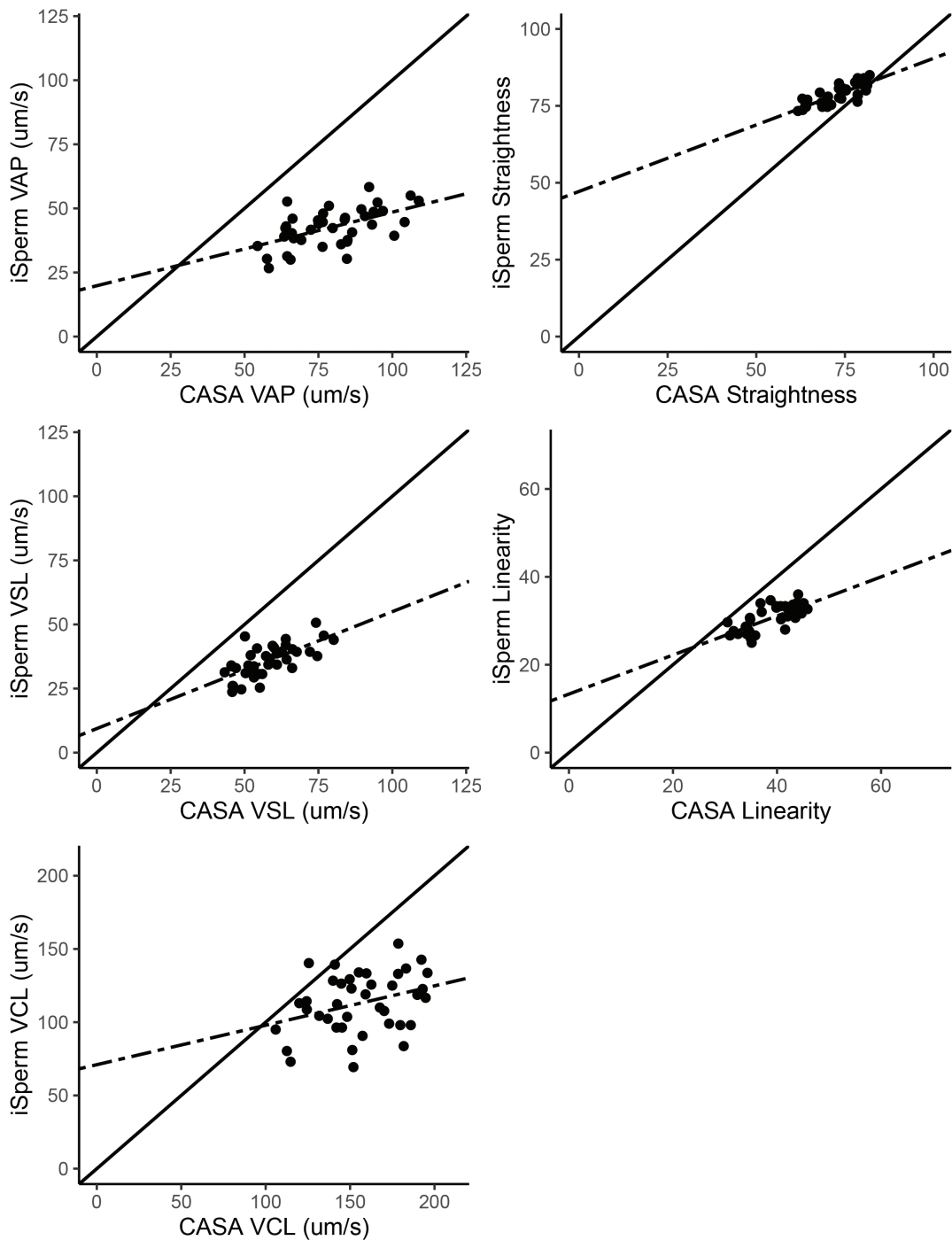
Linear mixed model analysis revealed no differences between means for iSperm and CASA methods for TM and PM ( $P = 0.0741$  and  $P = 0.7248$ , respectively) (Fig. 2). All other motility parameters, VAP, VSL, VCL, STR, LIN, were found to have differences between the means of each method ( $P < 0.001$ ) (Fig. 3). Further analysis of the CCC for each parameter revealed good agreement between methods for TM, CCC = 0.871 (0.788, 0.923) and PM, CCC = 0.916 (0.847, 0.955). The velocity measurements showed less agreement with the line of perfect concordance by having low CCC values. For VAP, the CCC was 0.0729 (0.0269, 0.118); for VSL, the value was 0.130 (0.0626, 0.196); and for VCL, the value was 0.115 (0.0953, 0.226). Measurements of LIN and STR, which are derivations of the velocity parameters, also had low CCC values. The CCC value for STR was 0.418 (0.274, 0.543), and the CCC value for LIN was 0.189 (0.0980, 0.278).

