Theriogenology 142 (2020) 67–76

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Evaluation of the effect of laboratory methods on semen analysis and breeding soundness examination (BSE) classification in stallions

Kristina Whitesell [1], Darko Stefanovski, Sue McDonnell, Regina Turner*

Department of Clinical Studies, New Bolton Center, University of Pennsylvania, School of Veterinary Medicine, 382 W. Street Rd., Kennett Square, PA, 19348, USA

ARTICLE INFO

Article history:
Received 2 March 2019
Received in revised form
9 August 2019
Accepted 21 September 2019
Available online 23 September 2019

Keywords:
Stallion
Fertility
Breeding soundness
Semen analysis

ABSTRACT

The stallion Breeding Soundness Examination (BSE), as proposed by the Society for Theriogenology, recommends that a stallion produce a minimum of one billion progressively motile, morphologically normal sperm (PMMNS) in the second of two ejaculates collected 1 h apart to be classified as a Satisfactory Prospective Breeder. With this in mind, the first objective of this study was to determine if the classification outcome of the traditional BSE differs depending on the methods used to evaluate sperm motility, morphology and concentration. We hypothesized that application of Computer Assisted Sperm Motion Analysis (CASA) and Differential Interference Contrast (DIC) microscopy to stallion semen evaluation would yield a more conservative estimate of the number of PMMNS. If this hypothesis is correct, then the use of CASA and DIC microscopy for semen evaluation would result in significantly fewer stallions meeting the historical standards for classification as a Satisfactory Prospective Breeder. Additionally, we determined whether the use of these modern technologies resulted in more accurate prediction of the actual fertility of a stallion compared to the use of more traditional technologies. Our results support the hypothesis that modern semen analysis techniques (including CASA and DIC microscopy) result in more conservative estimates of the number of PMMNS when compared to standard semen analysis techniques. As a result, the choice of methods used for semen analysis may impact the outcome of the traditional BSE. However, none of the methodologies used in this study reliably predicted different levels of fertility among this group of moderately to highly fertile stallions within the context of the traditional BSE. Additionally, the only individual semen measure that was significantly correlated with fertility was the percentage of morphologically normal sperm as determined using DIC microscopy. These results caution against strict use of the traditional 'cutoff' of 1 billion PMMNS for classification of breeding potential, particularly when attempting to differentiate between moderately and highly fertile stallions and regardless of the laboratory methods employed for semen analysis.

1. Introduction

Breeding Soundness Examination (BSE) of stallions for the purpose of prospective identification of fertile and subfertile individuals was formally introduced in 1975 [1]. Criteria for classification of a stallion as a Satisfactory Prospective Breeder for rendering pregnant at least 75% of 40 or more mares bred naturally or 120 mares bred artificially (considered a full book of mares at that time) included that the stallion demonstrate normal libido, have a normal penis, have no evidence of a reproductive tract infection, have normal testes and produce a minimum of one billion progressively motile, morphologically normal sperm (PMMNS) in the second of two ejaculates collected 1 h apart [2]. Since these criteria were published, the number of PMMNS has been a central component in the assessment of breeding soundness.

When the traditional BSE was first outlined, sperm motility was estimated visually, sperm morphology was examined using either light microscopy on stained semen smears, or phase contrast microscopy on unstained, fixed semen samples, and concentration of sperm in the ejaculate was most often estimated based on the transmittance of light through raw semen as determined by a spectrophotometer ("Standard" semen analysis techniques). Since then, technological improvements including computer-assisted

* Corresponding author.
E-mail address: rmturner@vet.upenn.edu (R. Turner).
[1] Present address: Independence Equine and Small Animal Clinic, 3709 E. Hwy 82, Gainesville, TX 76240.

https://doi.org/10.1016/j.theriogenology.2019.09.035
0093-691X/

sperm motion analysis (CASA), differential interference contrast (DIC) microscopy for evaluation of sperm morphology, and fluorescence-based automated nucleated cell counters for determination of sperm concentration have been introduced, and, in some cases, shown to be more repeatable and accurate than Standard semen analysis techniques [3–5]. The influence that these modern, advanced semen analysis techniques may have on estimation of the number of PMMNS in an ejaculate, and therefore on BSE classification, has not been objectively studied to our knowledge.

With this in mind, the first objective of this study was to determine if the use of Standard vs. Advanced semen analysis techniques alters the estimation of the number of PMMNS in the ejaculate. We hypothesized that application of CASA and DIC optics to stallion semen evaluation would yield a more conservative estimate of the number of PMMNS. If this is correct, then we also hypothesized that the use of CASA and DIC microscopy for semen evaluation will result in significantly fewer stallions meeting the historical standards for classification as a Satisfactory Prospective Breeder.

Although semen analysis is a central focus of the BSE, the predictive value of semen analysis in evaluating stallion fertility is limited. In most instances, semen analysis does reliably identify severely subfertile individuals based on poor semen quality. However, it does not reliably predict different levels of fertility among moderately to highly fertile animals, nor does good semen quality guarantee that an animal will prove to be sufficiently fertile to be economically successful in a commercial breeding program [6]. In particular, individual measurements of semen quality, such as the percentages of total and progressively motile sperm or the percentage of morphologically normal sperm are at best moderately predictive of fertility. Variations in these attributes account for only a small percentage of observed variations in fertility rates [7]. Collectively measuring a range of sperm attributes in each ejaculate, as is recommended for the BSE, does improve the predictive value of the examination. However, to our knowledge, the actual fertility of stallions that produce semen that meets or fails to meet the 1 billion PMMNS value for the BSE has not been determined.

Based on this, an additional objective of this study, was to determine if measurement of sperm attributes and semen quality using Advanced semen analysis techniques (CASA, DIC optics and fluorescent-based nucleated cell counting) was more or less predictive of different degrees of fertility in commercially successful breeding stallions than measurement of sperm attributes and semen quality using Standard semen analysis techniques (subjective estimation of motility, phase contrast optics and spectrophotometric estimation of sperm concentration), particularly in the context of the BSE and its historical minimum requirement of 1 billion PMMNS. Because the BSE recommendations were developed based on Standard semen analysis techniques, we hypothesized that the use of Standard techniques would be more predictive of actual fertility when applied within the context of the traditional BSE than would the use of Advanced techniques.

2. Materials and methods

2.1. Stallions

Breeding Soundness Evaluations were performed on 20 Thoroughbred and Standardbred stallions aged 6–22 years standing at stud regionally (PA, MD, NY) in commercial breeding programs. All stallions were managed by experienced, successful breeding farms and none had a history of poor fertility, based on farm standards. Examinations were conducted from December through February, prior to the 2013 & 2014 breeding seasons. Farm managers supplied actual fertility data for 19 stallions at the conclusion of the respective breeding season. Number of mares bred, seasonal pregnancy rates, per cycle pregnancy rates, and first cycle pregnancy rates were extracted from raw data provided. Data from each of 2 ejaculates collected from these 19 stallions (book sizes ranging from 2 to 140 mares, mean (+/− SD) = 46.8 (±47.9)) were included when comparing differences in individual sperm parameters among the three evaluation methods. However, to reduce the possibility of mare and/or management factors affecting end-of-season fertility data, only stallions with book sizes greater than 10 mares were included in analysis of end-of-season fertility data (n = 16 stallions with book sizes ranging from 13 to 140 mares, mean (+/− SD) = 54.6 (±48.4).

