Trajectory variance and autocorrelations within single sperm tracks as population level descriptors of sperm track complexity, predictability and energy generating ability.

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Abstract

The objectives of the present study were to develop an alternative theoretical approach to the analysis of sperm motility and to develop motility parameters that would complement those more commonly used in current CASA procedures. We have defined a set of parameters and have tested them using boar spermatozoa undergoing bicarbonate-induced motility activation. The new parameters were calculated for a series of (x,y) coordinates of sperm head positions recorded at each move along the trajectory. The parameters were: mean velocity (MV), immobility ratio (IMR), fractal dimension (FD), the variance of the step-lengths (VAR) and two autocorrelation function coefficients of the step-lengths time series for lags 1 and 2 (C_1 and C_2). MV measures the average speed along the trajectory, and VAR is a measure of displacement variability that can be related to the specific mean (per step) kinetic energy of the spermatozoon. All of the parameters except MV and FD were affected by the sampling frequency (25 vs 50 Hz); inappropriately high sampling frequency in relation to magnification resulted in step lengths between successive frames that were below the resolution threshold of the imaging system. The autocorrelation functions were especially informative; discrimination between sperm subpopulations was obvious within simple histogram formats and complex statistical analyses were not needed for their identification.

Key words

CASA, sperm velocity, Brownian motion, fractal dimension, sperm assessment

Short sentence

Short sentence: Sperm trajectories are analyzed here in terms of several within-track properties such as variance, autocorrelations and immobility ratio. These parameters efficiently highlight sperm subpopulations without the need for multivariate analysis.

Introduction

Over the last twenty years computer-assisted semen analysis (CASA) has gained recognition as an objective method of evaluating sperm motility characteristics, and continues to provide a major advantage over subjective assessment methods. In general, but not exclusively, CASA procedures use serially captured digital images of motile sperm heads to reconstruct individual trajectories. A suite of mathematical algorithms then describes the sperm motility in terms of speed (curvilinear velocity-VCL, average path velocity-VAP, straight line velocity-VSL), trajectory shape (amplitude of lateral head displacement-ALH, linearity-LIN, and straightness-STR) and flagellar beat frequency (BCF). One of the main advantages of CASA systems is the large amount of information they provide from the analysis of large numbers of spermatozoa in each sample. However, CASA procedures have been criticized because several of the head-centroid derived parameters are strongly dependent on the sampling frequency (Dunson, et al., 1999, Katz, et al., 1985) of the sequential images and on the software-specific procedures used to analyze the sampled-path. As an example, the CASA kinetic parameters VAP, STR, ALH and BCF are derived from the average path of the spermatozoon; calculation of this average path depends on the averaging or smoothing method used (Davis and Katz, 1992, Davis, et al., 1992, Mortimer and Swan, 1995). The shortcomings of the commonly used CASA analysis methods were highlighted by Davis, et al., (1992), who proposed that an alternative approach using principles of signal processing and harmonic analysis to analyze sperm tracks. This approach still, however, requires the calculation of an “average path” as a reference point from which other parameters can be derived.

The practical success of CASA as a sperm evaluation method has met with mixed degrees of receptiveness. Many laboratories report their data parameters in terms of mean and standard errors, and consequently often miss the important information present within their data (Holt, et al., 2007, Holt and Van Look, 2004). The use of statistical treatments based on multivariate cluster analyses has helped to improve the value of information obtained by CASA technology, but while there is now a wide consensus that CASA enables sperm subpopulation structures to be evaluated (e.g. Dorado, et

al., (2010), Holt and Harrison (2002), Quintero-Moreno, et al., (2007)) this approach has mostly been used in research, rather than clinical, settings.

The limitations of CASA analysis were also recognized by Dunson et al, (1999), who saw that the parameters normally reported by CASA systems are insufficiently able to discriminate between fundamentally different physiological states such as hyperactivation and cellular dysfunction, where both conditions may be described as showing low track linearity coupled with high curvilinear velocity. These authors argued that there is a need for alternative parameters that better describe the overall behaviour of the spermatozoa. They therefore developed a number of new measures, and demonstrated the value of a particular parameter that involved the predictability of the sperm trajectory.

Here we extend and explore these ideas and have aimed to develop and test alternative motion parameters. This should be especially relevant with the advent of CASA methods developed using open-source software (Tomlinson, et al., 2010, Wilson-Leedy and Ingermann, 2007) that are more amenable to modification by users, as well as being of potential value to computer scientists tasked with modeling the behaviour of “virtual” spermatozoa for the development of system-based approaches to reproductive science (Burkitt, et al., 2010). The objectives of the present study were to define and investigate parameters with which to characterize sperm motility, avoiding the need for the calculation of smoothed paths and the use of filtering methods. The new parameters were calculated for a series of (x,y) coordinates of sperm head positions recorded at each move along the trajectory; the more traditional parameters were also recorded for comparison.

Boar spermatozoa were chosen as the model for these experiments as their motility characteristics are highly sensitive to the composition of their environment. The addition of bicarbonate/CO_2 to boar spermatozoa stimulates various signaling pathways, resulting in alterations in the stimulation of protein kinase A, increased membrane fluidity (Harrison, et al., 1993, Harrison, et al., 1996, Harrison, et al., 1993), together with rapid (<2 minutes) and significant increases in velocity and linearization of sperm tracks (Holt and Harrison, 2002, Satake, et al., 2006). Bicarbonate is also known to stimulate motility,

capacitation and hyperactivation in spermatozoa from other species through the action of relatively well-defined signaling pathways (Gadella and van Gestel, 2004) and is also known to be an important component of female reproductive tract fluids (Rodriguez-Martinez, 2007).

Materials and methods

Collection and washing of spermatozoa

Sperm-rich fractions of semen were collected from 4 fertile boars from the colony kept by JSR Genetics Limited at Thorpe Willoughby (Selby, Yorkshire, UK) for commercial artificial insemination; various breeds were represented. The semen samples were supplied (via Royal Mail Special Delivery, arriving the day after semen collection) already diluted in BTS extender, and was stored for no more than one day.

