Choice of Operating Conditions to Minimize Sperm Subpopulation Sampling Bias in the Assessment of Boar Semen by Computer-Assisted Semen Analysis

CLARE HOLT, W. V. HOLT, AND H. D. M. MOORE*

From the Institute of Zoology, Zoological Society of London, Regent's Park, London, United Kingdom.

ABSTRACT: The performance of a computer-assisted semen analysis system was evaluated for use with washed boar spermatozoa. Accuracy was tested using a computer graphics-generated series of spots moving along horizontal, vertical, and diagonal paths, with both straight and sinusoidal trajectories. Observed and expected values agreed to better than ±5%, and there was exact agreement in many cases. Reproducibility was tested by making 10 measurements of a single prerecorded sequence of boar spermatozoa. Coefficients of variation were <3% for all sperm motion parameters tested. Setup conditions affecting the sample statistics of sperm populations were examined. Search radius (10 settings) and minimum track point (10 settings) were varied factorially to evaluate their bi-asing effects upon population sampling and accuracy. Low search radius (<12 μm) or high minimum track point values (>26 frames) precluded measurements of rapidly moving cells and thus led to selection of slow-moving cells. High search radius (>16 μm) and low minimum track point settings (<22 frames) led to erroneous tracking and poor data quality. Suitable settings for these setup parameters (search radius = 13 μm; minimum track points = 24) were chosen for use in subsequent fertility trials because they caused the least sampling bias.

Key words: CASA, computer semen analysis, tracking, semen evaluation, pig.

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As a model species for investigating the relationship between semen parameters and fertility, the pig offers some advantages over other species. Two types of fertility information can be obtained: 1) success or failure to conceive, and 2) the number of piglets born. The average litter size is typically between 8 and 12, but it can exceed 20. Furthermore, large numbers of inseminations are performed by commercial breeders using standardized semen doses and management practices aimed at maximizing success rates. However, when extended boar semen was previously assessed by computer-assisted semen analysis (CASA) in an effort to study the predictive value of objectively derived measures of sperm motion, the results were rather disappointing, in that no correlations were found (Aumuller and Willeke, 1988; Rath et al, 1988; Reibenwein, 1989). Since then, rapid advances have been made in computerized image analysis, and the present studies were undertaken to reexamine the hypothesis that objectively measured sperm motility parameters can provide an indication of fertility. The development of a novel type of CASA system, the Hobson Sperm Tracker (HST), aroused some optimism in this respect because in contrast to other CASA systems, the HST operates continuously for extended time periods and can thus accumulate data from relatively dilute samples. This attribute was particularly useful in our subsequent studies, in which prolonged incubations in capacitating media reduced the number of motile cells per field of view at any given time.

Before addressing the hypothesis directly in controlled artificial insemination trials, a number of preliminary technical issues had to be resolved. These included establishing the appropriate choice of setup parameters for measuring boar spermatozoa with the HST system.

Certain features of the measurement algorithms are common to all CASA systems that rely upon image processing for the identification of single spermatozoa and their trajectories. Once the optical conditions are satisfactory, sequential images must be analyzed by software designed to compute cell trajectories. Certain assumptions about the movement characteristics of single cells are, however, built into these analyses (see Boyers et al, 1989 for review). For example, when a system is operating at 25 images/second, a single cell with a straight trajectory and a velocity of 100 μm/second will be 4 μm from its corresponding position in the previous image. Thus the search for corresponding objects within a circle of radius

<4 μm would not detect it. In this paper this radius is termed the "search radius" (SR). The choice of an SR requires operator adjustment in the HST system, but deciding upon an appropriate value influences data quality. A low SR excludes rapidly moving cells from the data set, whereas an excessively high SR permits erroneous linking of objects in sequential images.

The progress of a continuously moving cell should also be recognizable through sequential images; thus a series of coordinates corresponding to its sequential positions will be acquired for analysis. Data quality can be controlled by imposing a minimum limit, here termed "minimum track points" (MTP), for the number of sequential coordinates in a series. Incorrectly identified trajectories will terminate abruptly, because no further positions are detected. Finding the best MTP value is a compromise, however, because low settings favor inclusion of short and possibly erroneously identified tracks, whereas excessively high settings exclude fast moving cell populations from the data. In this study, SR and MTP settings were varied systematically to investigate their effects upon the resultant data.

Immotile spermatozoa in a slide preparation can move randomly due to thermal currents and drift. CASA systems register these as slow-moving sperm, skewing sample data by the inappropriate inclusion of low-velocity tracks. An attempt was made to define a minimum threshold so that an objective basis could be used to discriminate and exclude such false tracks from the data. It is recognized that this will also eliminate genuinely slow trajectories from the data; a noncritical inclusion of all data, however, would be inappropriate.

Here we describe the preliminary study on the use of the HST, performed prior to undertaking fertility trials to validate the use of CASA with boar semen. The results are of relevance to CASA studies in general and are thus described here. Tests of accuracy and precision were undertaken using computer-graphics-generated objects moving with known characteristics to confirm the validity of the derived measurements. These differed fundamentally from other tests, in which the influence of setup parameters upon sperm population statistics were examined. It was found that inappropriate settings of these important variables would result in significantly biased sampling of sperm subpopulations and the acquisition of potentially misleading data.

Two independent fertility trials were performed subsequently, in 1992 and 1993, in which the motility of boar spermatozoa was assessed with the HST system using the methodology described here. Parallel samples of spermatozoa from the same ejaculates were used in on-farm inseminations. A standardized suboptimal sperm dose was used for inseminations to amplify possible differences in fertility between boars and ejaculates. The detailed results

will be published separately, but essentially it was found that maintenance of sperm survival over a 2-hour period *in vitro* was correlated with both conception rate and litter size.