2.2. Semen collection & analysis

A breeding soundness examination as described by the Society for Theriogenology, excluding microbial cultures, was performed on each stallion [2]. Two ejaculates were collected 1 h apart using a Missouri model artificial vagina. The gel fraction of the ejaculate was removed using an inline filter at the time of semen collection or via filtration subsequent to collection.

Semen analysis was performed in triplicate using “Standard” methods of semen analysis and each of two “Advanced” methods of semen analysis. (Table 1). Standard estimation of semen volume and sperm concentration relied on visual estimation of volume in milliliters using a graduated container and determination of sperm concentration using a modified spectrophotometer (Densimeter, Animal Reproduction Systems, Model 534B, Chino, CA). Advanced estimation of semen volume and sperm concentration included measurement of volume by weight based on gram to milliliter equivalence and determination of concentration using a fluorescence-based nucleated cell counter (Nucleocounter NC-100, Chemometec, Denmark).

Standard motility analysis involved visual estimation of total and progressive motility using a phase contrast microscope. Advanced motility analysis involved estimation of total and progressive motility using CASA (Hamilton-Thorne IVOS version 12, Beverly, MD). CASA analysis included measurement of average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN). CASA settings can be altered by the user and different settings can result in vastly different values for total and progressive motility [8]. We chose to compare total and progressive motility based on two different CASA settings currently popular within the industry (CASA1 [9] & CASA2 [10]) and then determined whether the choice of settings affected the outcome of the BSE. Complete machine settings for each of the two CASA analyses are listed in Table 1. Differences in the two settings relevant to total and progressive motility are as follows: CASA1: Progressively motile cells were defined as having STR >75%, and VAP >50 μm/s. The VAP cutoff for a static cell was 20 μm/s and the VSL cutoff was 0 μM/s; CASA2: Progressively motile cells were defined as having STR >50%, and VAP >30um/s. The VAP cutoff for a static cell was 15 μm/s and the VSL cutoff was 0.0 μM/s. Twenty micron chambered slides were used for all CASA motion analyses (Leja Products, Nieuw-Vennep, Netherlands). Because many raw semen samples were too concentrated for CASA analysis, all motility analyses were performed on semen diluted in a commercial semen extender (INRA96, IMV Technologies, Maple Grove, MN) to a concentration of 50 million sperm per ml. A single theriogenologist (RT) performed all visual and computer-based motility analyses.

Standard evaluation of sperm morphology was performed by examining 100 individual, buffered formalin-fixed sperm at 1000x magnification with phase contrast optics while Advanced

Table 1
Semen analysis methods.

Parameter	Standard Analysis	Advanced CASA 1	Advanced CASA 2
Volume	Graduated container	Weight (gram to milliliter equivalence)	Weight (gram to milliliter equivalence)
Concentration (million sperm per ml)	Densimeter®	Nucleocounter® NC-100™	Nucleocounter® NC-100™
Total and Progressive Sperm Motility (%)	Visual estimate, phase contrast optics	CASA1	CASA2
CASA Settings			
Frames Acquired	Not applicable	30	45
Frame Rate		60 Hz	60 Hz
Min. Contrast		80	80
Min. Cell Size		3 pixels	3 pixels
Min. Static Contrast		15	15
STR Threshold		75%	50%
VAP Cutoff		20 μM/s	15 μM/s
Prog. Min. VAP		50 μM/s	30 μM/s
VSL Cutoff		0.0 μM/s	0.0 μM/s
Cell Size		5 pixels	6 pixels
Cell Intensity		110	110
Static Head Size		0.59–2.99	0.72–8.82
Static Head Intensity		0.61–1.74	0.14–1.84
Static Elongation		0–47	0–90
Slow Cells Motile		No	No
Field Illumination		Dark Field	Dark Field
LED Illum. Intensity		2360	2295
Temperature		37 °C	37 °C
Morphologically Normal and Abnormal Sperm (%)	Visual estimate, phase contrast optics	Visual estimate, DIC optics	Visual estimate, DIC optics

evaluation of sperm morphology was performed by examining 100 individual buffered formalin-fixed sperm at 1000x using DIC microscopy (Olympus BX-53, Olympus Corporation, Waltham, MA). The percentages of normal and abnormal sperm were recorded for each method. Abnormal sperm were further classified based on the morphologic defect observed, including abnormal acrosomes, detached heads, proximal and distal droplets, bent midpieces, other midpiece defects, hairpin bent tails, coiled tails or other cells. If more than one abnormality was identified on an individual cell, all were recorded. A single theriogenologist (KW) performed all morphology analyses.

The above values were recorded and used to calculate total sperm number (volume x concentration) and number of progressively motile, morphologically normal sperm (total sperm number x percent progressively motile sperm x percent morphologically normal sperm) for each of the three methods of analysis (Standard, Advanced CASA1 and Advanced CASA2).

2.3. Testicular evaluation

For each stallion, scrotal contents were examined by palpation and ultrasonography and the length, width, and height of each testicle, as well as total scrotal width (cm) were obtained. Testicular length, width and height were used to calculate testicular volume and testicular volume was used to calculate expected daily sperm output, all as previously described [11].

2.4. Classification of 'actual fertility'

Pregnancy outcomes were determined via transrectal ultrasonography of mares typically performed between 14 and 20 days post ovulation by each farm's attending veterinarian.

All stallions in our sample population (n = 20) were commercially successful and were considered normally fertile by their managers (i.e., our population did not include any significantly subfertile individuals). As such, stallions (n = 19 stallions with for which data was available) were separated into "Highly Fertile" or "Moderately Fertile" groups based on each of two different justifiable definitions of Actual Fertility:

(1) Fertility Based on End-of-Season Data: Seasonal Pregnancy Rate (SPR, the number of mares pregnant divided by the total number of mares bred), Cycles per Pregnancy (CPP, the number of estrous cycles bred over the season divided by the number of mares pregnant) and First Cycle Pregnancy Rate (FCPR, the number of mares pregnant on the first breeding cycle of the year divided by the total number of mares pregnant at the end of the year) were calculated for each stallion [12]. Cutoff values to differentiate Highly from Moderately Fertile stallions were based on analysis of population distributions. For SPR, the population distribution was skewed to the right (Fig. 1). Based on this, and consistent with the Society for Theriogenology's definition of a Satisfactory Prospective Breeder [2], Highly Fertile stallions were defined as those with an SPR of ≥75% and Moderately Fertile stallions were defined as those with an SPR of <75%. For CPP, the population distribution was binary on either side of 1.9 (Fig. 2). Based on this, Highly Fertile stallions were defined as those with an average CPP of <1.9 and Moderately Fertile stallions were defined as those with an average CPP of >1.9. For FCPR, the population distribution most closely resembled a binary distribution on either side of 49.5% (Fig. 3). Based on this, Highly Fertile stallions were defined as those with an FCPR ≥50% and Moderately Fertile stallions were defined as those with an FCPR <50%. To be classified as Highly Fertile, a stallion was required to meet the above-described standards for all three parameters (SPR ≥ 75% and CPP < 1.9 and FCPR ≥ 50%). If a stallion fell below any one or more of these standards, it was classified as Moderately Fertile.

