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Validation of the iSperm for assessing rhinoceros Sperm

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ABSTRACT

This study's objective was to determine the feasibility of using a portable computer-assisted sperm analyzer to standardize the evaluation of rhinoceros sperm motility and concentration. Assessments were performed opportunistically as part of other ongoing studies and included both fresh and cryopreserved samples from black (*Diceros bicornis*; n = 3), white (*Ceratotherium simum*; n = 7), and greater one-horned (*Rhinoceros unicornis*; n = 5) rhinoceroses. Accuracy of the iSperm Rhino 5 software in identifying sperm cells was evaluated through manual scoring of sperm tracks (n = 48 videos from 12 samples). The number of sperm identified by the observer and the software were significantly correlated (Pearson $r = 0.9908$, $P < 0.0001$). Moreover, the bias between the observer to iSperm values was small (Bland-Altman test; mean ratio = 0.97 observer/iSperm). A strong correlation (Pearson $r = 0.9847$, $P < 0.0001$) between hemocytometer counting and the iSperm for sperm concentration (n = 50) was also observed with a bias of just 1.03 (mean ratio hemocytometer to iSperm). Reliability analysis was performed by comparing ten consecutive evaluations on four different samples using intraclass correlation coefficients (ICC). In this study, the reliability (aka repeatability) of measuring the concentration or total motility via iSperm was deemed excellent (ICC > 0.96). Results of this work demonstrate that the iSperm offers a reliable and semi-automated alternative to manual evaluations for assessing sperm concentrations and motility in the rhinoceros. The ability to determine wildlife sperm concentrations rapidly and motility objectively while working under field conditions would be a great asset to conservation biologists in both zoological institutions and wildlife reserves.

1. Introduction

The global decline of wildlife populations has led to an urgent need to preserve genetic diversity through the banking of biological material, including sperm. This is particularly important for endangered species such as the rhinoceros (henceforth referred to as rhino), in which low population sizes and high levels of poaching increase the risk of extinction [1,2]. The number of facilities banking rhino sperm has increased in recent years, reflecting the growing importance of genetic management in wildlife conservation efforts. By cryopreserving semen, zoological institutions overcome the constraints of distance and time, enabling strategic enhancement of genetic diversity in *ex-situ* rhino populations through artificial insemination (AI). Consequently, these institutions effectively mitigate reductions in genetic diversity caused by population growth restrictions of finite habitat space or male mortality [3]. To ensure the effectiveness of these efforts, it is essential to standardize the evaluation of sperm quality across different institutions [3]. Consistency in sample quality assessments is important not only for exchanging samples between institutions for assisted reproductive technology procedures but also for tracking fertility changes in individuals or generations of endangered species [4].

The two most important parameters used to evaluate sperm quality are concentration and motility. These measures of sperm quality dictate whether a sample, fresh or frozen, can be used for AI. Currently, the recommended minimum dose of sperm for rhino AI is 500 million motile sperm [2]. Concentration, which reflects the number of sperm in a sample, can be determined manually using a hemocytometer, a relatively simple, albeit time-consuming process. Motility refers to the portion of sperm swimming, which is essential for fertilization success [5]. Current manual evaluation techniques using phase-contrast microscopy are subjective and can lead to disparate values, especially when compared across institutions [6].

The bias and inaccuracy of visual estimation for motile sperm are well-established [7,8]. When studying sperm movements through a

Abbreviations: CASA, computer-assisted sperm analyzer; LIN, linearity; LoA, limits of agreement; M/mL, Million sperm per milliliter; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

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microscope, even experienced evaluators cannot avoid focusing on a moving object or, more importantly, when observing over the course of several minutes, the progressively motile sperm (i.e., those rapidly swimming forward) entering and leaving the field of view. This leads to inadvertent tracking of the motile over the non-motile portion of sperm and can result in an overestimation of the total motility. This overestimation gets even more prominent with samples at higher concentrations and/or higher velocities. And because each evaluator sees things differently, motility values from the same sample often vary as much as 10–30% from person to person depending on the experience and training of the evaluators [6].