Materials and Methods

Spermatozoa were received for laboratory assessment from a pig breeding center (JSR Healthbred Ltd., Thorpe Willoughby, UK) after 24 hours of storage in a holding medium (Beltsville thawing solution [BTS]). The samples were recovered from the BTS medium prior to evaluation and resuspended in a physiological medium. A Percoll washing technique developed for boar spermatozoa (Berger and Horton, 1988) was used for this purpose. For evaluation of sperm motility, spermatozoa were resuspended in a high-calcium Tris-buffered medium (Clarke and Johnson, 1987; Berger and Horton, 1988; Berger and Parker, 1989) at a concentration of 20 × 10⁶ cells/ml and allowed to equilibrate for 10 minutes at 39°C in an atmosphere of 5% CO_2 in air, with 100% humidity. The sperm concentration was chosen to minimize the chances of sperm–sperm collisions and thus the potential for inaccurate track identification.

Microscopy and Sample Recording

Difficulty was initially experienced with the tendency of washed boar spermatozoa to adhere to glass surfaces. A number of possible methods to overcome this problem were evaluated, and the best approach was that of coating glass slides and coverslips with agar before use. A solution of 0.4% agar (Sigma, Poole, UK) was used, as described by Suarez et al (1991). Twenty-five microliters of sperm suspension was placed on a slide and covered by a coverslip (22 × 22 mm), thus creating a sample preparation approximately 50 μm deep. With this sample depth, most of the sperm remained in focus as they crossed the field in view. There was considerable tolerance of focusing inaccuracy with these samples, however, and sperm could still be tracked when slightly out of focus.

This method resulted in approximately 50 spermatozoa being within the HST measurement area at any time. Sample temperature was maintained at 39°C using a warm stage and prewarmed slides. Drifting of cells was not a significant problem with these preparations, but the recognition of slight drifting was specifically investigated in this study. Images were recorded for 3–4 minutes using a shuttered (0.001 seconds/frame) monochrome video camera (Pulnix, UK), an Olympus BH-2 negative-high phase-contrast microscope (Olympus, Tokyo, Japan) fitted with an X3.3 camera ocular and ×20 objective, and a Panasonic VHS video recorder.

Determination of the Minimum Velocity Threshold

Boar spermatozoa were prepared as described above, and the concentration was adjusted to 40 × 10⁶ sperm/ml. The cells were immobilized by fixation (1:1 v/v dilution with 3.5% paraformaldehyde in phosphate buffer, pH 7.4), and a slide preparation was made, as described above. Five replicates were video recorded and analyzed for a total of 2 minutes each. For each of

the replicates the ranges of the motility measurements were evaluated and the inter-replicate variation evaluated.

Nomenclature and Definition of the Motion Parameters

The motion parameters obtained from HST measurements, and considered here, are listed below (see Boyers et al, 1989 for more detailed descriptions of these parameters): 1) Curvilinear velocity (VCL); velocity over the total distance moved, i.e., including all deviations of sperm head movement. 2) Average path velocity (VAP); velocity over a calculated, smoothed, path, i.e., a shorter distance than that used for calculating VCL. 3) Straight line velocity (VSL); velocity calculated using the straight line distance between the beginning and end of the sperm track. 4) Amplitude of lateral head displacement (ALH); the average value of the extreme side to side movement of the sperm head in each beat cycle. 5) Beat cross frequency (BCF); the frequency with which the actual track crosses the smoothed track. 6) Mean angular displacement (MAD); the time average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory. 7) Linearity (LIN); ratio of distances (as a percentage) of straight line track length/actual track length. (This value = 100% for a completely linear track).

Some parameters measured by the HST were not used in the evaluations. The numbers of spermatozoa tracked in each test were also recorded.

Determination of Accuracy and Precision of the HST

The calibration videotape used in this study was generated by a computer program written by Hobson Tracking Systems Ltd. (Sheffield, UK). The calibration tape showed four bright spots (each similar in size and brightness to the image of a sperm head obtained using negative-high phase-contrast microscopy), moving at known VSLs of 20, 40, 60, and 80 μm/second from one edge of the field to the other. The movements were either straight paths or sinusoidal waves (frequency 6 Hz, peak amplitude [A] 2 μm). With the software used, ALH is calculated as 2 × A, and expected ALH was therefore 4 μm. Different sequences of the tape showed the spots moving horizontally, vertically, or diagonally across the field of view. Each sweep of the video field lasted between 2 and 12 seconds, depending on velocity, and sinusoidal tracks therefore included between 12 and 72 complete cycles.

Theoretical VCL values for sinusoidal tracks were derived by using the path length along the sine wave in the following calculation: VCL = 2 × length along half cycle × frequency. Components of this equation were calculated from the following general expression for a sine wave: Y = a sin(2πt/T), where T is the period of the sine wave and t is a time variable. Expected LIN was calculated as net distance/total distance or λ/(2 × length along half cycle) × 100, where λ is the wavelength or straight line distance of a cycle between beat crosses.