(2) Fertility based on CPP: We concluded that CPP was likely to be the single most accurate indicator of Actual Fertility because (1) Principal Component Analysis (PCA) performed on end of breeding season fertility data showed that CPP accounted for 68% of the variance within our dataset and (2) CPP in this population appeared to be a binary categorical variable separated on either side of 1.9 (Fig. 2). We therefore were able to use CPP to clearly and objectively separate our population into two groups and (3) CPP is a measure of breeding efficiency and therefore is likely to be a more sensitive indicator of fertility than SPR [12]. Based on these findings, as well as results of both

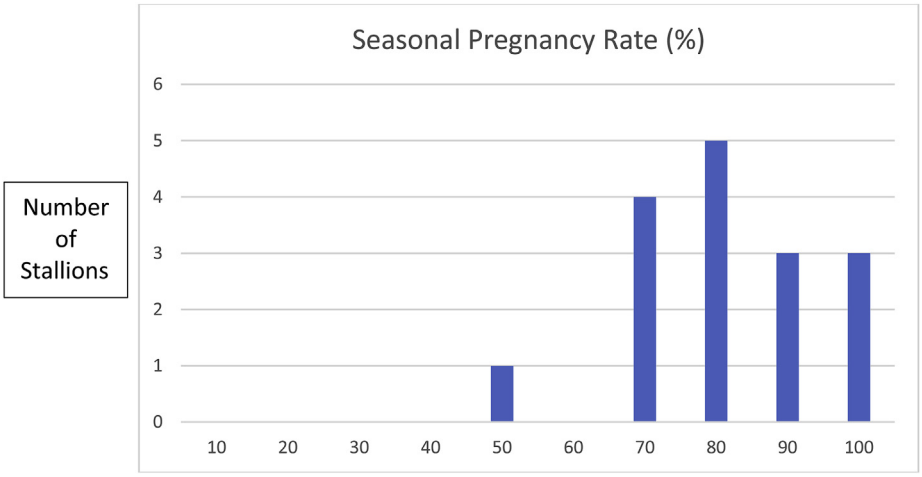

Fig. 1. Frequency distribution for Seasonal Pregnancy Rate for stallions with books of over 10 mares. The population is skewed to the right in this group of commercially successful stallions.

Pearson and Spearman Correlation Analyses, a stallion was defined as CPP Highly Fertile if it achieved an average CPP of <1.9 (mean 1.63, median 1.60) and CPP Moderately Fertile if it achieved an average CPP of >1.9 (mean 2.1, median 2.08), regardless of SPR and FCPR.

2.5. Classification of 'Predicted Fertility'

'Predicted Fertility' for each stallion was determined based on the results of its BSE using each of the three semen analysis methods described in Section 2.2, above (Standard, Advanced CASA1 and Advanced CASA2). Our sample set did not include any animals that would have been classified as Unsatisfactory Prospective Breeders based on the Society for Theriogenology guidelines. Therefore, for the purposes of this study, stallions were classified as either Satisfactory or Questionable Prospective Breeders for each of the three analysis methods. A stallion was classified as a Satisfactory Prospective Breeder if it 1) produced a minimum of 1 billion progressively motile morphologically normal sperm (PMMNS) in the second of two ejaculates collected 1 h apart based on the analysis method used and 2) had two normal testicles with a minimum total scrotal width of 8.0 cm and 3) produced total sperm numbers normal for its testicular volume. A stallion was classified as a Questionable Prospective Breeder if it failed to meet any one or more of the criteria for a Satisfactory Prospective Breeder [2].

If Standard, Advanced CASA1 or Advanced CASA2 analyses were able to correctly differentiate Highly Fertile animals from Moderately Fertile animals, then we hypothesized that those stallions classified as Satisfactory by any of the three analysis methods would be more likely to have High Actual Fertility based on one or both of the definitions of Actual Fertility listed in Section 2.4, above. Similarly, those stallions classified as Questionable by any of the three analysis methods would be more likely to have Moderate Actual Fertility based one or both of the definitions of Actual Fertility listed in Section 2.4, above.

2.6. Statistical analysis

All analyses were conducted with Stata 15 MP (StataCorp, State College, TX) with one- or two-sided tests of hypotheses (as appropriate) and a p-value < 0.05 as the criterion for statistical significance.

Descriptive statistics were reported as means (with 95% confidence intervals [95%CI]), standard deviations, medians, interquartile

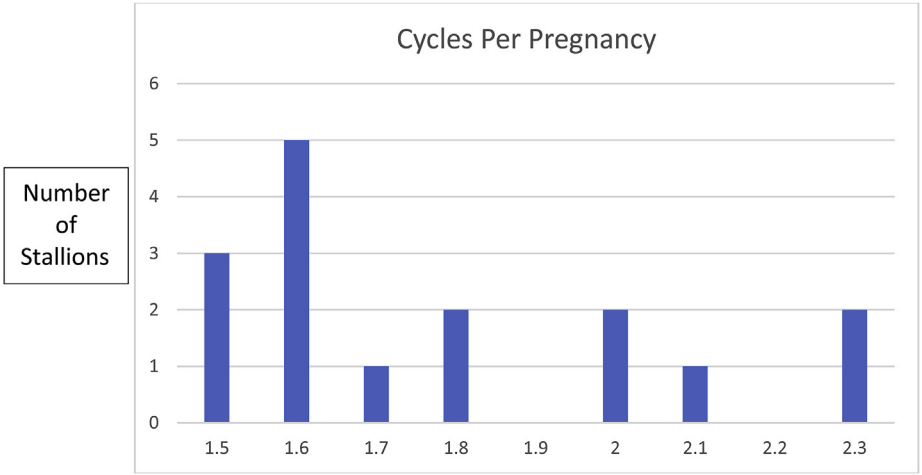

Fig. 2. Frequency distribution for Cycles Per Pregnancy for stallions with books of over 10 mares. The population is binary on either side of 1.9 cycles per pregnancy.

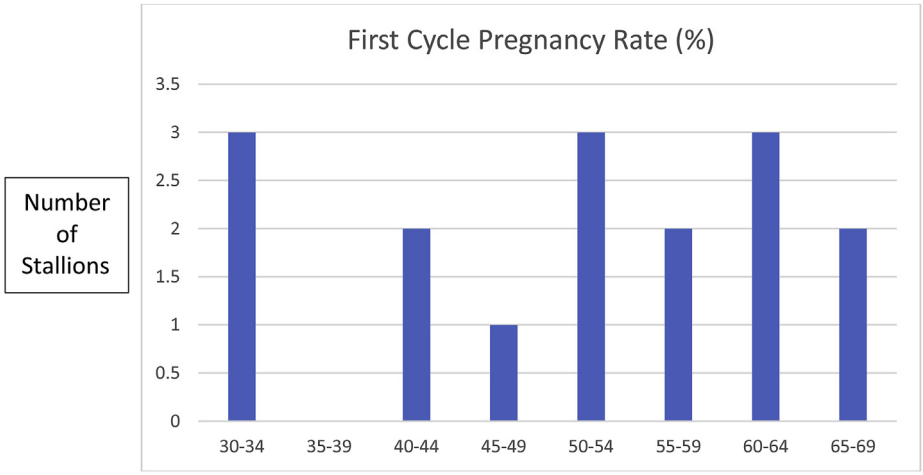

Fig. 3. Frequency distribution for First Cycle Pregnancy Rate for stallions with books of over 10 mares. The data most closely approximate a binary distribution around 49.5%.

ranges (IQR) of continuous variables and tabulation of categorical variables. Tests of normal distribution were performed to determine extent of skewness, and transformation methods (e.g., logarithmic) were used when needed to normalize the distribution of seriously skewed variables. Frequency counts and percentages were used for categorical variables (e.g., gender, breed).