Computer-assisted sperm analyzers (CASA) offer more objective and standardized evaluations, which allow for accurate and reproducible assessments of sperm concentration and motility [7,8]. These systems use an optical device, typically a built-in microscope, and algorithms to accurately track the movement of each individual sperm within the frame of view. After less than a minute of analysis, a CASA system can determine how many sperm are motile, what percent are progressively motile, and the total concentration of sperm within the sample. An additional advantage is that video and outcome data can be downloaded and easily shared electronically. For over 40 years, CASA technology has been used on sperm of different mammalian species and provided increasingly accurate and repeatable quantitative information [8–10]. When used correctly, a CASA system can reduce subjectivity and human error [7,8].

Many CASA systems are designed to be utilized in a laboratory setting and thus are not easily transportable to be used for fieldwork. Recently, a portable and relatively inexpensive tablet-based system, the iSperm, has been validated for use in several domestic animals [11–14]. This study aimed to determine whether the iSperm could be used to standardize the evaluation of rhino sperm. As per the recommendations for validating a CASA system in Amann et al. (2014; [7]), we evaluated the accuracy of the iSperm system to detect and track rhino sperm while correctly excluding objects or particles that are not a sperm head or complete spermatozoa using idealized (i.e., washed) and 'real world' (i.e., neat and extended) samples for the assessments. Efforts primarily focused on assessing the reliability of sperm concentration and motility determinations.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise stated, all chemicals were obtained from MilliporeSigma (Burlington, MA, USA). An in-house prepared buffer (153 mM α-lactose monohydrate, 76 mM D-glucose, 3.2 mM trisodium citrate dihydrate, 3.2 mM ethylenedinitrilotetraacetic acid (EDTA), 3.6 mM sodium bicarbonate, 15.4 μg/mL penicillin-G, and 33.3 μg/mL streptomycin sulfate; pH 7.1, mOsm/kg 310–320) based on a diluent previously described to be compatible with rhino sperm [15] was utilized for diluting samples. An iSperm clip-on microscope and associated components were generously provided by Aidmics Biotechnology (Taipei City, Taiwan), whereas the iPad Mini 5 and the iSperm chips were purchased from Apple Inc. (Cupertino, CA, USA) and Breeder's Choice (Irwindale, CA, USA), respectively.

2.2. Semen samples

As evaluations required only small amounts of sperm, assessments were performed opportunistically as part of other ongoing studies and included both fresh (n = 12) and cryopreserved (n = 19) samples from black (*Diceros bicornis*; n = 3), white (*Ceratotherium simum*; n = 7), and greater one-horned (*Rhinoceros unicornis*; n = 5) rhinos. All sperm samples had been collected with the approval of the Cincinnati Zoo and Botanical Garden's Institutional Animal Care and Use Committee (protocols # 20–163, 22–173, and 22–175) and by the zoological institutions from whence the samples originated.

Frozen-thawed samples had been cryopreserved in 0.5 cc straws after dilution with either OptiXcell (IMV Technologies U.S.A., Maple Grove, MN, USA) or a lactose-egg-glycerol extender and stored in liquid nitrogen (range 2 months to 20 years). Extender constituents and cryopreservation methodologies are described in detail in Wojtusik et al. (2019; [16]).