Prior to experimentation, spermatozoa were isolated from the diluted semen by sedimentation through a two-step Percoll gradient. Seven-ml aliquants of diluted semen were layered over 2 ml 35% Percoll on 2 ml 70% Percoll; the Percoll suspensions were iso-osmotic, prepared according to Vincent and Nadeau's method (Vincent and Nadeau, 1984) in a saline medium: 137 mM NaCl, 10 mM glucose, 2.5 mM KOH, 20 mM HEPES, pH 7.4 at 20°C. Centrifugation was performed for 15 min at $200g_{max}$ followed by $900g_{max}$. After centrifugation, the supernatant layers were removed by aspiration to leave about 0.4 ml of the 70% Percoll, in which the loose sperm pellets were resuspended. These preparations (final concentration approximately 4×10^8 sperm/ml, viability > 90%; estimated using propidium iodide staining (Harrison and Vickers, 1990)) were kept at ambient temperature (approximately 18˚C) protected from draughts and used within 2 h of washing.

Media and reagents

A basal Tyrode's-based incubation medium ('M' solution) (Harrison, et al., 1996) consisted of 116 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 1 mM EGTA, 20 mM HEPES (adjusted with NaOH to pH 7.6 at 20°C), 3 mg BSA ml^{-1}, 100 µg kanamycin ml^{-1}, and 20 µg phenol red ml^{-1}; its final pH at 38°C was 7.4 and its osmolality 300 mOsmol kg^{-1}. Bicarbonate/CO_2 was added (8.3µl /ml Tyrodes's solution) in the form of a 300 mM aqueous solution of $NaHCO_3$ saturated with 100% CO_2 (a ratio of bicarbonate: CO_2 yielding pH 7.4 at 38°C after dilution). To prevent loss of CO_2 during subsequent incubation, the bicarbonate-containing suspensions were maintained under a CO_2 -containing atmosphere.

Experimental protocol

Incubations were carried out following a procedure that has been reported previously as part of an experimental study of bicarbonate-induced sperm activation (Holt and Harrison, 2002). Some of the video recordings from this experiment were reanalyzed for this study. In brief, 1 ml of "M" solution was pre-warmed to 38°C in a capped 15-ml polystyrene tube (Sterilin, Stone, Staffs, UK). An aliquant (1-3 µl) of washed sperm (final concentration approximately 2 x 10^6 cells ml^{-1}) was added, and the suspension incubated at 38°C for 10 min. A 60 µl sample was then removed for motility analysis. Next, half the remaining suspension was transferred to an empty pre-warmed tube and to it was added the "control" treatment (7.3 µL 300 mM NaCl as control for bicarbonate addition). Twelve minutes after the initiation of incubation, 8.3 µL of the bicarbonate/ CO_2 mixture ("activator") was added to the rest of the sperm suspension (in the first tube), achieving a final bicarbonate concentration of 15 mM. Incubation of both tubes was continued, and further 60 µl samples were removed from the "bicarbonate" tube for video recording at 5 min intervals. In the original experiments these samples were taken until 27 min after the addition of bicarbonate, whereupon the original "control" tube was resampled. In this study we did not analyses all the original time points.

Sperm trajectories were recorded by videomicroscopy as described by (Holt and Harrison, 2002). 60 µl samples were taken from the sperm suspensions, placed on electropositive glass slides and covered with a 22 x 22 mm glass coverslip. Small amounts of vacuum grease containing 100 µm diameter glass beads (Sigma-Aldrich, UK) were placed on each corner of the coversip to provide a chamber depth of 100 µm. Microscopy was performed using a x10 negative-high phase contrast objective and an Olympus BH-2 microscope. Sperm video sequences of approximately 2 min each were recorded on CDs using a VCD recorder (VDR-3000; Datavideo UK Ltd, Manchester, UK).

More than one thousand individual spermatozoa trajectories were analyzed quantitatively for each boar treatment combination, using a Hobson Sperm Tracker (Hobson Tracking Systems, Sheffield, UK) operating at 50 Hz controlled through an IBM-compatible computer. The 'search radius' used was 5.9 µm, and the 'minimum track points' setting was 50 frames. Details regarding the use of the Hobson Sperm Tracker and discussion of these parameters may be found in an earlier publication (Abaigar, et al., 1999).

Detailed exploration of individual trajectories was undertaken using purpose-written software (GET XY) supplied by Prof. G. S. Hobson (Hobson Tracking Systems, Sheffield, UK). This software provides series of (x, y) coordinates of sequential sperm head positions at a resolution of 50 Hz, and with a spatial resolution of 600 x 600 pixels, from video recordings. Individual trajectories were reconstructed using IDRISI software (IDRISI version 132.22, USA) and special software designed for this purpose (Data Track Analysis-DTA); DTA uses a frame of 512 x 512 pixels.

Experimental design

An experiment was conducted using semen samples from 4 different boars. The main original objective of the experiment was to study the effects of bicarbonate upon sperm activation; that aspect of the data has been reported previously (Holt and Harrison, 2002). The present analysis was undertaken using the video recordings prepared during that experiment.

Data analysis

The parameters were derived from trajectories that included a minimum of 40 frames (0.8 s): this is consistent with the fractal dimension definition cited by Mortimer, et al., (1996).

A series of parameters was derived from the unsmoothed raw data; these were MV = mean velocity, IMR = immobility ratio, FD = Fractal dimension, VAR = variance of the step-lengths and the two autocorrelation function coefficients of the step-lengths time series for lags 1 and 2, namely C_1 and C_2. Their derivation is described below and their independence and uniqueness were tested by correlation analysis.

To analyze the dependence of the defined parameters on sampling frequency, we reconstructed and recalculated all the track parameters for 25 Hz tracking, by selecting every second XY coordinate. This simulated an experiment with 0.04 s time-steps instead 0.02 s.

Results

Theoretical basis for the definition of parameters

The spermatozoon**,** *in vivo* **and** *in vitro*, is propelled by the flagellar beat and describes a wave-like movement which causes the sperm head to turn. A series of sperm head positions (represented to data analysis software by centroids) is commonly used to define the movement trajectory of a spermatozoon. The actual trajectory can be recorded (using video equipment) and then sampled at a fixed sampling rate (or time). The spatial position of the sperm head in each sampled time may be defined by its planar (x, y) coordinates so that the sampled trajectory is represented by a series of moves, each move being represented graphically as a short line. The successive (x_i, y_i) coordinates of the head-centroid permit reconstruction of the trajectory at a defined sampling rate and from this series of moves we may define the motility parameters.