Comparisons were made between values for individual semen and sperm parameters obtained by each of the three analysis methods (Standard, Advanced CASA1, Advanced CASA2) using a paired *t*-test (for normally distributed data) or a Wilcoxon Signed Rank Test (for data that was not normally distributed). Analyzed values included semen volume, sperm concentration, the percentage of total motile sperm, the percentage of progressively motile sperm, the percentage of morphologically normal sperm, the percentages of sperm with abnormal heads, abnormal acrosomes, detached heads, proximal droplets, distal droplets, bent/coiled midpieces, other midpiece abnormalities, hairpin/bent principal pieces, coiled principal pieces, percentage of non-sperm ('other') cells, total number of sperm, and number of progressively motile, morphologically normal sperm (18 different characteristics for each of the three analysis methods for a total of 54 different values). These analyses were run on data from both the first and second ejaculates from all 20 stallions (n = 40 ejaculates). The objective was to determine if analysis method significantly affected the values.

To account for repeated measures, mixed effects regression modelling was performed on the value of PMMNS as the outcome to determine if the number of PMMNS is influenced by method of analysis (Standard vs. CASA1 vs. CASA2, fixed effects) and potentially confounded by breed and book size. Random effects were set on the level of farm, horse, or ejaculate number.

Based on this mixed effects regression modeling, we also determined whether or not estimation of the number of PMMNS using any of the three analysis methods (Standard, CASA1 or CASA2) in conjunction with the 1 billion PMMNS in ejaculate 2 'cutoff' is predictive of whether a stallion will actually be Highly vs. Moderately Fertile (as determined by either end-of-season data or CPP alone). This analysis was performed on all 19 stallions for which fertility data was available. Additionally, to minimize the potential effects of individual mare fertility on pregnancy outcomes, we repeated this analysis including only stallions with book sizes >10 mares (n = 16 stallions).

Actual Fertility (as determined by each of the two methods defined in Section 2.4, above) was compared to Predicted Fertility (as determined by each of the three analysis methods defined in Section 2.2 above). The Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value was determined for each of the three analysis methods used to determine Predicted Fertility. The objective was to evaluate each analysis method for its ability to correctly predict Actual Fertility in the context of the traditional BSE (n = 16 stallions).

Values for 55 individual semen and sperm parameters were compared to Actual Fertility based on the binary categorical value of CPP using Univariate Logistic Regression analysis. Evaluated parameters included semen volume based on visual estimation or weight, sperm concentration based on spectrophotometric or fluorescence-based nucleated cell counting analysis, percentage of normal sperm based on phase contrast or DIC microscopy, percentage of sperm with each abnormality (abnormal heads, abnormal acrosomes, detached heads, proximal droplets, distal droplets, bent/coiled midpieces, other midpiece abnormalities, hairpin/bent principal pieces, coiled principal pieces) based on either phase contrast or DIC optics, percentage of non-sperm ('other') cells based on either phase contrast or DIC optics, percentage of total motile sperm based on visual, CASA1 or CASA2 evaluation, percentage of progressively motile sperm based on visual, CASA1 or CASA2 analysis, total sperm numbers based on Standard, Advanced CASA1 or Advanced CASA2 analysis, number of progressively motile sperm based on Standard, Advanced CASA1 or Advanced CASA2 analysis, number of progressively motile, morphologically normal sperm based on Standard, Advanced CASA1 or Advanced CASA2 analysis and individual CASA parameters for the CASA1 and CASA2 settings (VAP, VSL, VCL, ALH, BCF, STR and LIN). These analyses were performed using data from the first ejaculate from the 19 stallions with known fertility outcomes both with and without adjusting for Breed as a random effect. The objective was to determine which, if any, individual parameters correlated with Actual Fertility.

3. Results

3.1. Testicular examination

Testicular volume ranged from 142.67 cm^3 to 467.69 cm^3 with a mean of 289.29 cm^3. All stallions produced normal sperm numbers for testicular size and all had a scrotal width of over 8 cm. Because examination of scrotal contents was normal for all stallions, these parameters were not considered further.

3.2. Effect of analysis method on semen and sperm parameters

Semen volume measured by visual estimation using a graduated container (50.1±23.1 ml) was not significantly different from that obtained by weighing (50.8±23.3 ml; $p = 0.29$, two-sided paired *t*-test). Concentration estimated using spectrophotometry (276.3±174.1 million cells per ml) was not significantly different from that obtained by nucleated cell count (263.5±166.2 million cells per ml; $p = 0.09$, two-sided paired *t*-test). However, estimated total sperm number when calculated based on visual estimation of volume and spectrophotometric measurement of concentration ('Standard' analysis; 13.2±9.2 billion cells) was significantly greater than when based on weight estimation of volume and nucleated cell count estimation of concentration ('Advanced' analysis; 12.3±8.7 billion cells; $p < 0.02$, two-sided paired *t*-test).

The percentage of normal sperm was significantly higher when morphology was evaluated with phase contrast microscopy (73.4±11.9%) compared to DIC microscopy (65.8±11.7%; $p < 0.001$, one-sided Wilcoxon Signed Rank Test). DIC microscopy resulted in significantly greater percentages of abnormal sperm heads (DIC: 10.7±8.2%, phase: 3.2±3.2%; $p < 0.0001$) and abnormal acrosomes (DIC: 6.3±7.6%, phase 3.2±7/3%; $p < 0.01$, both one-sided Wilcoxon Signed Rank Test), than did phase contrast microscopy. For the percentage of detached heads, proximal droplets, distal droplets, bent/coiled midpieces, other midpiece abnormalities, hairpin/bent principal pieces, coiled principal pieces, or other cell types, differences between DIC and phase contrast microscopy were not significant.

The percentage of total motile cells was not different with visual analysis of motility than with CASA1 analysis. However, the percentage of total motile cells was significantly greater with visual analysis of motility than with CASA2 analysis (visual: 75.8±10.8%, CASA2: 69.4±13.5%; $p < 0.0001$, one-sided Wilcoxon Signed Rank Test). Similarly, the percentage of total motile cells was significantly greater with CASA1 analysis than with CASA2 (CASA1: 74.6±12.4%, CASA2: 69.4±13.5%; $p < 0.0001$, one-sided Wilcoxon Signed Rank Test).