2.3. Sperm assessments

Semen samples were either assessed 1) directly in extender and seminal plasma (n = 8), 2) after dilution with prewarmed buffer (4- to 100-fold; n = 17), or 3) after washing (centrifuge 600 x g for 5 min and the pellet resuspended in buffer prior to assessment; n = 6). As per manufacturer guidelines, the optimal concentration for iSperm assessments is > 10 Million sperm per milliliter (M/mL), and < 75 M/mL. Samples at higher concentrations can be evaluated, but the results will lack trajectory mapping and velocity outputs, with only concentration and total motility calculated. Sperm samples (~10 μL) were loaded onto an iSperm chip according to the manufacturer's recommendations. Frequently, a sample assessed on the iSperm was also evaluated subjectively on a phase-contrast microscope to confirm the total motility of the sample was unaffected by the dilution, handling, and sample chip loading necessary for iSperm evaluations (subjective data not recorded). On one occasion, an experienced observer manually assessed total motility on a conventional phase contrast microscope simultaneous to iSperm assessments being performed (n = 10 samples). In this instance the evaluator was kept blind to iSperm values during assessments.

2.4. Rhino-specific iSperm application development

Videos of white and greater one-horned (GOH) rhino cryopreserved semen (sourced from 2 different individuals per species) were captured with the iSperm and provided to Aidmics for the development of algorithms to recognize rhino sperm. Aliquots of each sample were diluted in water 10–1000-fold (adjusted appropriately to allow enumeration of individual sperm) and then assessed on a double improved Neubauer hemocytometer (Fisher Scientific, Waltham, MA, USA). Ten microliters of diluted sample were loaded into each chamber and counts were performed using a phase-contrast microscope at 400X. The average between the two chambers was utilized to calculate the concentration in the undiluted sample. Semen samples were then diluted to three different concentrations (high: ~100 M/mL, medium: ~50 M/mL, and low: ~25 M/mL) and each concentration was re-confirmed by hemocytometer counting before samples were assessed in triplicate (total of 72 videos per species) using the iSperm.

The resulting software application, iSperm Rhino 5 (ver. 5.7.9), was used subsequently to assess fresh and frozen-thawed semen. The software analyzed 1.5 s video recordings (45 frames at 30 frames/s) from 4 random fields of view for each chip and then automatically stored video recordings and data (concentration and motility values [Supp Fig 1]) to the tablet. A chip was discarded and a new one was prepared in instances where fluid dynamics were observed to be affecting sperm (i.e., drifting) during the assessment. The software parameters were adjusted to use the values from O'Brien et al. (2015; [15]) for evaluating rhino sperm on an HTM-IVOS CASA system and were as follows: spermatozoa with average path velocity (VAP) > 5 μm/s were considered motile and those with straight-line velocity (VSL) > 25 μm/s and straightness of sperm movement (calculated as VSL/VAP) > 75% were considered progressively motile. The software also calculated the concentration of the measured sample and the raw sample based on user provided dilution factor; values were reported as M/mL.

2.5. iSperm validation

To verify the software's accuracy in identifying rhino sperm, an

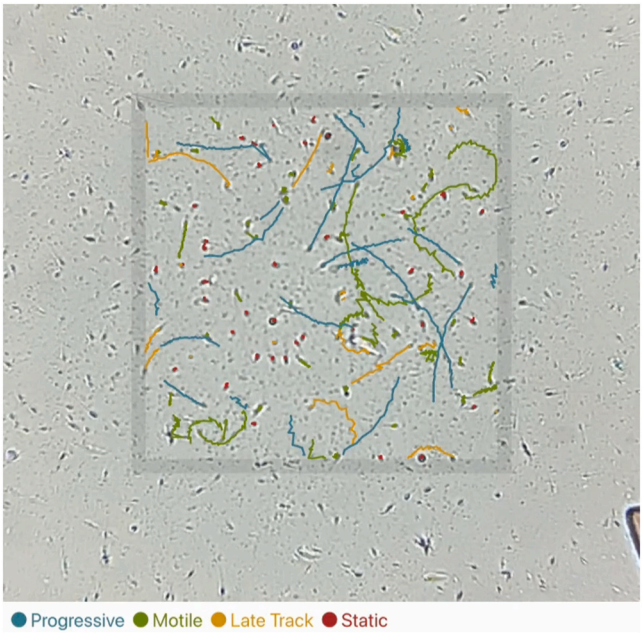

Fig. 1. Sperm trajectory mapping of an egg-based extended frozen-thawed sample (non-diluted). The image frame was extracted from a representative video generated by the iSperm Rhino 5 application and used for analyses. Blue, green, and yellow tracks denote the movement of individual sperm over 1.5 s (color-coded based on software classification), and red dots indicate the sperm classified as immotile.