To describe the reconstructed track geometrically, here we define an “oriented step-length”. The step-length is calculated as the distance between two consecutive points:

$$l_i = \sqrt{(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2}$$

But the actual step-length is “oriented” in relation to the previous position of the spermatozoon head, in such a way that the step-length is considered:

positive when dx (= x_{i+1} - x_i) > 0 and negative when dx < 0. In the unusual case when dx = 0, the step length is considered positive if dy (= y_{i+1} - y_i) > 0 and negative when dy < 0

The speed of the spermatozoon within each step is calculated as the ratio between the absolute value of the step-length and the sampling time interval. This procedure allows the definition of effective “immobility” when there is no detectable change in the position of the sperm head because the displacement is small enough to be below the limits of the spatial resolution used at the selected sampling frequency. Step-lengths and speeds have been converted from the “image” units, pixels and pixel/sec, to the corresponding spatial and physical units, μm and μm sec^{-1}, through the conversion factor derived for the whole spatial sampling process. As the frame dimensions are 600 x 600 μm (translated to 512 x 512 pixels by the Idrisi software), the factor for changing spatial to image size is 600 μm / 512 pix = 1.17 μm s pix^{-1}. This results in 1 pixel = 1.17 μm. In our case, using a sampling frequency of 50 Hz, the minimum detectable velocity of the spermatozoon in one step was 25 μm sec^{-1}. Here we define the parameters to characterize sperm movement in terms of: velocity, mobility, complexity of trajectories, step-length variability and autocorrelation coefficients.

The velocity is measured as the mean velocity (MV); the mobility by its complement, the immobility ratio (IMR); the complexity of a trajectory is measured using fractal dimension (FD), the variability (VAR) by means of a step-length variance parameter, and two coefficients of the autocorrelation function. Each parameter definition is presented with its symbol and its physical units.

Mean velocity (MV) (μm s^{-1})

MV = Total displacement along the path / total track time

$$MV = \frac{L}{T} = \frac{1}{N\tau}\sum_{i=1}^{N}|l_i|$$

Where: *T* = total track time

L = total displacement

N = total number of steps in the track

τ = sampling time (s)

$|l_i|$ = length (absolute value) of the step.

As defined, *MV* measures the spermatozoon mean velocity along its sampled path. This parameter is equivalent to the curvilinear velocity (VCL) obtained from CASA procedures.

Immobility ratio (IMR) (% total tracking time)

IMR is defined as the fraction of the total track time (T) (%) in which the actual displacement of the spermatozoon is less than the spatial resolution available through the measuring system and we cannot therefore detect any movement. In terms of velocity, and for the experiment sampling time and resolution, this is less than 25 μm s^{-1}.

Fractal dimension (FD) (non-dimensional).

Fractal dimension of a trajectory measures the complexity of the trajectory and is a simple and practical method for classifying and comparing planar curves composed of connected line segments.

Several definitions of the fractal index or fractal dimension may be found in the literature, but in our case we have used that proposed by Katz and Georges (1985).

$$FD = \frac{\log N}{\log N + \log \frac{d_{\max}}{L}}$$

Where:

N and *L* have been defined previously and

d_{max} is the planar diameter of the path (i.e. maximum distance between any two points along the trajectory).

Variability or Step-length variance (VAR), (μm^2)

We define this parameter as the variance of the step lengths, in its statistical definition, but without reducing the values to the mean value of the step-lengths. The parameter is defined as:

$$VAR = \frac{1}{N} \sum_{i=1}^{N} l_i^{\,2}$$

From the point of view of sperm kinematic behaviour, this parameter can be related to the specific (per unit mass) mean (per step) kinetic energy of the spermatozoon, as follows:

$$VAR = \frac{2\tau^2}{Nm} Ec \,,$$

Where, *Ec* is the total kinetic energy developed by a spermatozoon along the track, and *m* a mean characteristic spermatozoon mass.

Autocorrelation function coefficients: C_1 and C_2 (dimensionless)

The properties of a time series can be derived from a set of values called autocorrelation coefficients (or time-step lag coefficients), which measures the statistical correlation between observations that are different “distances” (in time) apart, thereby representing a sort of “memory” of the process. In our case, the autocorrelation function coefficients of the step-lengths time series assesses the presence of serial statistical correlation within the series of moves, which were defined both by length and orientation.

Given N observations on a discrete time series, (z_t), we define the autocorrelation coefficient between observations separated by “time distance” *k* time-steps as:

$$C_k = \frac{\sum_{t=1}^{N-k} z_t \; z_{t+k}}{\sum_{t=1}^{N} z_t^{\,2}}$$

C_1 measures the statistical correlation between consecutive observations ($k = 1$), and C_k the same for observations *k*-lags apart. From the definition, all the autocorrelation coefficients are in units of the variance of the time series and should have values ranging between -1.0 and 1.0. The first autocorrelation coefficient, C_0, (which corresponds to time-step lag 0) as defined, is equal to 1.0 in all cases. The graph of the autocorrelation function versus the time-step lags is termed a correlogram.

Autocorrelation values between the limits $\pm 2/\sqrt{N}$ are not statistically significant.

If a time series is completely random (a purely random process or white noise), all the coefficients, except C_0 will be nearly zero or will lie between $\pm 2/\sqrt{N}$. In other words, if the time series reproduces the behaviour of a random phenomenon, the unique significant value is for time-lag 0, being negligible (or below the statistical significance value) for the coefficients of the remaining time-lags. In the case of a time series which shows a tendency to alternate, then the autocorrelation function also tends to alternate, and time lag coefficients tend to show plus and minus signs as the time lag increases.

Sperm behaviour may be inferred from the correlogram of its step-length time series. To characterize the behaviour of a time series of a short-term memory process, only a limited set of coefficients should have statistical significance. In our case, we have found that only the first two coefficients (apart from the C_0 coefficient) have statistical significance, when considering the whole sample, and we therefore keep only the coefficients, C_1 and C_2.

Validation and behaviour of the parameters

To illustrate the relevance of these parameters of sperm motion, we show, in Fig. 1, series of graphs for three individual spermatozoa (Fig 1a – d, a' – d' and a'' – d'') (corresponding to boar 1; time 0 min). The reconstructed trajectories (series a), the time series of the calculated step-lengths (series b), the histogram of the step-lengths (series c) and the correlogram, for up to 10 time step lags, of the oriented step-lengths (series d).

Careful observation of the trajectories, and the time series of oriented step-lengths in Figs 1a and 1b shows the behaviour of spermatozoa in terms of: 1) activity and "resting" time, 2) positive and negative displacements, as previously defined, and 3) the step-length values of each displacement. Periods of movement (activity) are followed by others where a spermatozoon seems to be immotile; the appearance and duration of the resting times are rather variable within the trajectory of the spermatozoa. Alternatively, it is possible to see episodes where the moves have the same sign

(positive or negative), as evidence of some degree of directional persistence during the considered time interval.