The percentage of progressively motile cells was significantly greater with visual analysis of motility than with either CASA1 or CASA2 analysis (visual: 55.3±17.0%; CASA1: 27.4±12.2%; CASA2: 40.9±11.7%; $p < 0.0001$, one-sided Wilcoxon Signed Rank Test). The percentage of progressively motile cells also was significantly greater with CASA2 analysis than with CASA1 ($p < 0.0001$, one-sided Wilcoxon Signed Rank Test).

Mixed effects modelling indicated that breed had no effect on the number of PMMNS. Farm contributed minimally to the variance in the number of PMMNS. The contribution to the overall variance of the effects set on the level of horse and ejaculate was several levels of magnitude higher and had a significant influence on the number of PMMNS. Random effects were assigned to farm, horse and ejaculate number and normalized for breed and book size. Using this approach, use of Standard semen analysis resulted in a significant increase in the estimated number of PMMNS compared to CASA1 analysis (the Model Adjusted Difference indicates that Standard is 3295.272 ± 450.951 billion higher than CASA1; $p < 0.001$). Similarly, use of Standard analysis resulted in a significant increase in the estimated number of PMMNS compared to CASA2 analysis (the Model Adjusted Difference indicates that Standard is 2031.557 ± 397.748 billion higher than CASA2; $p < 0.001$). Finally, the use of CASA2 analysis resulted in a significant increase in the estimated number of PMMNS compared to CASA1 analysis (the Model Adjusted Difference indicates that CASA2 is 1263.715 ± 295.743 billion higher than CASA1; $p < 0.001$; Fig. 4).

3.3. Effect of analysis method on Predicted Fertility

Table 2 summarizes the classification results for all 19 stallions using the three different analysis methods in the context of the traditional BSE. Ten stallions were classified as Satisfactory by all three methods, one stallion was classified as Questionable by all three methods, and eight stallions were classified differently among the three methods.

Mixed effects regression modelling indicated that breed and book size had no effect on classification outcome. Farm contributed significantly to the variance. Random effects were assigned to farm and normalized for breed and book size. Analyses were run including either all 19 stallions, regardless of book size, or including only the 16 stallions with books of over 10 mares. The results were the same for both approaches and showed that Predicted Fertility as determined by any of the three analysis methods was not associated with the binary outcome of Actual Fertility (Highly vs. Moderately Fertile) as defined by either end-of-season data or by CPP. Receiver Operating Characteristic (ROC) analysis also was used to determine that none of the three analysis methods were any better than any other at predicting Actual Fertility. All three analysis methods performed similarly poorly.

3.3.1. Evaluation of analysis methods for prediction of actual fertility

Tables 3 and 4 present the data in a slightly different way by showing the Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for each of the three analysis methods and based on each of the two definitions of 'Actual Fertility' (End-of-Season Data Fertility (Table 3) and CPP-Based Fertility (Table 4).

Using End-of-Season data as the gold standard to define a Highly vs. Moderately Fertile stallion, and using each of the three analysis methods in the context of the traditional BSE cutoff of 1 billion PMMNS, none of the three analysis methods were both highly sensitive and highly specific for differentiating Highly from Moderately Fertile animals.

Using the binary nature of CPP as the gold standard to define a Highly vs. Moderately Fertile stallion and using each of the three analysis methods in the context of the traditional BSE cutoff of 1 billion PMMNS, none of the three analysis methods were both highly sensitive and highly specific for differentiating Highly from Moderately Fertile animals.

3.4. Exploratory analysis of individual semen and sperm parameters for prediction of actual fertility

Logistic regression analysis was used to determine if any of the 55 *in-vitro* assessed indices of sperm quality could be used to differentiate Highly from Moderately Fertile animals (as defined using the 1.9 cutoff for CPP). Regardless of analysis method (Standard, Advanced CASA1 or Advanced CASA2), if breed was not included as a fixed effect, the only value that was significantly associated with degree of fertility was the percentage of normal sperm based on DIC microscopy. For each 1% point increase in the percentage of normal sperm based on DIC microscopy, there was a 26% increase in the chance that the horse would be classified as Highly Fertile vs. Moderately Fertile ($OR = 1.234$; $p = 0.029$). The percentage of normal sperm based on DIC microscopy accurately predicted whether a stallion would be classified as Highly or Moderately Fertile 80% of the time with a sensitivity of 71.43% and a specificity of 84.62%. The area under the Receiver Operating Characteristics (ROC) curve (threshold 0.5) was 0.8407, indicating that this value was discriminatory [13]. However, when the analysis was

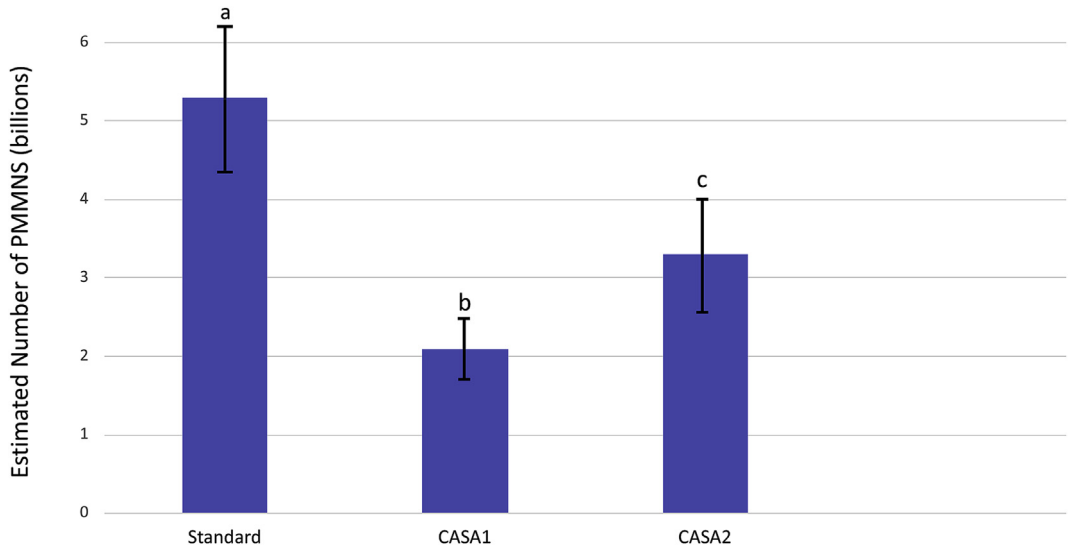

Fig. 4. Estimated number of progressively motile, morphologically normal sperm (PMMNS) based on semen analysis using either Standard, Advanced CASA1 or Advanced CASA2 methodology. Different superscripts indicate significant differences ($p < 0.001$).

adjusted for breed, the percentage of normal sperm based on DIC microscopy loses its significance.

When these analyses were repeated using End-of-Breeding-Season data to define Actual Fertility, there was no correlation with any of the *in-vitro* assessed parameters.