Table 1
Details on semen samples utilized for video analysis.

Type	No. Rhinos by Species			Conc Range (M/mL)	Motility Range (%)
	Black	White	GOH		
Fresh (n = 8)	2	5	1	9.0 – 69.3	0 – 94.3
Frozen-Thawed (n = 4*)	0	1	2	38.9 – 62.9	7.9 – 71.1

* Two of the four samples came from a single individual; each had been frozen with a different extender type. Abbreviations used: Conc, concentration; GOH, greater-one horned; M/mL, Million per milliliter.

observer manually scored iSperm videos for the number of sperm tracked and untracked (Fig. 1, Video S1). The number of non-sperm cells and debris mislabeled by the software as sperm was also recorded. The total numbers of sperm identified manually (i.e., tracked + untracked by the observer) and by the iSperm software (i.e., all objects marked) from each video were calculated. Scoring was performed on 48 videos in total; details on the samples from whence the videos were derived are described in Table 1. To validate the software's concentration calculations, a hemocytometer was used to manually assess a separate aliquot of the sperm sample after it had been evaluated on the iSperm (n = 50; sperm from 1 black, 5 white, and 2 GOH rhinos). Samples starting concentrations ranged from 15 to 950 M/mL.

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To determine the repeatability of the iSperm concentration and motility assessments, ten consecutive measurements of four different samples (frozen-thawed semen from 1 white and 2 GOH rhinos) were performed; a new chip was used for each measurement and samples were not diluted before evaluation because their original concentrations fell within the iSperm recommended range. Although the system outputs numerous motility measures (Supp Fig 1), the CV was only determined for total motile. The repeatability in hemocytometer counting was ascertained by performing ten consecutive measurements on the same four samples. For consistency, all assessments were performed by the same individual. Any samples containing a large amount of non-sperm debris similar in size to sperm heads (Video S2) were omitted from the validation process due to their documented interference in CASA system accuracy [8].

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2.6. Statistical analysis

For exploratory and statistical analyses, GraphPad Prism 9 (ver. 9.5.1; Dotmatics, Boston, MA, USA) was utilized unless otherwise noted. Pearson correlation and Bland-Altman [17] analyses were performed to assess the accuracy of the software in identifying sperm cells and to determine the agreement between concentration assessment methods. For the correlation analysis, the data were plotted, and the correlation coefficient reported to provide a measure of the strength in the linear relationship between the values obtained by the different methods (iSperm versus manual assessment). A P-value ≤ 0.05 was considered statistically significant. A post-hoc power analysis using G*Power (ver. 3.19.6; [18]) confirmed that sample sizes provided sufficient power for the correlation outcomes ($\alpha = 0.05$, $\beta = 0$). The Bland-Altman test was used for assessing the agreement ratio between methods (i.e., a ratio equal to 1 indicates complete agreement). The ratio (manual/iSperm) versus the average for each matched assessment, the bias (mean ratio), and the limits of agreement (LoA; 95% confidence interval) were plotted. Shading for a ± 10% difference between the outcomes of each method was incorporated into plots to denote the variation considered acceptable for an expert andrology laboratory [19]. The CV for the ten consecutive measurements of an individual sample was calculated and the average reported as the mean percent variance (± SEM). Reliability of the methods was assessed by intraclass correlation coefficients (ICC; [20]) with SPSS Statistics for Windows version 29 (IBM Corp., Armonk, NY, USA) using the 2-way mixed effects model and absolute agreement setting. The ICC interpretation was as follows: poor (ICC < 0.5), moderate (0.5–0.75), good (0.75–0.9), and excellent reliability (ICC > 0.9) [20].