In general, the histograms for step-lengths of spermatozoa (Figs. 1c) are symmetrical, but it is not unusual to find histograms with some degree of skewness, thereby revealing an overall directional persistence. In the histograms shown in Figs. 1c there is moderate to strong presence of null values (step-length less than the spatial resolution), corresponding to the “immobility” periods. But despite the presence of the immobility values, the histogram of step-lengths found in all the analyzed cases showed a concentration of low displacement values and a moderate to strong decrease in high or very high step-length, in a bell-shaped curve, as in the case of a Gaussian or near-Gaussian distribution.

The correlograms of the step-length time series (Figs. 1d), show that only the first and/or second autocorrelation coefficients may have significant values (greater than the statistical significance value), being negligible for the remaining time-lags. The analysis of the autocorrelation function of all the spermatozoa in the sample shows that this is the norm.

Independence of parameters

Correlations between the parameters defined here showed that a low Pearson correlation coefficient exists, except for the pairs (MV, IMR) and (MV, VAR).

The correlation ($R^2 = 0.63$, SD = 15.1, $P < 0.001$) between MV and IMR should be understood as a “logical” relationship rather than a correlation; as long as the spermatozoon remains immotile for a significant amount of time, its MV should, necessarily also decrease. However, we stress that the standard deviation of fitting both parameters is very poor, and the ratio between the standard deviation and the mean value of the average velocities reaches 30% (15 $\mu m\ s^{-1}$ / 50 $\mu m\ s^{-1}$). As an example, for an IMR value of 60% we may obtain average sperm velocities between 15 and 80 $\mu m\ s^{-1}$.

Although a high R^2 value was obtained for the correlation between MV and VAR ($R^2 = 0.94$, SD = 0.53, $P < 0.0001$), the ratio between the standard deviation parameter and the mean VAR value for all tracks

also reaches a value as high as 15%. We keep both parameters because we think they are measuring different properties: MV measures the average speed along the trajectory, and VAR is related to the mean square velocity and is a measure of displacement variability.

Dependence of parameters on the sampling frequency

The dependence of the derived parameters on sampling frequency was analyzed. We compared, in the different sections of Fig 2 (a to f), histograms for the selected parameters at two sampling frequencies in the range of typical CASA analyzers: 50 Hz and 25 Hz (sampling times of 0.02 sec/frame v 0.04 sec/frame, respectively), with the 50 Hz data placed at the bottom of each pair of graphs.

As expected by the definition of parameters, histograms for the mean velocity (MV) and for fractal dimension (FD) were not affected by the sampling frequency; however histograms for the immobility ratio (IMR) and for variance of the step-length (VAR) were significantly affected by the frequency of sampling used. In the case of VAR, the histogram for 25 Hz is wider that in the case of 50 Hz, and in general, individual values should be greater, because the measured step-lengths are larger when considering a lower frequency. Moreover, this lower frequency implies that we have a higher number of steps with “non-zero” step-lengths. Both arguments contribute to explain the observed differences. The IMR (50 Hz) histogram was highly skewed to the right, indicating that large numbers of sperm tracks contained individual step lengths that were below the detection limit for spatial resolution. In contrast, the IMR (25Hz) histogram was left skewed, showing that the majority of sperm tracks did not contain these small step lengths. We interpret these differences as showing that the higher sampling frequency should be matched by the use of higher magnification microscopy if IMR is to be used with confidence.

The case for the autocorrelation coefficients deserves a more detailed commentary. Figure 2e shows that the number of spermatozoa with significant C_1 values is greater for a sampling frequency of 25 Hz than for 50 Hz. On the other hand, the number of spermatozoa with significant C_2 values is almost

negligible when using a 25 Hz sampling frequency in comparison to 50 Hz (figure 2f). This confirms that both autocorrelation parameters are, by definition, frequency-dependent.

Effect of incubation on sperm motility as revealed by the new parameters

The response of boar spermatozoa to bicarbonate addition is illustrated in the dot plots shown in Fig. 3a and b). These show the control (0 min: bicarbonate absent; Fig 3a) *v* added bicarbonate (7 min after addition; Fig 3b) derived by routine use of the Hobson Sperm Tracker. These plots are useful in that they illustrate graphically the appearance of more rapid and linear (increased VAP and LIN) within the sample.

Consistent with the dot plots, the new parameters showed the expected responses to the bicarbonate incubation-experiment with boar spermatozoa (figure 3c and 3d. The addition of bicarbonate (results for 0 min are for spermatozoa in the absence of bicarbonate, while the other graphs show data at 2 and 7 minutes after addition of 15 mM bicarbonate) increased the mean velocity (Fig 3d) and reduced the fractal dimension (Fig 3e) of the sperm trajectories. The variance of the step-lengths and mean velocity was increased markedly by bicarbonate addition (Fig 3c), with the appearance of a new cohort of high variance and high velocity spermatozoa. There were, nevertheless, unequal effects on the individual spermatozoa: the presence of a low velocity and low variance population persisted throughout the time course of the experiment (figures 3c and 3d).

We also found that the C_2 coefficient, which statistically correlates steps that are 0.04 sec (two steps) apart, seems to be relevant in 40 ± 10 % of cases, independent of both the incubation time and the boar. Figure 3g shows that almost all the “significant” spermatozoa in the control treatment (0 min) show negative C_2 values. The fraction of spermatozoa demonstrating positive C_2 coefficients increases abruptly with bicarbonate addition, and highlights the existence of two sperm subpopulations within the samples. The two subpopulations remained unchanged with further incubation for 12 minutes (data not shown).

The graphs in Fig 3c reveal that two distinct sperm subpopulations became visible 2 and 7 minutes after bicarbonate addition, with the value VAR = 4.5 μm^2 providing a useful dividing point between them. To see in greater detail how the new parameters reflect these apparent subpopulations we show a series of plots (Figs. 4a-f) that were prepared by dividing the data at VAR = 4.5 μm^2 for a specific boar (boar 4), 7 minutes after bicarbonate addition, and then replotting some of the histograms for >4.5 and <4.5 μm^2.

It is evident that the mean velocity histograms representing the VAR< and >4.5 μm^2 populations (Fig. 4b; upper and lower panels respectively) are effectively separated with a boundary at about 60 $\mu m\ sec^{-1}$. The IMR histogram (Fig 4c) for the population with the lower variance is wide and flat (extending from 40% to about 95%), while the higher VAR population exhibits a maximum IMR value of about 60% and a narrower distribution of values. Correspondingly, the FD histogram for the low VAR population is also much wider than that for the high VAR population (Fig. 4d). This difference indicates that the subpopulation showing lower variance is also exhibiting more complex and erratic movement patterns. The autocorrelation coefficient C_1 was not particularly useful in differentiating the different subpopulations (Fig 4e); on the other hand, the C_2 coefficient produced markedly different histograms for the low and high VAR populations. The VAR >4.5 population was almost entirely concentrated within the right hand side (positive) of the histogram (Fig 4f); the lower VAR plot also revealed two subpopulations, where the greater proportion of trajectories were in negative region. This analysis, based on the differential values of VAR, therefore revealed three sperm subpopulations.