4. Discussion

Although semen volume did not differ when measured from a graduated cylinder vs. by weight, the relative objectivity of using a scale to measure volume may be advantageous. Similarly, spectrophotometric estimates and direct nucleated cell counts of sperm concentration were not different. Although the relationship between optical density of a semen sample and sperm concentration is linear for most stallion ejaculates [14], at lower sperm concentrations spectrophotometry may overestimate concentration compared to the 'gold standards' of flow cytometry and hemocytometry. In contrast, fluorescent-based nucleated cell counting remains in close agreement with both the flow cytometer and the hemocytometer even at more dilute ejaculate concentrations [5,15,16]. Our dataset contained relatively few ejaculates with concentrations less than 100 million cells per ml (5 out of 38 ejaculates) and therefore was less likely to be affected by the loss of accuracy of the spectrophotometer at these lower concentrations. Nonetheless, consistent with a tendency for the spectrophotometer to overestimate sperm concentration compared to the nucleated cell counter ($p = 0.09$), the calculated total number of sperm was significantly higher when it was determined based on visual estimation of volume and spectrophotometric estimation of concentration vs. weight estimation of volume and nucleated cell count estimation of concentration.

Table 2
Effect of Analysis Method on Predicted Fertility (n = 19 stallions)

Analysis Method	Predicted Fertility	
	Satisfactory	Questionable
Standard	16	3
Advanced CASA1	10	9
Advanced CASA2	14	5

The percentage of normal sperm was significantly lower when morphology was evaluated with DIC vs. phase contrast microscopy. This difference was due to a significantly greater number of head and acrosome abnormalities identified with DIC microscopy. These findings suggest that the use of DIC microscopy is a more sensitive method of identifying sperm defects than phase contrast microscopy, and are consistent with previous reports indicating that DIC microscopy offers superior morphologic resolution of sperm [17]. Although DIC and phase contrast microscopy both are designed to improve contrast in an unstained cell, DIC offers the advantage of having the cell appear bright against a dark background and eliminates the diffraction halo artifact associated with phase contrast. The resulting enhanced surface detail and sharpened edges of the cell likely contribute to the increased identification of sperm head and acrosomal defects.

We identified no difference in total motility between visual and CASA1 analysis, indicating that, depending on the computer settings, subjective visual assessment of total motility can correlate well with objective computer measurements. CASA2 analysis resulted in a significantly lower value for total motility than either visual estimation or CASA1. The reason for the difference in total motility between the CASA2 and CASA1 settings is not at first apparent since the CASA2 setting uses a slightly lower VAP cutoff to define a motile cell. However, since the CASA system defines the percentage of total motile sperm as the number of motile sperm divided by the total number of motile + immotile sperm x 100, the value for percentage of total motile sperm will be affected by a change in the denominator (total number motile + immotile sperm). CASA1 and CASA2 settings differ in how each differentiates an immotile (static) sperm from debris based on static elongation gate settings. Elongation is a measure of the roundness of an object, with higher elongations indicating a more round object (a perfectly circular object would have a static elongation of 100). CASA1 settings define only elongated objects as static sperm (upper limit for static elongation = 47), while CASA2 settings allow for rounder and more elongated objects to qualify as a static sperm (upper limit for static elongation of 90). As a result, CASA2 settings identify a larger number of static objects as immotile sperm compared to CASA1. The resulting increase in the denominator apparently offsets CASA2's lower VAP cutoff, thus lowering the value for percentage of total motile sperm as determined by CASA2.

Table 3
Actual fertility defined by end-of-season breeding data.

	Highly Fertile	Moderately Fertile	Sensitivity	Specificity	PPV	NPV
Satisfactory Standard	6	7	23%	86%	67%	46%
Questionable Standard	1	2				
Satisfactory Advanced CASA1	3	4	56%	43%	56%	43%
Questionable Advanced CASA1	4	5				
Satisfactory Advanced CASA2	3	8	12%	43%	23%	27%
Questionable Advanced CASA2	4	1				

The percentage of progressively motile sperm was significantly lower for both CASA1 and CASA2 compared to visual estimation. This supports the hypothesis that CASA evaluation of sperm progressive motility is more stringent than visual estimation. Visual evaluation of progressive motility requires that the observer follow randomly chosen individual sperm moving across a microscope slide to determine if the path followed by the cell is straight or curved and of sufficient velocity. This can be very difficult, particularly for concentrated samples. Additionally, estimation of the speed of a sperm cell is highly subjective. Finally, relatively few cells are counted visually, compared to hundreds of cells rapidly evaluated with most CASA systems. Taken together, these limitations of visual estimation render this method highly subjective, and contributes to substantial inter and intra-observer variability compared to computer-assisted evaluation [18].

The calculated total number of sperm, the percentage of morphologically normal sperm and the percentage of progressively motile sperm all were significantly greater with Standard than Advanced methods. Therefore, consistent with our hypothesis, Standard methods of semen analysis resulted in significantly higher estimates for the number of PMMNS compared to either of the two Advanced methods. Additionally, CASA2 settings resulted in significantly higher estimates of the number of PMMNS than did CASA1. Therefore, laboratories that employ Advanced semen analysis techniques will produce more conservative estimates of the number of PMMNS than those relying on Standard methods. Additionally, laboratories using CASA1 settings will estimate lower numbers of PMMNS than laboratories that choose to use the CASA2 settings. Given these significant differences, and given that a near infinite number of CASA setting combinations are possible, it seems critical that industry standards be established to provide consistency and a common language among Theriogenologists.

An objective of this study was to determine if the classification outcome of the traditional BSE is altered depending on which laboratory methods are used for semen evaluation. Because the definition of a Satisfactory Prospective Breeder is based in part on the presence of a minimum number of PMMNS, the number of stallions that were classified as Satisfactory differed depending on which analysis method was used. Because the application of Advanced semen analysis techniques to stallion semen evaluation yields a more conservative estimate of the number of PMMNS, fewer stallions will be classified as Satisfactory Prospective Breeders when semen is analyzed with Advanced vs. Standard methods. The objectivity and repeatability of Advanced methods are quickly making their use the standard of care, particularly at referral institutions. If the future of stallion fertility evaluation continues to include recommendations regarding the number of PMMNS in an ejaculate, that recommendation may need to be adjusted from 1 billion to account for the more conservative numbers generated by Advanced techniques.

Semen analysis typically can reliably differentiate between severely subfertile individuals and fertile individuals, but not between different degrees of fertility in a fertile population [6,7,12,19–23]. Our dataset consisted only of commercially successful stallions and therefore we were able to apply our analyses only to differentiating moderately from highly fertile animals. Additionally, all stallions in our dataset were of similar body size (only Thoroughbreds and Standardbreds were examined) and had clinically normal testes (based on total scrotal width, testicular volume and the number of sperm produced per unit of testicular volume). Taken together, this resulted in a relatively homogeneous population for which we observed subjectively less variation in semen parameters compared to what might be observed in the broader population. Therefore it is not surprising that, consistent with previous reports, none of our three analysis methods (Standard, Advanced CASA1 or Advanced CASA2) were able to accurately differentiate Moderately from Highly fertile stallions (classified based on either of our two definitions of Actual Fertility) when these methods were applied in the context of the traditional BSE (i.e., using a 'hard cutoff' of 1 billion PMMNS). These data caution against the use of the 1 billion PMMNS hard cutoff as the sole or even the main predictor of fertility, regardless of laboratory method.