### Comparison of Concentration With Hemocytometer, NC, and iSperm Methods

iSperm concentration values were compared to both NC and hemocytometer concentration counts (Fig. 4). In the comparison of iSperm with NC concentration, there were no differences between means for both methods ( $P = 0.748$ ). When compared to hemocytometer concentration estimation, there were also no differences between means for iSperm and hemocytometer counts



**Figure 2.** Comparison of total and progressive motility between methods (two ejaculates from five stallions were used to construct the plots). The dashed line represents the linear relationship between each method and the solid line is the line of perfect concordance (slope = 1; intercept = 0). Means between CASA and iSperm methods were not significantly difference for total and progressive motility.



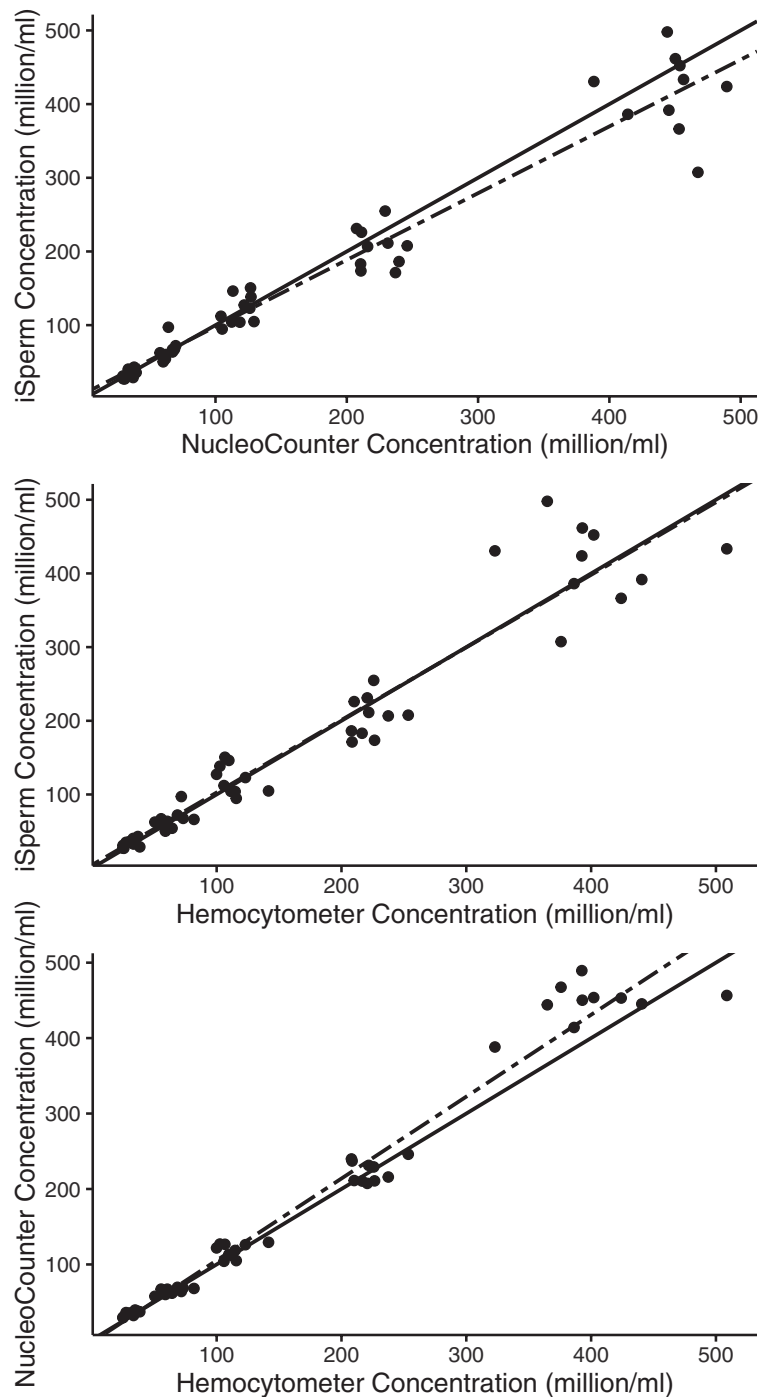
**Figure 3.** Comparison of velocity parameters between methods (two ejaculates from five stallions were used to construct the plots). The dashed line represents the linear relationship between each method and the solid line is the line of perfect concordance (slope = 1; intercept = 0). Means between CASA and iSperm methods were significantly different for all velocity parameters (VAP, VCL, VSL) and the derivations of these parameters (LIN, STR).

( $P = 0.953$ ). NC and hemocytometer counts were also compared to each other and no differences between means were detected between methods ( $P = 0.703$ ). All methods showed good agreement with the line of perfect concordance by having CCC values close to 1. In the comparison of iSperm and NC values the CCC was 0.970 (0.949, 0.982). For the comparison of iSperm and hemocytometer the CCC was 0.962 (0.934, 0.978). Finally, in the

comparison of hemocytometer with NC values the CCC was 0.979 (0.965, 0.987).