Discussion

The objectives of the present study were to develop an alternative theoretical approach to the analysis of sperm motility and to develop motility parameters that would complement those more commonly used in current CASA procedures. Some of the parameters enhance the biological meaning of data gained during sperm motility studies, while others actually help to define the validity or limitations of the equipment being used. This was particularly evident in the demonstration that the 50 Hz frame, rate combined with the relatively low powered objective (x10) used in these experiments, resulted in a

377 significant proportion of the individual step lengths along a trajectory being below the resolution
378 threshold; in our case, “immotile” means that the instantaneous speed of a spermatozoon was less
379 than 25 μm s^{-1}. This effect was particularly evident because the parameters were being derived
380 entirely from raw (x, y) coordinates and without the application of smoothing or interpolating
381 algorithms. A relatively easy solution to this problem would be to use a higher magnification objective
382 (x20), although this would be at the expense of track length. In the present study we set minimum
383 acceptable track duration of 40 video frames, i.e. slightly less than one second, although many tracks
384 significantly exceeded this duration. There might be some difficulty in maintaining this minimum
385 standard when trying to measure rapidly moving spermatozoa with a higher magnification objective.

386 One outcome of this study was the demonstration that several sperm motility parameters can be
387 calculated from simple (x, y) coordinate information. These are largely complementary to the more
388 commonly used parameters, and in some cases, especially the autocorrelation C_2 and the variance,
389 were able to provide population level information that would normally require the application of
390 multivariate cluster analysis. Mean velocity (MV) is derived from a procedure that was previously used
391 by Mortimer and Swan (1995) to study human sperm motility. As defined, MV measures the sperm
392 mean velocity along the sampled path. This parameter is equivalent to the curvilinear velocity (VCL)
393 obtained from standard CASA procedures.

394 Although it should be clear from the preceding text, we emphasize that the IMR as developed here, is
395 not the same as the parameter, percentage motility (MOT) that is usually included as one of the
396 standard outputs in commercial systems that measure sperm motility. MOT is calculated by assessing
397 the proportion of spermatozoa that do not show any forward progression, while IMR is a “within-track”
398 parameter that describes the proportion of steps in a single trajectory that fall below the limit of system
399 resolution. Provided the spatial and time resolution are appropriate, the IMR represents one of the
400 simpler and, to some extent, logical, parameters to define when studying sperm motility. It provides
401 information about what is happening during the observed sperm displacement, in terms of the fraction
402 of time in which the spermatozoon has apparently not changed its position. In the context of the
403 bicarbonate-induced activation, this parameter was not, however, particularly informative.

Nevertheless, it could be useful in characterising highly erratic sperm tracks that are not adequately described by the more traditional suite of measures. A recent study of sperm motility in the whitefish, *Coregonus albula* L, (Dietrich, et al., 2010), presented CASA parameters and mentioned the erratic behaviour of spermatozoa from this species. Nevertheless, the graphs presented in the paper did not adequately convey these apparent erratic behaviours. Other qualitative descriptions of sperm behaviours have also revealed that sperm tracks can be erratic under certain conditions, and that different physiological situations induce varied track responses. For example, in a study of rabbit spermatozoa (Suarez, et al., 1983) tracings of sperm tracks were used to illustrate these effects, but it would be of interest to see whether IMR might provide an additional method of describing sperm tracks under these conditions.

The fractal dimension is a simple way to measure the complexity of a trajectory and has been previously used as a measure of human sperm motility (Davis and Siemers, 1995, Mortimer, et al., 1996, Schoevaert-Brossault and David, 1984). Its definition, as confirmed in the present study, is significantly independent of the image sampling frequency. Its utility was previously suggested by Mortimer, et al., (1996) who applied fractal analysis to sperm motility evaluation, and who proposed that “Brownian motion” theory should be introduced into sperm kinematics. In the present study the bicarbonate-induced sperm activation treatment resulted in the conversion of a broadly-based and right-skewed histogram (control treatment) that spanned FD values between 1 and 2, to histograms in which the right-skewed tails became diminished, especially after 7 minutes of incubation. These observations confirm that bicarbonate activation rapidly reduces track complexity, an observation that would otherwise be reflected by increased linearity or straightness.

The variance of the step-lengths is related to the variability of each displacement (step) along the trajectory. As the calculation of variance involves a term that directly represents kinetic energy (*Ec*) and reflects the mean kinetic energy developed per step by the spermatozoon along the track, the variance term stands out as being more directly informative about the sperm status than other parameters. As Figure 3c shows, the variance of the sperm population increased with the incubation time, with a particularly pronounced right shift in histogram structure being induced by the bicarbonate

treatment. This indicates an increase in the mean energy developed by the moving spermatozoa; the broad base of the histograms together with the left skews that remain apparent after the bicarbonate treatment must therefore reflect sperm heterogeneity, caused by the unequal abilities of spermatozoa to develop kinetic energy. This observation led to the hypothesis that partition of the histograms on the basis of variance differences might represent another useful strategy for analyzing aspects of sperm heterogeneity, and this idea was supported by the various outcomes presented in Fig 4. It was of particular interest that the combination of variance partitioning and autocorrelation analysis was able to identify at least three sperm subpopulations within the bicarbonate-treated sample. Subjective examination of these histograms was sufficient to recognise the presence of these subpopulations; although the two-dimensional dot plots of VAP v LIN effectively and graphically illustrate how the sperm trajectories change in response to bicarbonate, it is not possible to distinguish subpopulations with any certainty.

The autocorrelation functions revealed some interesting features with respect to the motility of individual spermatozoa, although inappropriate sampling frequency was shown to present a problem. When sampling at 50 Hz in “control” media, most spermatozoa showed step-length time series with non-significant C_1 values. This can be interpreted as a lack of statistical “memory” between successive steps separated by 0.02s. However, when the spermatozoa are incubated in media that stimulates their movement, i.e. containing bicarbonate, they display more deterministic behaviour, where successive moves are related to previous moves. Significant values for C_1 were negative in almost all cases; in practical terms, this means that, incubation increased the number of spermatozoa for which the next step would be in a contrary direction to the step just taken. In this context it is important to remember that the steps represent successive positions of the sperm head, and therefore the negative values for C_1 probably represent the side-to-side deviations of the sperm head in response to the flagellar action.