Note that the Society for Theriogenology guidelines suggest that a Satisfactory Prospective Breeder should be able to achieve a minimum of 75% SPR when booked to up to 40 mares (when breeding by natural cover) or 120 mares (when breeding by artificial insemination), but that stallions not meeting the criteria for a 'full book' may still achieve a high SPR when bred to fewer mares. One limitation of the present study is that many of the stallions in our dataset were bred to fewer than the 'full book' of 40/120 mares and therefore would likely be able to achieve a 75% SPR even without reaching the 1 billion PMMNS target. To compensate for this, we included a requirement for a minimum CPP (a more sensitive measure of breeding efficiency) in both of our definitions of Actual Fertility.

Table 4
Actual fertility defined by CPP.

Satisfactory Standard	9	4	20%	82%	34%	69%
Questionable Standard	2	1				
Satisfactory Advanced CASA1	4	3	40%	36%	23%	57%
Questionable Advanced CASA1	7	2				
Satisfactory Advanced CASA2	7	4	20%	64%	20%	64%
Questionable Advanced CASA2	4	1				

Part of the problem with attempting to identify individual variables that are predictive of fertility is that the fertility of an individual sperm cell is a multivariate trait. Nonetheless, in the course of this study, we accumulated data on 55 different sperm and semen traits and determined whether any of these traits were, in themselves, able to accurately differentiate Moderately from Highly Fertile stallions as defined by our binary cutoff of 1.9 CPP. As expected, the vast majority of these traits (54 out of 55) were not predictive of whether a stallion would be classified as Moderately or Highly Fertile based on CPP. The only individual parameter to reach significance was the percentage of morphologically normal sperm as determined by DIC microscopy. This is consistent with previous work describing significant correlations between the percentage of normal sperm and fertility [7,12,24]. However, even this one parameter loses its significance when Breed is included as a fixed effect. This indicates that either our study lacks sufficient power to detect significance once adjusted for Breed, or that Breed is a true confounder and the percentage of normal sperm based on DIC microscopy is not significantly associated with Actual Fertility. Pending further analysis with larger data sets, our findings suggest that the evaluation of sperm morphology, particularly using DIC optics, may be one of the most important elements of semen analysis for the prediction of fertility.

Our results suggest that the method used for analysis of morphology (phase contrast vs. DIC microscopy) can affect the correlation between the percentage of morphologically normal sperm and fertility. In this study, the percentage of normal sperm as assessed with phase microscopy optics was unable to differentiate Moderately from Highly Fertile stallions. Differences in analysis method might explain why some earlier studies that relied on stained semen smears or phase contrast microscopy described only weak or no correlations between the percentage of normal sperm and fertility [19,21].

Although we concluded that the percentages of different individual morphologic abnormalities were not predictive of Moderate vs. High Fertility, we did identify trends towards inverse relationships between the percentage of abnormal heads (DIC) and Actual Fertility and the percentage of coiled tails (DIC) and Actual Fertility ($p > 0.05$ and < 0.1). In this regard, a previous report found inverse relationships between the percentages of abnormal sperm heads, abnormal midpieces, detached sperm heads, coiled tails and premature germ cells and fertility in a group of commercially fertility stallions [12]. Therefore, not only the percentage of normal sperm, but also the types of defects that are present, are likely to be relevant in fertility analysis. Because we identified an association of the percentage of normal sperm as determined by DIC (but not phase) microscopy, and because DIC provides better resolution of head (and acrosomal) defects, we recommend the use of DIC microscopy for sperm morphology analysis in the stallion whenever possible.

Love et al. also found associations between several CASA-based motility parameters and fertility [12] whereas our study did not. These differences can be explained in part by the use of different CASA settings, the use of different definitions of fertility, and variations in mare and stallion management. Additionally, our study had a relatively low sample size and so was limited in its ability to detect statistically significant differences.

How one chooses to define fertility will have a significant impact on any study attempting to determine the predictive value of semen analysis. In this regard, one of the main limitations to accurate laboratory-based prediction of fertility may not be the laboratory testing methods themselves, but rather the lack of a true measure of Actual Fertility. Without a 'gold standard' for Actual Fertility, any BSE will be imperfect, no matter what methodology is used. It has been suggested that a minimum of 100 females per individual stallion would be required to achieve a fertility estimation with a 95% confidence interval [25]. The ideal sample population also would include only stallions managed by experienced farms bred to well managed fertile mares, and only those for which detailed fertility records are available. Finally, the population would need to include individuals with a broad range of fertility, including a wide range of subfertile animals. All of these factors are difficult to find in a commercial breeding population and creating this population in a research setting is cost and labor prohibitive. As such, the definition of Actual Fertility remains elusive and its absence remains a common limiting factor to identifying the best laboratory approaches to fertility estimation.

In the absence of a gold standard, fertility can be estimated based on a variety of endpoints or combinations of endpoints including embryo flush rate, pregnancy rate once the conceptus can be identified ultrasonographically, foaling rate, average cycles per pregnancy, and first cycle pregnancy rate. Additionally, the cutoffs for each value that are used to differentiate among different levels of fertility can be debated. We based our definitions of Moderate and High Actual Fertility on each of two objective approaches; (1) based on three end-of breeding season data endpoints (SPR, CPP and FCPR) and (2) based on the single most discriminatory data endpoint (CPP). Consistent with our observations, others have suggested that CPP, and in some studies FCPR, are better at discriminating between highly and moderately fertile individuals than is SPR. We selected CPP over FCPR because CPP was binary and accounted for 68% of the variance in our dataset. We also noticed an unexpectedly low FCPR for some stallions with very large books, in spite of these same stallions achieving relatively high SPRs and low average CPPs. Closer evaluation of raw breeding records revealed that many mares being bred to these stallions were presenting very early in the breeding season for our region (February and early March). These mares often were bred on multiple occasions over a prolonged period of time during their first breeding cycle but did not become pregnant. These same mares did become pregnant on subsequent breedings later in the year in the absence of any interim diagnostics or treatments. This pattern suggested many of these mares were being bred during the transitional period and that this might have contributed to the low FCPR. If true, then FCPR, rather than correcting for mare factors, might have inadvertently introduced a management factor unfairly biasing the data towards a lower FCPR.

It is interesting to note that, in spite of the range of CPP seen in the stallions in this study, all animals were considered fertile in the opinions of their respective farm managers and all were commercially successful. While several individual stallions in this study were highly efficient breeders (CPP $\leq$ 1.5), the higher average CPP for our total sample population (1.76) indicates that a significant degree of breeding inefficiency is tolerated in the industry, at least in the regions in which these stallions were marketed. Lower average CPPs have been described for a group of large-book Thoroughbred stallions involved in dual-hemisphere breeding programs [26] suggesting either that only the most highly fertile stallions are able to succeed in dual-hemisphere markets and/or that mare and stallion management practices in those markets (including the exclusive use of natural cover in Thoroughbreds) result in improved outcomes compared to the regions included in the present study which included both Standardbreds and Thoroughbreds.