3. Results and discussion

The first step in validating the iSperm was to determine if the system was correctly detecting and identifying each sperm in a field of view as well as excluding objects that were not actually sperm. There was a significant correlation between the total number of sperm identified manually by an observer and automatically by the iSperm software ($r = 0.9908$, $P < 0.0001$; Fig. 2A). The bias between the observer and the iSperm identified number of sperm was 0.97 (Fig. 2C). In other words, for every 100 sperm identified by the observer on the screen, the software on average correctly identified 97 sperm. Underestimation occurred when sperm were insufficiently in the plane of view for software to recognize or when sperm clumped together (even as few as two) or with debris. Overestimation happened when debris and/or cells similar in size to a sperm head were present, a known issue and limit in using a software-based system for sperm analysis [8]. Most of the identifications fell between the limits of agreement (44/48; LoA: 0.86 – 1.06) and the acceptable 10% variation (46/48; Fig. 2C). With idealized sperm samples (i.e., washed), one would expect all identifications to be within the LoA but with 'real world' samples (neat or extended), as was utilized for the video analysis, a lower accuracy can be tolerated [7]. In cases where samples contain an abundance of debris and particles that resemble sperm heads (Video S2), there may be a need for manual assessment of motility. Previous studies have shown that the iSperm system can be utilized as a portable microscope for such manual motility assessments in the field, as demonstrated with stallions [21] and jaguar [22]). It is worth noting that occurrences requiring manual assessment have been infrequent, and our laboratory has found the iSperm system to