In the case of C_2 coefficients, the sperm fraction showing a significant temporal pattern seemed to be constant (around 40 ±10 %). Incubation caused some changes to the temporal pattern as follows: in the control sample lacking bicarbonate the spermatozoa possess a statistical “memory” making the

458 sperm heads move in a direction that is opposite to the move made 2 time-steps previously. This
459 could only happen with complex and non-linear tracks. However, after bicarbonate addition the
460 situation has changed noticeably and a new cohort of spermatozoa with positive C_2 values appears.
461 These spermatozoa are making moves that go in the same direction as those two steps previously,
462 thus producing an overall linear and progressive direction of travel. The clarity with which these
463 negatively and positively correlated tracks are separated within the histogram (Fig. 3g) suggests that
464 they represent clear and simple criteria with which to dissect and describe the subpopulation structure
465 within sperm suspensions and their responses to treatments.

466 Both parameters, C_1 and C_2, also provide information about the frequencies underlying the sperm
467 motility. Within the non-activated spermatozoa (control samples lacking bicarbonate), the low fraction
468 of spermatozoa with significant C_1 and those with the higher fraction of negative C_2 values can be
469 interpreted as if these fractions were moving with a specific frequency. However, the change in pattern
470 of movement which follows incubation (higher fraction of spermatozoa with significant C_1 combined
471 with positive C_2 values) could be also interpreted as indicating a shift to higher frequency, therefore
472 representing another aspect of sperm activation.

473 The alternative analytical approach to sperm track analysis suggested by Davis, et al., (1992) involved
474 the use of signal processing techniques to produce a new, average, track from the individual series of
475 coordinates, followed by the identification of major harmonic frequencies and amplitudes in the
476 curvilinear trajectories of the spermatozoa. In their paper Davis, et al., (1992) demonstrated that BCF
477 and ALH are only accurate if there is a periodic, progressive and symmetrical average path. The
478 parameters derived in this paper overcome this dependence on the average path, and offer another
479 useful alternative approach to the analysis of sperm tracks. It is nevertheless worth pointing out that
480 the autocorrelation parameter $\boldsymbol{C_2}$ derived here indicated the existence of dominant frequencies in the
481 range 10 to 20 Hz as found by Davis, et al., (1992).

482 One reason for deriving and investigating a different set of parameters relates to current interests in
483 the development of "*in silico*" models of sperm interactions with the female reproductive tract (Burkitt,

et al., 2010; Gefen, 2010). The intention of such models is that they will produce computer-generated representations that reflect the behaviour of sperm populations when, for example, they encounter different physical and chemical environments during their journey towards the oocyte. To achieve this aim, computer models include “virtual” sperm populations whose behaviour and responses have to be represented within specific algorithms. Assigning different parameter values to these virtual spermatozoa is likely to result in more realistic heterogeneous behaviours when the models are run. Some of the parameters described here, especially the autocorrelations, the fractal dimension and the variance should be useful in this respect as they possess deterministic, rather than purely descriptive, qualities.

This study was not specifically undertaken in order to examine the effects of bicarbonate on boar spermatozoa, as the rapid stimulatory effects of bicarbonate on boar sperm motility have been described previously (Holt and Harrison, 2002). Nevertheless, the analyses presented here underline the previous observations and reinforce the observations that bicarbonate induces a heterogeneous response within single populations of spermatozoa. This heterogeneity is best recognized here from the autocorrelation histograms. From a biological perspective it seems that the stimulatory events taking place after bicarbonate addition may involve two or more distinct processes. Studies that involved exposing mouse spermatozoa to bicarbonate (Wennemuth, et al., 2003) showed that the presence of bicarbonate significantly increased the flagellar beat and its symmetry within 30 seconds and facilitated the opening of voltage-gated calcium channels. The present results with boar spermatozoa confirm the initiation of increased flagellar beat symmetry, which would generate linear, and highly autocorrelated tracks, as well as increased mean velocity and increases in variance.

A recent biomechanical study of sperm motility (Gefen, 2010) explored the relationships between human sperm velocity, sperm head density and flagellar stiffness and the predicted ability of an individual spermatozoon to traverse the zona pellucida. This theoretical modelling study confirmed that the ability to generate higher velocity should be more influential in this respect than the other parameters, and the author interpreted the results as supporting previous observations that sperm velocity, especially in a viscous environment, is positively correlated with higher conception rates and

fertilizing ability (see, for example: Aitken, et al., (1992) and Cox, et al., (2006)). As sperm transport in the female reproductive tract is such a complex process, where sperm velocity is positively and negatively regulated by the environmental milieu (Holt, 2009), it is likely that sperm velocity in semen samples probably represents a proxy for energy production and mechanical transduction capabilities of spermatozoa when they eventually meet the oocyte. The heterogeneity of sperm motion characteristics within single semen samples complicates this relationship, but also means that recognising and quantifying the relative proportions of high and low velocity sperm populations should be an advantage in predicting the probability that spermatozoa will ultimately reach the vicinity of the oocyte. The analyses presented in this paper should therefore help to develop and improve predictive tools for the more effective assessment of semen quality than is currently possible.

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Figure legends

Figure 1.

Graphs of the sperm motion parameters of three individual spermatozoa. In rows: a) Reconstructed tracks; a, a' and a''. b) Step-length time series are shown in panels b, b' and b''. c) Step-length histograms are shown in panels c, c' and c''. d) Correlograms are shown in panels d, d' and d''. Calculated parameters are shown at the foot of each column.

Figure 2.

Histograms for the selected parameters at 25 Hz (upper panel) and 50 Hz (lower panel) sampling frequencies for boar 1, control sample (0 minutes). a) IMR (%), b) Variance (μm^2), c) Mean velocity ($\mu m/s$), d) Fractal Dimension, e) C_1, and f) C_2.

Figure 3

Figure 3a and b. Dot plots (VAP v LIN) showing the effects of 15 mM bicarbonate addition on the behaviour of individual spermatozoa (each dot represents a single sperm trajectory). Panels 3a and 3b show the plots for boar 4 "control" (0 min) and "added bicarbonate" (7 min) treatments.

Figures 3c-g are histograms for the selected parameters for boar 4 and different experimental times (in minutes after 15 mM bicarbonate addition). Panel c) Variance (μm^2), Panel d) Mean Velocity ($\mu m/s$), Panel e) Fractal Dimension, Panel f) C_1, and Panel g) C_2. Ordinates are, in all cases, number of spermatozoa. As labelled, experimental time (0, 2 and 7 min) increases from top to bottom.