Sampling the path length of a sine wave along its length at a frequency of 25 Hz introduces a systematic error into calculations of expected VCL values, because the observed path length is slightly shorter than the real path length. With a peak amplitude of 2 μm and a frequency of 6 Hz the introduced error varies between 3% and 8% within the VSL range of 20–80 μm/second. The exact error introduced by this discrepancy can be calculated

Table 1. *Comparison of expected and observed motion measurements with artificial objects; straight (horizontal and vertical) movement at 20, 40, 60, and 80 μm/second*

	N*	Expected value	Observed mean ± SD
VCL (μm/second)	76	20	20.8 ± 1.51
	78	40	40.0 ± 0
	78	60	59.8 ± 0.52
	80	80	80.5 ± 0.59
VAP (μm/second)	76	20	19.9 ± 0.32
	78	40	40.0 ± 0
	78	60	59.5 ± 0.55
	80	80	80.1 ± 0.74
VSL (μm/second)	76	20	19.5 ± 0.50
	78	40	39.6 ± 0.49
	78	60	59.1 ± 0.13
	80	80	79.0 ± 0.42
Expected VAP and VSL 20 μm/second			
MAD (°)	76	0	2.57 ± 3.85
BCF (Hz)	76	0	0.39 ± 1.17
ALH (μm)	76	0	0.18 ± 0.56
LIN %	76	100	94.5 ± 6.57
Expected VAP and VSL 40 μm/second			
MAD (°)	78	0	0.23 ± 0.89
BCF (Hz)	78	0	0 ± 0
ALH (μm)	78	0	0 ± 0
LIN %	78	100	98.4 ± 0.49
Expected VAP and VSL 60 μm/second			
MAD (°)	78	0	2.25 ± 4.1
BCF (Hz)	78	0	0.08 ± 0.47
ALH (μm)	78	0	0.05 ± 0.32
LIN %	78	100	97.8 ± 0.58
Expected VAP and VSL 80 μm/second			
MAD (°)	80	0	1.52 ± 2.45
BCF (Hz)	80	0	0 ± 0
ALH (μm)	80	0	0 ± 0
LIN %	80	100	97.6 ± 0.49

* *N*, number of measurements; SD, standard deviation. See the Materials and Methods section for an explanation of the other abbreviations.

for any sampling frequency if the peak amplitude and wavelength are known, using procedures derived from standard digital processing theory (Jong, 1982). These corrected VCL values are shown in Tables 3 and 4.

Each tape sequence was evaluated using the SR and MTP settings (13 and 24, respectively) derived within this study. Means and standard errors for each type of movement were calculated and compared with theoretical expected values, and with expected values allowing for path sampling error. Because single values were expected, it was not possible to evaluate the performance of the HST by standard significance tests in which distributions are compared. Accuracy must therefore be judged by comparison of the observed and expected means, and precision by inspection of the observed standard deviations. Attempts to impose acceptable tolerance limits are entirely subjective and are therefore avoided in this paper.

To further evaluate the precision achieved with real samples,

Table 2. *Comparison of expected and observed motion measurements with artificial objects; straight (diagonal) movement at 20, 40, 60, and 80 µm/second*

	N*	Expected value	Observed mean ± SD
VCL (µm/second)	36	20	20.8 ± 0.91
	36	40	39.9 ± 0.23
	40	60	59.1 ± 0.36
	40	80	85.2 ± 0.48
VAP (µm/second)	36	20	19.4 ± 0.50
	36	40	39.9 ± 0.23
	40	60	59.1 ± 0.36
	40	80	83.3 ± 2.98
VSL (µm/second)	36	20	19.4 ± 0.50
	36	40	39.9 ± 0.23
	40	60	59.1 ± 0.36
	40	80	78.9 ± 0.303
Expected VAP and VSL 20 µm/second			
MAD (°)	36	0	5.72 ± 2.1
BCF (Hz)	36	0	4.05 ± 4.05
ALH (µm)	36	0	0.833 ± 0.38
LIN %	36	100	93.2 ± 3.85
Expected VAP and VSL 40 µm/second			
MAD (°)	36	0	3.0 ± 1.47
BCF (Hz)	36	0	0 ± 0
ALH (µm)	36	0	0 ± 0
LIN %	36	100	100.0 ± 0
Expected VAP and VSL 60 µm/second			
MAD (°)	40	0	3.0 ± 1.55
BCF (Hz)	40	0	0 ± 0
ALH (µm)	40	0	0 ± 0
LIN %	40	100	100.0 ± 0
Expected VAP and VSL 80 µm/second			
MAD (°)	40	0	2.10 ± 2.14
BCF (Hz)	40	0	0.3 ± 0.46
ALH (µm)	40	0	8.15 ± 12.7
LIN %	40	100	91.9 ± 0.22

* N, number of measurements; SD, standard deviation.

Table 3. *Comparison of expected and observed motion measurements with artificial objects; sinusoidal (horizontal and vertical) movement. Known amplitude 4 µm, known frequency 6 Hz*

	N*	Expected value	Corrected VCL for 25-Hz sampling†	Observed mean ± SD
VCL (µm/second)	76	53	49	43.9 ± 0.87
	80	65	61	56.4 ± 0.74
	80	80	76	81.6 ± 0.97
	78	96	93	97.0 ± 2.01
VAP (µm/second)	76	20	—	20.1 ± 0.32
	80	40	—	39.5 ± 0.50
	80	60	—	59.2 ± 0.35
	78	80	—	78.9 ± 0.32
VSL (µm/second)	76	20	—	19.6 ± 0.50
	80	40	—	39.5 ± 0.50
	80	60	—	59.1 ± 0.32
	78	80	—	78.9 ± 0.32
Expected VAP and VSL 20 µm/second				
BCF (Hz)	76	6	—	5.37 ± 0.48
ALH (µm)	76	4	—	3.26 ± 0.44
LIN %	76	37	—	44.7 ± 0.97
Expected VAP and VSL 40 µm/second				
BCF (Hz)	80	6	—	5.75 ± 0.44
ALH (µm)	80	4	—	3.07 ± 0.26
LIN %	80	62	—	69.8 ± 0.91
Expected VAP and VSL 60 µm/second				
BCF (Hz)	80	6	—	5.57 ± 0.49
ALH (µm)	80	4	—	4.97 ± 0.15
LIN %	80	75	—	72.2 ± 1.06
Expected VAP and VSL 80 µm/second µm/second				
BCF (Hz)	78	6	—	5.69 ± 0.46
ALH (µm)	78	4	—	4.58 ± 0.49
LIN %	78	84	—	80.9 ± 1.59