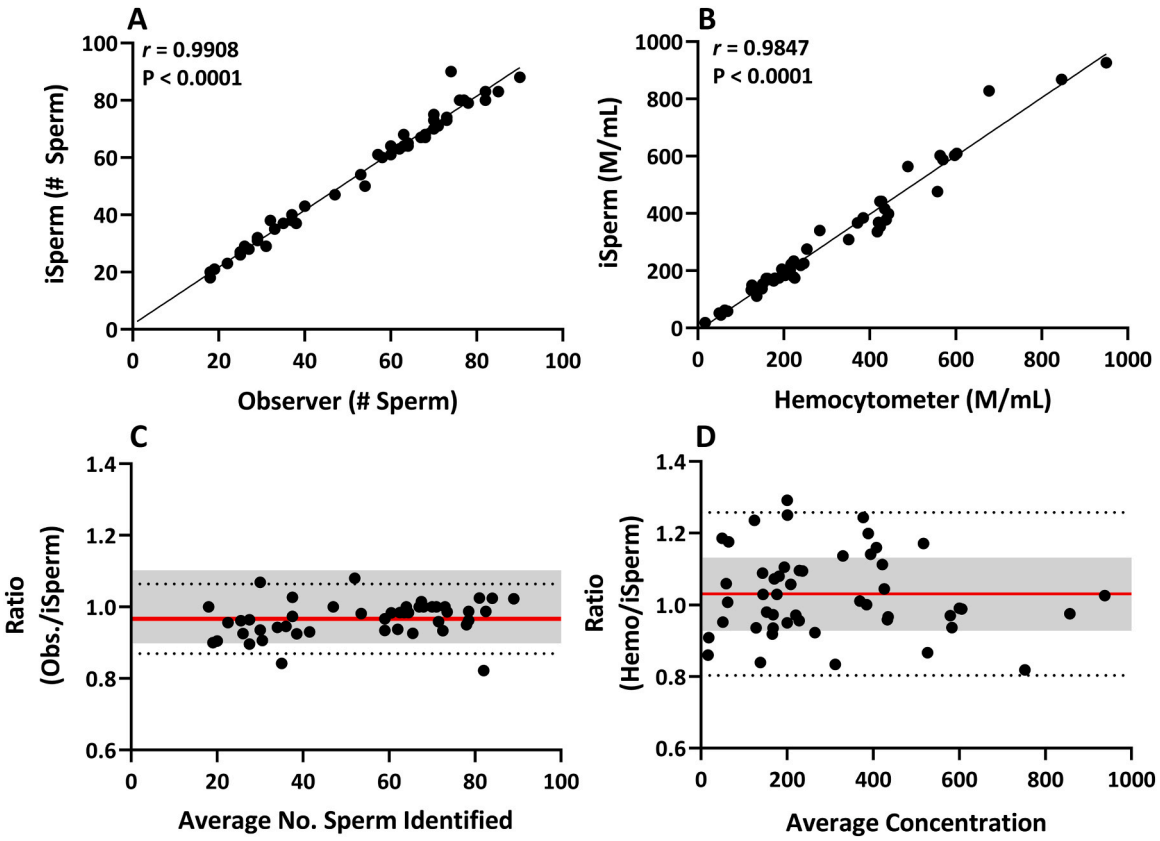

Fig. 2. Comparing manual evaluated (Observer; [Obs.]) to iSperm obtained values to assess the accuracy of the software in identifying rhino sperm (panels A and C, respectively), and the agreement between concentration assessments (panels B and D, respectively). Pearson's statistical test was utilized for the correlation plots (panels A and B). For the Bland-Altman plots (panels C and D), the red line denotes the bias, the dashed lines the limits of agreement, and the shaded area 10% variation around the bias.

be accurate for evaluating semen diluted in different extenders, including egg- and milk-based formulations.

Establishing that the iSperm is accurately identifying the sperm within a field of view supports the notion that the software is measuring the sperm kinetic parameters correctly. The system outputs numerous motility measures, though total and progressively motile may be the two best indicators of fertilization potential [5]. Total motile refers to the percentage of moving sperm in the sample and typically is estimated subjectively, to the nearest 5%, after examining several fields of view on a phase contrast light microscope without dilution [23]. Progressive motility refers to the number of motile sperm swimming forward linearly and with good speed. Both total and progressive motile calculations by the iSperm are based on the velocity and trajectory paths of tracked sperm heads. This differs from the manual scoring system often utilized for rhino sperm assessments [16,24,25], in which total and progressive motility are assigned based not only on the forward movement of the sperm head but also on flagellar side-to-side beating regardless of forward momentum. While the algorithms of iSperm can detect subtle changes in sperm motion that cannot be identified by conventional, human observation, the software cannot analyze flagellar beating directly and must rely on tracking the spatial displacement of the sperm head. The inability to discern the stationary motile sperm (i.e., sperm with flagellar side-to-side movement only) and the reliance on specific velocity parameters for calculating motility results in total motile values being more stringent than any subjectively assigned. In the single instance where total motility observations from an evaluator were compared to iSperm assessments, the iSperm values were consistently lower (9 out of 10; average difference 10 ± 3%) than the manual observations. However, the iSperm assessments were significantly correlated ($r = 0.8271$, $P = 0.0016$) with those of the experienced evaluator. The progressively motile values calculated by the iSperm only reflect the portion of sperm swimming rapidly and linearly forward. This criteria appears to be more restrictive compared to the manual systems previously used for rhino sperm, which are based on a 0–5 progressive score assessment [26] or on the percentage of sperm which cross at least two-thirds of a field of view in a virtually progressive manner at 200-fold magnification [27]. Whereas the iSperm values may be lower than a technician's assessment due to the stringent criteria used, the iSperm's assessment of the percentage of progressively motile sperm is likely to be of higher quality.