Figure 4

Histograms show that dividing the data for boar 4 (7 min after bicarbonate addition) into two subsets defined by the variance (VAR < and > 4.5 μm^2) readily distinguishes sperm subpopulations; upper panels represent <4.5 and lower panels represent >4.5 μm^2). Panel a shows the variance; panel b shows mean velocity; panel c shows IMR; panel d shows fractal dimension and panels e and f show the autocorrelation coefficients C_1 and C_2 respectively.

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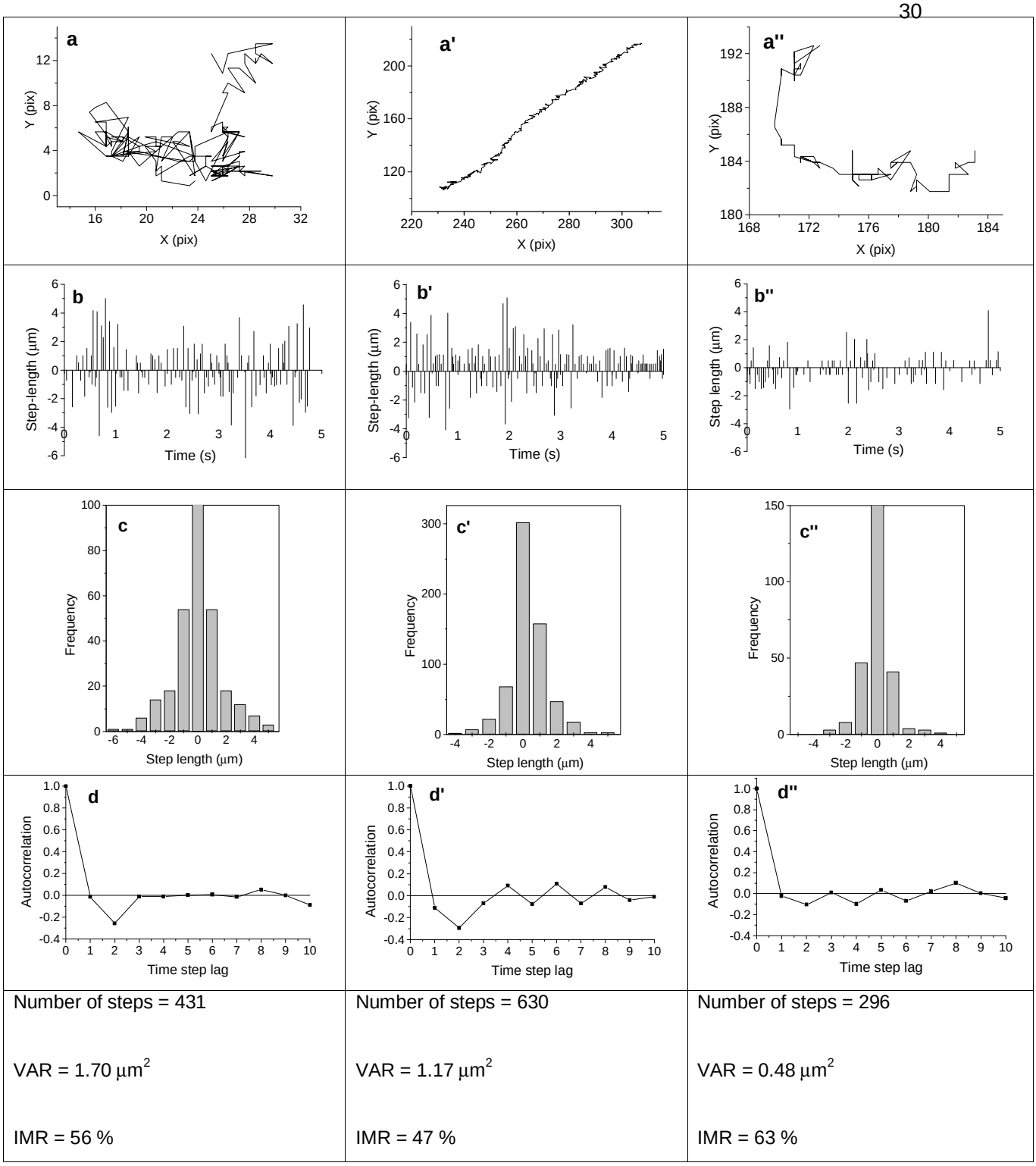

Number of steps = 431	Number of steps = 630	Number of steps = 296
VAR = 1.70 μm^2	VAR = 1.17 μm^2	VAR = 0.48 μm^2
IMR = 56 %	IMR = 47 %	IMR = 63 %