* N, number of measurements; SD, standard deviation.
† Theoretical VCL values corrected for 25-Hz sampling error. See text for explanation.

one 2-minute segment of video recorded spermatozoa was analyzed 10 times without changing any settings. The videotape was electronically marked to provide a signal to begin measurement. The coefficient of variation (CV) for each measured motility parameter was determined from the means and standard deviations of unedited data.

Evaluation of Optimal SR and MTP Settings

Ten copies of a video recording of boar spermatozoa, recorded as described above, were made sequentially on a videotape. This procedure did not significantly degrade video image quality and avoided the need for 100 replays of a single video sequence.

Contrast threshold and image filter settings were identified subjectively to provide the best detection of moving sperm heads. One hundred repetitive measurements of the identical, but copied, 2-minute videotape sequence were performed following a randomized scheme, where 10 SR values (5, 7, 8, 9, 10, 11, 13, 14, 15, and 16 µm) were each tested against 10 MTP values

(10–28 inclusive, in steps of 2). Data were analyzed using CSS/Statistica software (Statsoft UK, Letchworth, UK). Data generated in this part of the study were not edited in any way.

Results

Determination of the Minimum Velocity Threshold

The mean number (± standard error of the mean [SEM]) of killed spermatozoa tracked in the 2-minute period was 19 ± 18.9, and it ranged randomly between 0 and 49. These tracks were caused when cellular drift occurred within the slide preparations. Approximately 99% of the VAP measurements and 100% of the VSL measurements were <20 µm/second (mean ± SEM; VAP, 7.54 ± 3.87;

Table 4. *Comparison of expected and observed motion measurements with artificial objects; sinusoidal (diagonal) movement. Known amplitude 4 μm, known frequency 6 Hz*

	N*	Ex-pected	Correc-ted VCL for 25-Hz sam-pling†	Observed mean ± SD
VCL (μm/second)	36	53	49	45.1 ± 0.62
	36	65	61	58.1 ± 0.53
	40	80	76	74.3 ± 0.77
	40	96	93	93.4 ± 1.02
VAP (μm/second)	36	20	—	20.0 ± 0.48
	36	40	—	41.8 ± 0.37
	40	60	—	59.1 ± 0.22
	40	80	—	80.8 ± 1.14
VSL (μm/second)	36	20	—	19.6 ± 0.48
	36	40	—	40.0 ± 0
	40	60	—	59.1 ± 0.22
	40	80	—	78.9 ± 0.36
Expected VAP and VSL 20 μm/second				
BCF (Hz)	36	6	—	5.27 ± 0.45
ALH (μm)	36	4	—	3.88 ± 0.32
LIN %	36	37	—	43.3 ± 0.77
Expected VAP and VSL 40 μm/second				
BCF (Hz)	36	6	—	6.0 ± 0
ALH (μm)	36	4	—	3.05 ± 0.23
LIN %	36	62	—	67.9 ± 0.75
Expected VAP and VSL 60 μm/second				
BCF (Hz)	40	6	—	6.0 ± 0
ALH (μm)	40	4	—	3.9 ± 0.30
LIN %	40	75	—	79.5 ± 0.87
Expected VAP and VSL 80 μm/second				
BCF (Hz)	78	6	—	5.35 ± 0.48
ALH (μm)	78	4	—	3.9 ± 0.30
LIN %	78	84	—	84.2 ± 0.88

* N, number of measurements; SD, standard deviation.
† Theoretical VCL values corrected for 25-Hz sampling error. See text for explanation.

Table 5. *Evaluation of the precision of the Hobson Serm Tracker using 10 repeated measurements of a video sequence of boar spermatozoa*

Measurement	Mean ± SEM (n = 10)	CV (%)
VCL (μm/second)	138.97 ± 0.14	0.32
VAP (μm/second)	118.43 ± 0.36	0.95
VSL (μm/second)	93.99 ± 0.46	1.56
MAD (°)	85.46 ± 0.14	0.52
BCF (Hz)	6.38 ± 0.02	0.89
ALH (μm)	5.96 ± 0.05	2.76
LIN	67.35 ± 0.33	1.53

A single sequence of video recorded spermatozoa was repeatedly analyzed using the Hobson Sperm Tracker. The coefficient of variation (CV) for each measurement was calculated using the means of the 10 repeated measurements. SEM, standard error of the mean.

VSL, 5.16 ± 3.16), and therefore this was the threshold value adopted when analyzing data from subsequent fertility trials.

Determination of Accuracy and Precision of the HST

The results for horizontal and vertical motion showed few differences and have therefore been combined. For straight tracks (Tables 1 and 2) the observed VCL, VAP, and VSL results typically deviated by <1 μm/second from the respective expected values. The diagonal estimation of VCL at 80 μm/second produced a greater deviation (±5.2 μm/second); this was associated with a low-frequency deviation from the average path (BCF = 0.3) but a relatively high lateral movement (ALH = 8.15 μm) when it occurred. This effect would have caused a per-

ceived increase in distance traveled and hence influenced the calculation of VCL. Spots moving at 20 μm/second also showed evidence of slight deviation from perfectly straight tracks, because their observed MAD, BCF, and ALH values were slightly above zero and LIN was depressed.