## DISCUSSION

In this study, we determined that iSperm measurement of concentration, TM, and PM all had acceptable agreement with current technologies available to assess sperm concentration and motility.



**Figure 4.** Comparison of sperm concentration between methods (two ejaculates from five stallions were used to construct the plots). The dashed line represents the linear relationship between each method and the solid line is the line of perfect concordance (slope = 1; intercept = 0). Means between NucleoCounter, hemocytometer, and iSperm methods were not significantly different.

A recent abstract also found iSperm motility and concentration to be positively correlated with CASA and NC results, as determined by Pearson correlation statistical methods (Crabtree et al., 2018). Data analysis in our study took into account both the agreement between methods as well as hypothesis testing for differences between the means in each method. Although the correct method for comparing the repeatability and agreement between

two sets of results, Lin's correlation coefficient is not commonly used in animal andrology for comparison between methods. Often and incorrectly, a simple Pearson correlation is used to compare two sets of data which is incorrect as it does not assess agreement in any way (Watson and Petrie, 2010). Lin's correlation coefficient uses a modified Pearson correlation coefficient by also assessing how close the data points of the scatterplot of the

methods plotted against each other are to the line of best fit as well as how far that line is from the line of equality or the 45° line through the origin (Lin, 1989; Watson and Petrie, 2010). The 45° line through the origin represents exact or perfect agreement between methods when the two scales are the same and gives a CCC value of 1, meaning that all points lie on the 45° line.

In performing the semen analysis for this experiment, the authors found sample mixing to be essential to the success of the iSperm method. As the samples for iSperm are taken from the top of the sperm suspension in the sampling cup, it was crucial that the sperm suspension be mixed properly immediately prior to sampling. Samples were mixed thoroughly by agitating and swirling the sample several times before inserting the sampling chip. Gentle pipetting can also be used to thoroughly mix the semen before taking the sample. If the samples were not mixed well enough prior to analysis the authors could immediately see differences in the concentration between samples. In addition, this could be a potential source of error in giving inflated motility values as the dead sperm will sink to the bottom of the sampling cup faster than the live, motile sperm. It is well known that sperm aggregation and clustering occur in sperm suspensions and proper mixing is always necessary for precision and accuracy of analysis in any species (Schoeller and Keaveny, 2018). In order to avoid this issue of improper mixing, the manufacturer now recommends two alternative sampling methods, which are detailed in the product manual, but were not tested in this research.

The authors found the concentration data to have less variance when concentration was less than or equal to the 240 million sperm/ml dilution. A similar preliminary study also found that iSperm concentration measurements for samples greater than 300 million sperm/ml were not correlated with NC concentration estimates (Crabtree et al., 2018). The recommendation of our laboratory is to use the iSperm for determining concentration in the range of 30 million/ml to approximately 240 million/ml. As the samples should be diluted in extender prior to analysis, which is the standard for most semen analysis, the average equine semen sample should fall within the desired range if diluted 1:1 without the need for centrifugation.

Currently, iSperm is not recommended for use in research as the measurements for velocity, which are often used to differentiate sperm motion characteristics between treatments (Plaza Davila et al., 2015; Darr et al., 2016), did not have good agreement

between CASA and iSperm methods. We recommend that the manufacturer continue to work on this aspect of the software. Differences in velocity measurements are likely due to differences in how well the software is able to track and analyze the sperm movements. CASA systems have proprietary software that detect the sperm heads and establish a “centroid,” or center of the sperm head, which is then used to track the trajectory of the sperm’s movement (Brito et al., 2016). iSperm software also uses the detection of the sperm head in order to track the sperm, but with different algorithms, microscope optics, camera type and resolution, image acquisition rates, and track sampling time, just to name a few possible sources of differences between the two methods.

In addition to helping to standardize shipped semen and insemination doses due to the accuracy of measurement of concentration and TM and PM, the iSperm could also assist in field analysis of semen samples, including breeding soundness exams and certification and diagnosis of reproductive disorders (Brito et al., 2016). With a much lower cost than the NC and CASA systems, the iSperm is more accessible to smaller farms and veterinarians in private practice. With improvements to the software, it could also prove to be a powerful tool for use for research in the field. In addition, with the availability of software for numerous different species, the application could also extend far beyond just the equine breeding industry.

In conclusion, iSperm is an appropriate method for concentration determination of equine sperm with results that do not differ statistically from the gold standards of the hemocytometer and NC. iSperm TM and PM measurements additionally provide accurate measurement of motility that are not different from CASA values, making it a portable, simple, and affordable method for use in the field. Finally, more work is needed to improve the iSperm software for sperm velocity measurements in order for this method to be acceptable by research standards. The format of the software and ease of access through the Apple Store, allows users to download updates as the manufacturer makes them available. This will ensure that all users have access to the most up-to-date and accurate software without needing to purchase additional materials. Accessibility of this type of technology will help to standardize quality of equine ejaculates prepared for shipment, hopefully leading to better fertility outcomes through these assisted reproductive technologies.

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*Conflict of interest statement.* None declared.

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