The next step in validating the iSperm was to evaluate the agreement between manual and automated methods for calculating sperm concentration. The iSperm-derived values were significantly correlated with values obtained using a hemocytometer ($r = 0.9847$, $P < 0.0001$; Fig. 2B). The bias was determined to be 1.03 (Fig. 2D) and denotes that a sample determined to have a concentration of 200 M/mL on the hemocytometer would be 206 M/mL on the iSperm. It is evident from the plot that the iSperm count 'agrees' with the hemocytometer most of the time; a single outlier outside the limits of agreement was observed (LoA: 0.80 – 1.26; Fig. 2D). Numerous values fell outside the limit of acceptable variation (18/50; Fig. 2D). However, if a ± 20% variation limit were to be applied, a limit that would be adequate for general diagnostic laboratories [19], only 8% of the values fall outside the limits. Notably, there is no obvious relationship between the two methods for concentration determination when starting concentrations were less than 550 M/mL, i.e., the iSperm-derived values were not skewed in one direction compared to hemocytometer counts. Samples with concentrations greater than 550 M/mL, required serial dilutions to allow the manual enumeration of individual sperm by hemocytometer and may have contributed to values being lower compared to the iSperm. For the

Table 2
Intraclass correlation coefficients (ICC) and 95% confidence intervals from the repeated evaluations.

Method	Endpoint	ICC	95% Confidence Interval
iSperm	Concentration	0.964	0.873 – 0.997
iSperm	Total Motile	0.997	0.988 – 1.000
Hemocytometer	Concentration	0.984	0.946 – 0.999

iSperm assessment, a single dilution was typically only required to bring the sample within a readable concentration range.

A system's ability to be consistent in its evaluation of samples is fundamental to sperm analysis. The reliability (aka repeatability) of measuring the concentration or total motility via iSperm was deemed excellent (ICC $>$ 0.96; Table 2). The hemocytometer gave a slightly higher degree of repeatability compared to the iSperm for concentration (0.984 vs 0.964, respectively; Table 2). The CVs for concentration and total motile by the iSperm were 6.1 $\pm$ 1.7% and 12.0 $\pm$ 2.3%, respectively, whereas the CV in hemocytometer counting was 6.7 $\pm$ 1.3%. Although manual motility assessments were not recorded for this study, it is worth nothing that the CV in the iSperm values is lower than the 26.2% intra-individual variability observed in a study by Auger et al. (2000, [6]). Their study involved 12 technicians and biologists from 10 different human sperm banks performing blind evaluations of the same frozen-thawed semen samples repeatedly. Auger et al. (2010) also observed that motility evaluations varied greatly between evaluators, inter-individual CV was 21.8% [6]. Some participants' results were superimposable with the group mean motility values, while others consistently evaluated low or high. Whether using a CASA system or manual assessments, the outcomes will be influenced by the basic tenets of sperm handling, adequate sample mixing, good pipetting techniques, and precise loading of sample chambers. Auger and others [6] observed marked differences between expert and novice participants. As with the hemocytometer, some training by the sample collector is required to ensure the correct assembly of the sample chip so that technical artifacts do not mar the values calculated by the iSperm.

4. Conclusions

The results of these tests demonstrate that the iSperm offers a reliable, objective method for evaluating rhino sperm concentration and motility in the field. Semi-automated analysis of rhino sperm motility has the potential to be more accurate, consistent, and repeatable than subjective analyses. The iSperm analysis of rhino sperm includes a more detailed assessment of kinetic movement than the standard human evaluation. In addition to contributing to the conventional evaluation of sperm quality, this more comprehensive biological information could be incorporated into multivariate analyses to predict fertility potential. The ability to measure concentration rapidly and motility objectively would be a great asset to zoological institutions biobanking and incorporating assisted reproductive technologies into their management of rhinos and other endangered animal populations. These advantages of iSperm can, however, only be achieved by employing technicians familiar with sperm handling. The need for human input cannot be eliminated from rhino sperm analysis with the use of the iSperm but could provide a more standardized and objective evaluation of sperm quality across operators and institutions.

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Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2023.100048.

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