Measurements of sinusoidal tracks (Tables 3 and 4) agreed closely with the expected values; in some cases the observed values were exact. The CV values for motion parameters measured from video recordings of boar spermatozoa were all <3% (Table 5).

Evaluation of Optimal SR and MTP Settings

The number of spermatozoa tracked was positively correlated with SR values up to 10 μm (r = 0.74; P < 0.0001), but it showed no further increase above this value (Fig. 1a). Conversely, increasing MTP values decreased the number of spermatozoa tracked (r = −0.718, P < 0.001; Fig. 2a). Mann-Whitney U-tests were performed to see whether the number of sperm tracked decreased significantly when MTP was increased. Pairwise comparisons were performed between each adjacent data set defined by MTP values (i.e., 22 vs. 24, 24 vs. 26, etc.); it was found that each stepwise decrease was statistically significant (P < 0.05). None of the other parameters showed significant differences between pairs of data sets when tested in this manner.

Median VAP and VCL values declined by about 10% as MTP was increased from 10 to 28 (Fig. 3a,b). When SR was set at 5 μm, rapidly moving cells were generally excluded from the resultant data sets. Although this effect was evident with VCL and VSL, where the minimum range limit decreased with increases in MTP, it was particularly apparent for VAP (Fig. 3b), where these data points formed a discrete set of outliers.

Similarly, median VCL values increased with SR as rapidly motile sperm became included in data sets (Fig. 4a); this effect was highlighted when pairwise statistical
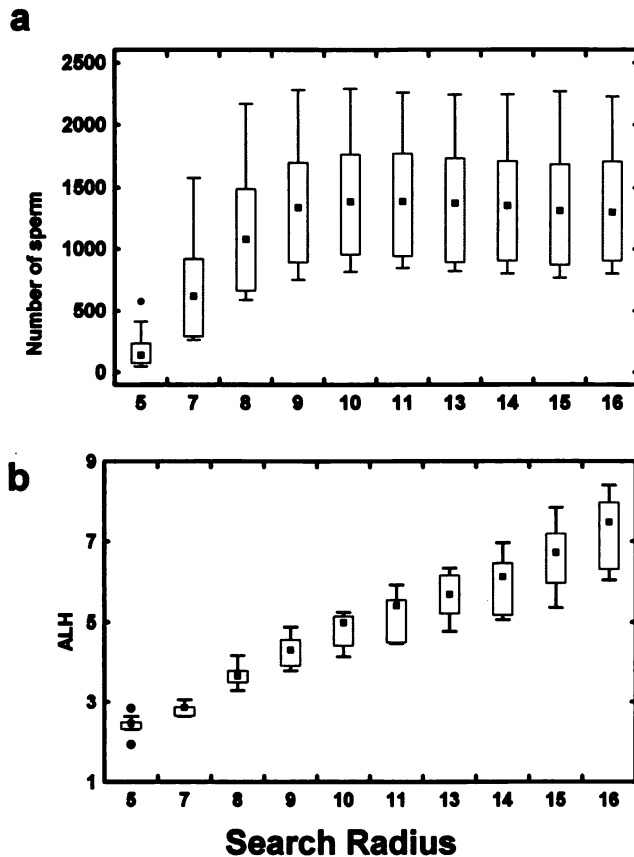
a

b

Search Radius

FIG. 1. (a, b), Box-whisker plots showing the effects of changing search radius (SR) on the number of sperm tracked (a) and ALH (b) during repeated measurements of the same 2-minute videotape sequence. Each plot shows the median value (plotted point), 25th and 75th percentiles (box), and highest and lowest points in the range (whiskers).

a

b

Minimum Track Points

FIG. 2. (a), Box-whisker plots showing the effects of changing minimum track points (MTP) on the number of sperm tracked during repeated measurements of the same 2-minute videotape sequence. Each plot shows the median value (plotted point), 25th and 75th percentiles (box), and highest and lowest points in the range (whiskers). (b), Scatterplot showing the effects of changing MTP upon mean ALH values obtained during repeated measurements of the same 2-minute videotape sequence. Data points for different search radius (SR) values are systematically distributed; points for three individual SR series (SR = 5, 13, and 16 μm) are shown as regression lines.

comparisons were made using Mann-Whitney U-tests between every adjacent data set defined by SR. VCL values were significantly increased ($P < 0.05$) by every increase in SR, whereas ALH values only showed significant increases when SR was raised from 8 to 9 ($P = 0.0006$) and 9 to 10 ($P = 0.0126$). None of the other parameters showed significant changes when tested in this way. Median VAP values were essentially constant once SR exceeded 8 μm (Fig. 4b). However, after initially reaching a maximum when SR = 8 μm, VSL values declined as SR increased (Fig. 4c).

Systematic relationships among VCL, VSL, and SR were evident. Different SR settings produced grouped sets of highly correlated data points (Fig. 5). VSL measurements within these data sets varied more than VCL; this variation was attributable to changes in the MTP settings. Within each data set, the highest VSL measurement corresponds to the lowest MTP setting (10); the remaining points are systematically distributed, with the highest MTP setting producing the lowest VSL data.