Sperm Chromatin Structure Analysis (SCSA) is one *in vitro* analysis method that has been shown to be well-correlated with fertility and with breeding efficiency [27]. Because our initial goal was limited to comparing Standard vs. Advanced semen analysis methods within the context of the traditional BSE, we did not include SCSA data in our dataset (i.e., SCSA is not described as part of the traditional BSE). However, SCSA would be an important

parameter to evaluate, especially when attempting to estimate degrees of fertility among moderately to highly fertile individuals.

5. Conclusions

Our results support the hypothesis that advanced semen analysis techniques result in more conservative estimates of total sperm numbers and the number of PMMNS compared to standard semen analysis techniques. As a result, the method of analysis can affect the classification outcome of the traditional BSE. Additionally, our data show that a significant number of stallions that fail to meet the minimum of 1 billion PMMNS are commercially successful, even when bred to large books of mares. This supports the opinions of the authors of the original Society for Theriogenology Manual for Fertility Evaluation of the Stallion who wrote that the criteria described in the manual are intentionally "conservative criteria which assure the owner or buyer of a stallion that there will be adequate quality of sperm to give each mare… a reasonable chance to become pregnant" [2]. Although advanced, automated methods of semen analysis offer many advantages over standard techniques including standardization, speed, precision, and objectivity, their use may not improve the sensitivity or specificity of the traditional breeding soundness examination. Our data also support the need for the development of standardized analysis methods for the evaluation of stallion fertility. Finally, regardless of the method of analysis used, our data are in agreement with previous reports and indicate that semen analysis performed as part of a BSE does not reliably predict different levels of fertility among moderately to highly fertile animals. Given that SCSA was not included in our dataset, the only individual parameter that was correlated with fertility was the percentage of morphologically normal sperm as determined using DIC microscopy. This study highlights the problems associated with attempting to reduce fertility evaluation to a set of values or cutoffs and emphasizes the importance of judicious interpretation of laboratory data.

On a final note, the prescience of the authors of the original Manual for Fertility Evaluation of the Stallion, written in 1983, is acknowledged. In spite of the many advances in knowledge and technology that have occurred in the intervening decades, almost all of the points brought out in the original Manual still remain valid. As demonstrated by the data presented here, as well as data presented in numerous other manuscripts, statements including, "It is realized that this examination may not invariably and reliably predict the level of fertility any particular stallion will achieve…", "There is no single physical or seminal parameter which is satisfactorily correlated with fertility of the stallion and the best combination of measures remains to be determined," and "The best measure of stallion fertility is the foaling rate achieved with mares of normal fertility under optimal management conditions," all remain true today.

Acknowledgement

The Raymond Firestone Research Fund supported this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.theriogenology.2019.09.035.

References

[1] Kenney RM, editor. Clinical fertility evaluation of the stallion. Annual Meeting of the American Association of Equine Practitioners; 1975.
[2] Kenney RM, Hurtgen JP, Pierson RH, Witherspoon D, Simons J. Clinical fertility evaluation of the stallion. J Soc Theriogenol 1983;9:7–62.
[3] Varner DD. Developments in stallion semen evaluation. Theriogenology 2008;70(3):448–62.
[4] Jasko DJ, Little TV, Smith K, Lein DH, Foote RH. Objective analysis of stallion sperm motility. Theriogenology 1988;30(6):1159–67.
[5] Comerford KL. Validation of commercially available fluorescence-based instrument to evaluate stallion spermatozoa concentration and comparison to photometric systems. Texas A&M University; 2008.
[6] Colenbrander B, Gadella BM, Stout TA. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod Domest Anim 2003;38(4):305–11.
[7] Jasko DJ, Little TV, Lein DH, Foote RH. Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). J Am Vet Med Assoc 1992;200(7):979–85.
[8] Hoogeqijs KM, Govaere JL, Rijsselaere T, DeSchauwer C, Vanhaesebrouch EM, deKruif A, et al., editors. Influence of technical settings on CASA motility parameters of frozen thawed stallion semen. Annual Meeting of the American Association of Equine Practitioners; 2009.
[9] Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008;105(1–2):119–28.
[10] Waite JA, Love CC, Brinsko SP, Teague SR, Salazar Jr JL, Mancill SS, et al. Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. Theriogenology 2008;70(4):704–14.
[11] Love CC, Garcia MC, Riera FR, Kenney RM. Evaluation of measures taken by ultrasonography and caliper to estimate testicular volume and predict daily sperm output in the stallion. J Reprod Fertil 1991;44:99–105. Supplement.
[12] Love CC. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76(3):547–57.
[13] Hosmer DW, Lemeshow S, Sturdivant RX. Applied logistic regression. third ed. Hoboken, NJ: John Wiley & Sons, Inc.; 2013.
[14] Haag FM. Determination of the approximate sperm concentration of horse semen with the aid of a spectrophotometer. J Am Vet Med Assoc 1959;134(7):314–6.
[15] Anzar M, Kroetsch T, Buhr MM. Comparison of different methods for assessment of sperm concentration and membrane integrity with bull semen. J Androl 2009;30(6):661–8.
[16] Love CC. Measurement of concentration and viability in stallion sperm. J Equine Vet Sci 2012;32:464–6.
[17] Cortner GV, Boudreau AJ. Phase contrast microscopy versus differential interference contrast microscopy as applicable to the observation of spermatozoa. J Forensic Sci 1978;23(4):830–2.
[18] Tejerina F, Morrell J, Petterson J, Dalin AM, Rodriguez-Martinez H. Routine assessment of motility of ejaculated stallion spermatozoa using a novel computer-assisted motility analyzer (QualispermTM). Anim Reprod 2009;6(2):380–5.
[19] Dowsett KF, Pattie WA. Characteristics and fertility of stallion semen. J Reprod Fertil Suppl 1982;32:1–8.
[20] Kenney RM, Kingston RS, Rajamannon AH, Ramberg CF, editors. Stallion semen characteristics for predicting fertility. Annual Meeting of the American Association of Equine Practitioners; 1971.
[21] Voss JL, Pickett BW, Squires EL. Stallion spermatozoal morphology and motility and their relationships to fertility. J Am Vet Med Assoc 1981;178(3):287–9.
[22] Haag FM. Evaluation of dismount semen in thoroughbred horse breeding. J Am Vet Med Assoc 1959;134(7):312–4.
[23] Jasko DJ, Moran DM, Farlin ME, Squires EL, editors. Pregnancy rates utilizing fresh, cooled and frozen-thawed stallion semen. Annual Meeting of the American Association of Equine Practitioners; 1992.
[24] Jasko DJ, Lein DH, Foote RH. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1988). J Am Vet Med Assoc 1990;197(3):389–94.
[25] Amann RP. Can the fertility potential of a seminal sample be predicted accurately? J Androl 1989;10(2):89–98.
[26] Walbornn SR, Love CC, Blanchard TL, Brinsko SP, Varner DD. The effect of dual-hemisphere breeding on stallion fertility. Theriogenology 2017;94:8–14.
[27] Kenney RM, Evenson DP, Garcia MC, Love CC. Relationship between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. Biol Reprod 1995;VI:647–53. Monograph Series 1 Equine Reproduction.