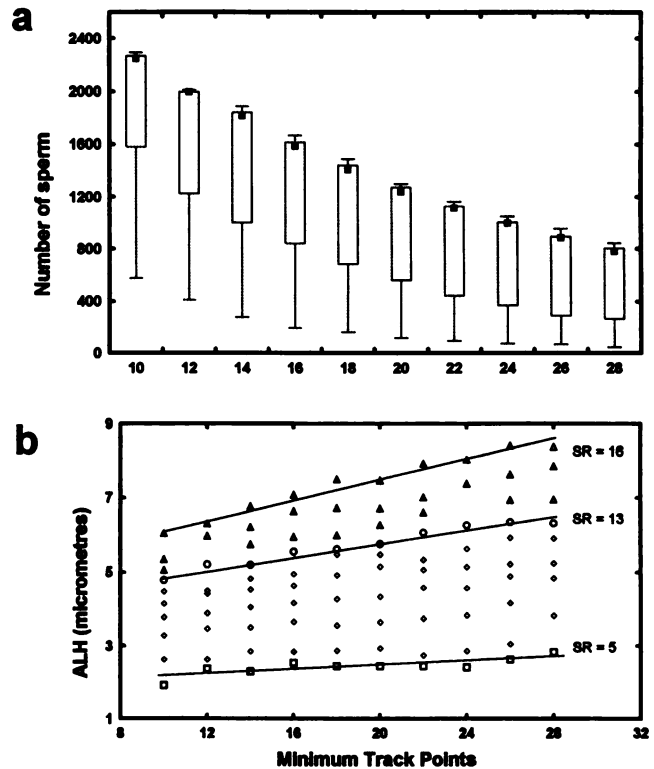Mean ALH values were positively correlated with SR

($r = 0.943$, $P < 0.001$; Fig. 1b). This effect was difficult to interpret; although the low SR settings would have excluded slow-moving cells, the number of cells tracked stabilized at SR values >8 μm. For boar spermatozoa it appears that the more rapidly motile spermatozoa exhibit greater lateral movement of the head. At low SR values (5–9 μm), mean ALH measurements were constant across all MTP settings, whereas at higher SR values ALH increased as MTP increased (Fig. 2b). This may be caused by a higher incidence of erroneous tracking at the higher SR values, where the probability of two tracks meeting, and being identified as a long track with more lateral components, is increased. When the SR setting is too high, this effect is easily recognized on the HST visual display, and unexpectedly large ALH values appear within the data.

. Mean values for BCF ranged from 5.89 to 7.32 Hz, and they were negatively correlated with MTP ($r = -0.757$, $P < 0.001$). MAD values were also negatively correlated with MTP ($r = -0.366$, $P < 0.001$, range of

means = 77.2–88.3°), whereas SR was positively correlated to MAD (r = 0.661, P < 0.001; data not shown).

From these data, MTP = 24 and SR = 13 μm were chosen for routine use. This particular combination represented the best compromise among error-free analysis, the decrease in numbers of sperm tracked that occurs as MTP value is increased, and the need to avoid biased sampling.

Discussion

Careful optimization of CASA system settings is clearly important before embarking upon studies of sperm behavior. Relatively few laboratories have undertaken such validations for animal species (e.g., bull: Anzar et al, 1991; rat: Yeung et al, 1992; Slott et al, 1993), but the requirement for standardized conditions for CASA measurements has been emphasized several times with respect to the human (Knuth et al, 1987; Mack et al, 1988; Davis and Katz, 1993). Davis and Katz (1993) regarded sperm concentration and digitization threshold to be especially important in affecting data quality. However, although they recommended that cell concentration should be <50 × 10⁶/ml, they pointed out that no objective method currently existed for setting digitization threshold (Davis and Katz, 1993). The results reported here were all measured under constant conditions, and the present data highlight the serious potential for erroneous measurements if other parameters are incorrectly adjusted.

The tests to establish a minimum velocity threshold for spermatozoa confirmed previous evaluations. It was found that cellular drifting of dead cells could generate spurious data with VAP values up to 20 μm/second, as shown by Mortimer and Mortimer (1988); cases in which VAP fell below this limit were excluded from data sets in subsequent fertility trials. Clearly, this policy may exclude genuinely slow but progressive cells. Inspection of velocity distributions suggests that this is less of a problem in the boar, where mean velocities of 80–120 μm/second are common, than would be the case, for example, in the human, where such slow-moving cells are more likely to form part of a normal population.

The HST performed well in tests of precision. Previously Davis et al (1992) performed tests of precision using the CellTrak system and obtained similar results (CV ≤3% for trajectory data).

The use of artificially created graphic objects moving with known motion patterns was useful in demonstrating that under ideal conditions the HST could produce highly accurate measurements. Systematic errors due to calibration were not a problem in these tests, although inaccurate calibration is clearly a potential problem that must be recognized when it occurs. The deviations between expected
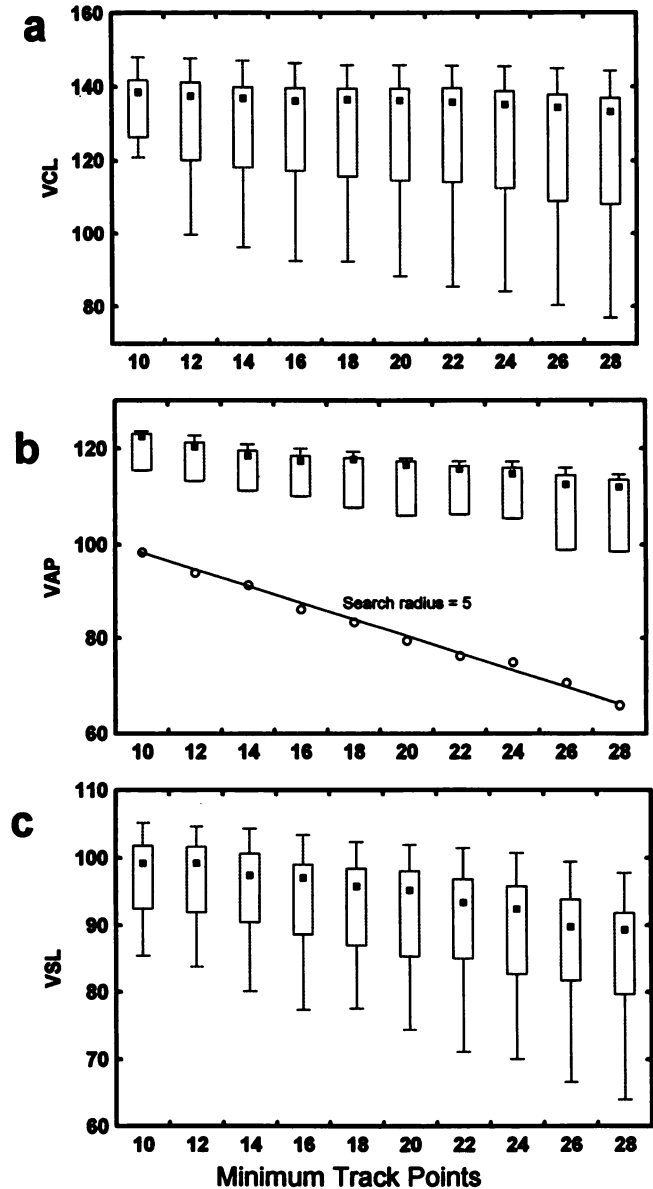
FIG. 3. (a–c), Box-whisker plots showing the effects of changing minimum track points (MTP) on VCL, VAP, and VSL values during repeated measurements of the same 2-minute videotape sequence. Each plot shows the median value (plotted point), 25th and 75th percentiles (box), and highest and lowest points in the range (whiskers). Values for search radius (SR) = 5 μm produced a linear subset of velocity data values in each case; this is specifically illustrated in b.

and observed results were probably caused by the limitation that measurements can only be made to an accuracy of 1 pixel. This limitation, termed pixellation, mainly affects ALH and VCL measurements that involve the identification of turning points. For example, measurement of ALH requires measurement of two turning points; the error on this measurement will therefore be $\sqrt{2}$ pixels. If 1 pixel is calibrated to represent 1.1 μm, the error will be approximately 1.5 μm. This source of error applies to all image-based CASA systems.
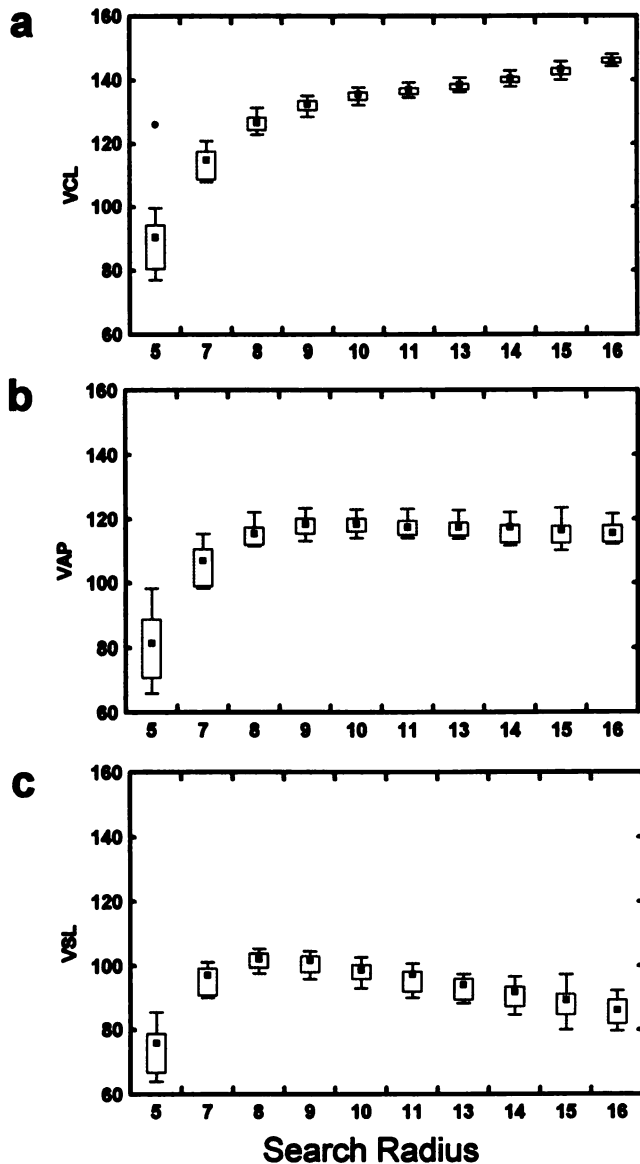
FIG. 4. (a–c), Box-whisker plots showing the effects of changing search radius (SR) on VCL, VAP, and VSL values during repeated measurements of the same 2-minute videotape sequence. Each plot shows the median value (plotted point), 25th and 75th percentiles (box), and highest and lowest points in the range (whiskers). An outlier point is shown in a; this is an extreme and possibly erroneous value.
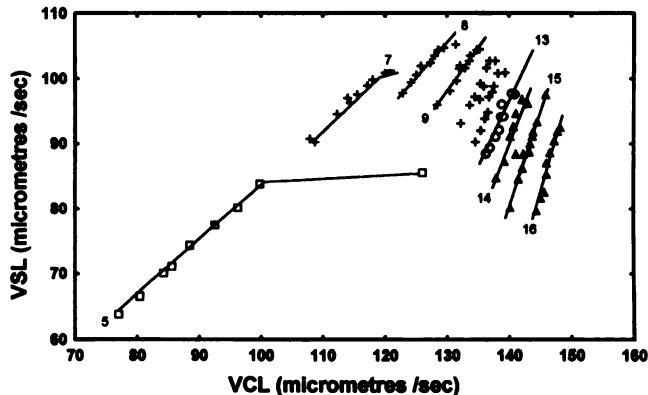

FIG. 5. Scatterplot of VSL vs. VCL values derived from repeated measurements of the same 2-minute videotape sequence. Systematic relationships between subsets of data points derived using common minimum track point (MTP) values are evident; MTP values are indicated within the figure.

of sine waves, it is likely that the real error introduced by the wave sampling effect is also greater than calculated values suggest.

Sperm population sampling errors, systematic image processing errors, and calibration errors differ fundamentally in concept. Although calibration errors are relatively easy to check with model objects, and the image processing errors can be recognized, but not necessarily eliminated, it is difficult to find a definitive solution to the sperm sampling problem. Here it is easy to inadvertently bias the selection of sperm for inclusion into data sets. Choosing a single combination of setup parameters (SR and MTP) for the HST system involved a compromise between the requirement to prevent errors within the data and the need to avoid excessive biasing in data acquisition. It is clear from the present study that major biases in sampling are introduced by extreme SR or MTP settings. When SR is too low, or MTP too high, only slow-moving spermatozoa are selected, and the data are not representative of the real population.

Attempts to optimize the performance of a CASA system always encounter the problem of obtaining a "standard" sperm sample, where the motility parameters are known accurately and in detail. Manual analysis—hand-tracing the paths of sperm from videotapes or time-lapse photographs—has been used to produce standard values for comparison with CASA results (Mack et al, 1988; Olds-Clarke et al, 1990). This process is laborious, and the number of sperm analyzed is typically small (<50 sperm per sample). In addition, there is no proof that the values produced this way are more accurate than those established using the computer systems. Sample data generated in this laboratory using manual analysis (Holt and Palomo, 1996) showed that the human eye preferentially included cells that showed clear movements between

Another source of error in CASA systems is that imposed by being unable to make continuous and uninterrupted measurements. As noted earlier, sampling the sine wave at 25 Hz causes slight miscalculation of its true length. Although this problem can be overcome mathematically when using sine waves, the only way to minimize it for real spermatozoa is to increase the sampling frequency. For this reason the routine use of 50- and 60-Hz CASA systems should enable better approximations to kinetic sperm behavior to be made. Because spermatozoa do not behave with the mathematical regularity

frames. Thus sample data were biased against the slow-moving cells.

The question "what is an acceptable minimum number of spermatozoa for reliable measurement?" is sometimes raised, and there is no single answer. The most satisfactory approach involves considering the purpose for which the data are being gathered. If mean or median values are all that is required, reference to established statistical sampling theory (Snedecor and Cochran, 1956) demonstrates that the number required depends upon the tolerance limits that are acceptable and the standard deviation of the measurements being made. For boar semen, it can be demonstrated that sampled mean VAP values do not change significantly once more than 25 spermatozoa have been measured, although the 95% confidence limits remain relatively far apart until more spermatozoa have been measured. If more detailed population analysis is required, it is probably necessary to exceed 60 spermatozoa before the results become meaningful. It should, however, be recognized that these arguments do not apply if the measurement technique itself introduces bias into the sampling method, and hence it is essential to recognize such bias when it occurs.

The sampling problem was solved pragmatically in the present study. The chosen SR and MTP values minimized, but probably did not eliminate, the inclusion of erratic track data. This combination was subsequently used, with the optical system described in the Materials and Methods section for routine measurements on boar spermatozoa, although the data were subsequently rejected if <25 spermatozoa were sampled. It is, however, recognized that the particular combination of SR and MTP chosen would not necessarily be the best for 1) spermatozoa from another species, 2) measurements made with a different optical system, or 3) measurements made with a CASA system operating at other than 25 Hz. It is therefore recommended that evaluations similar to that presented here be performed prior to working with new species or different conditions. One encouraging observation, however, is that the systematically derived SR value obtained here would also have been obtained subjectively by observing tracking quality on the HST video display. The MTP result could not, however, have been determined in this way.

Mack et al (1988) examined the effects of varying the number of trackpoints upon CASA (CellSoft Version 3.21c, Cryoresources Ltd., New York) parameters from five individual human spermatozoa. Although this differs from the present study, where effects upon populations were evaluated, their recommendation that 23 frames should be analyzed is almost identical to the present conclusion. Mack et al (1988) also studied a parameter similar to SR, known as "maximum velocity," which excluded cells from the data if they were moving faster than

a given threshold value. With the CellSoft system, and unlike the measurements made here, cells were also excluded from the analysis if two or more centroids appeared within the relevant circle described by the search radius. This made the parameter behave quite differently from the SR value used here, and the data are not, therefore, comparable.

The main purpose of this study was to establish a standard technique for boar sperm measurement as a prelude to performing field and laboratory trials to evaluate correlations with fertility. The methodology developed during this preliminary study was used in two fertility trials, both of which indicated that such correlations were indeed detectable. The detailed results of those studies will be published separately.

Acknowledgments

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Department of Urology, Salzburg General Hospital
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Tel: +43 662 4482 Ext. 2950 or 2951; Fax: +43 662 4482 880