Figure 1

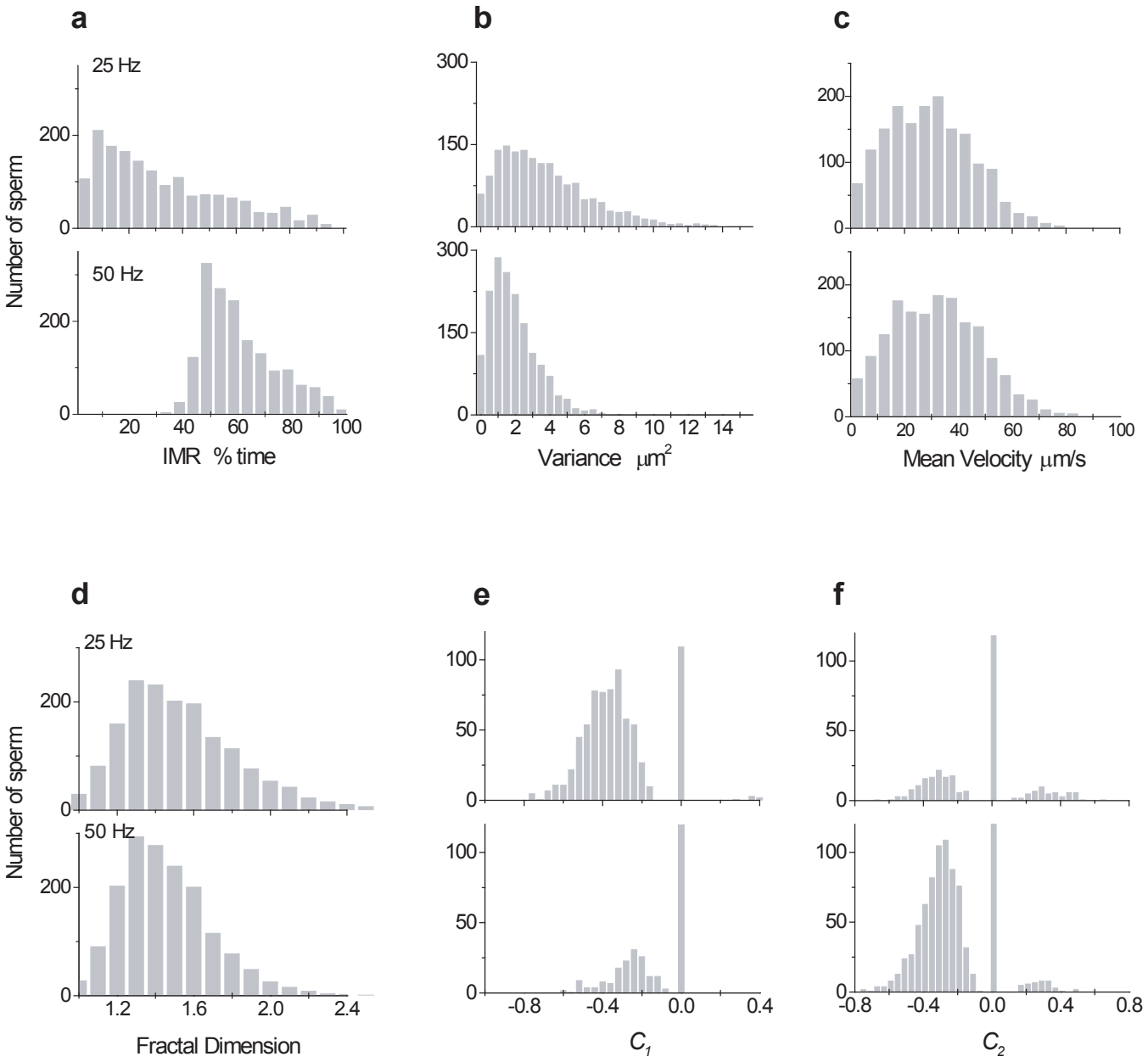
a
b
c
d
e
f
25 Hz
50 Hz
Number of sperm
IMR % time
Variance μm²
Mean Velocity μm/s
Fractal Dimension
C1
C2

Figure 2

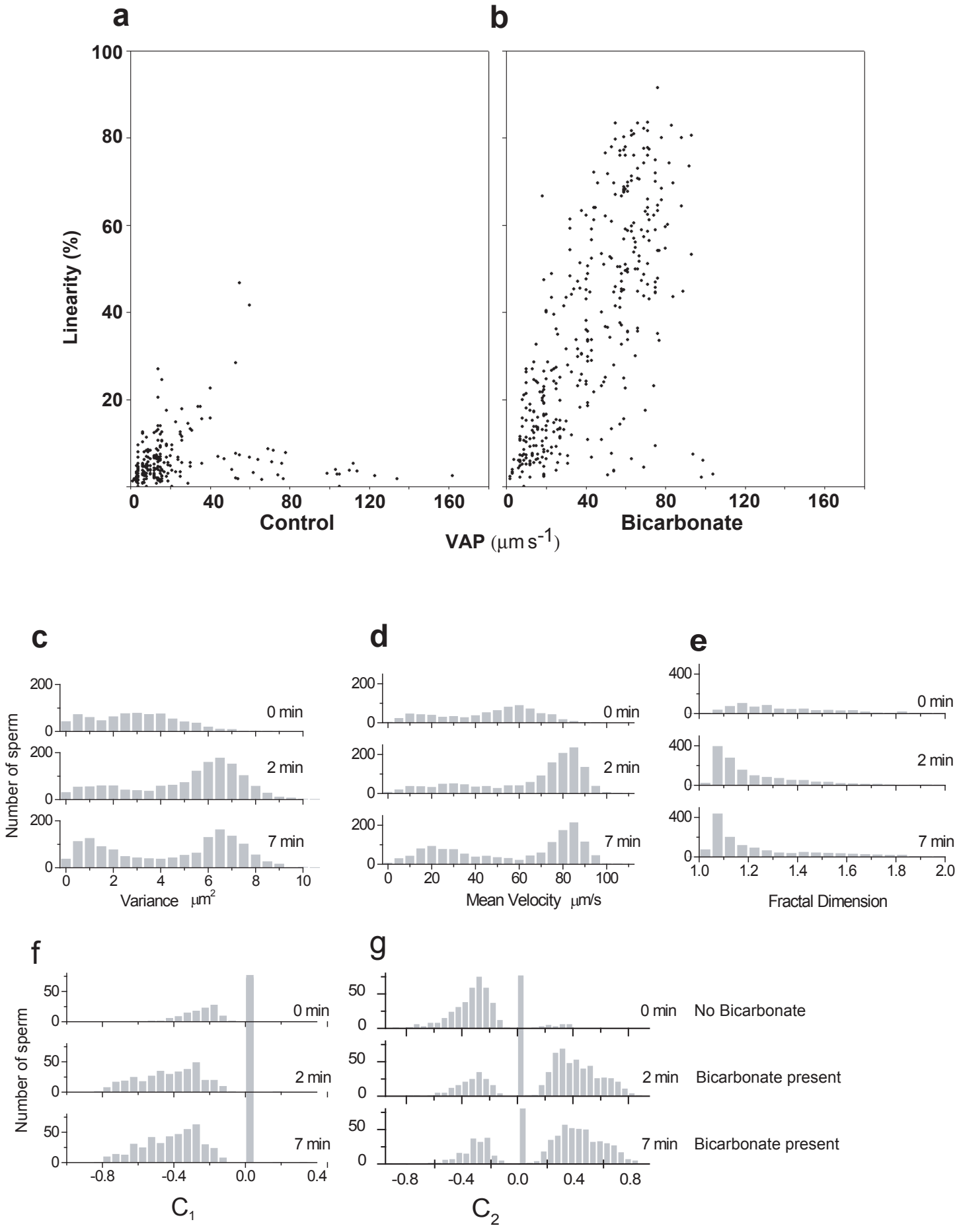
a
b
Linearity (%)
100
80
60
40
20
0
40
80
120
160
Control
Bicarbonate
VAP (μm s-1)
c
d
e
Number of sperm
0 min
2 min
7 min
Variance μm2
Mean Velocity μm/s
Fractal Dimension
f
g
C1
C2
No Bicarbonate
Bicarbonate present
Bicarbonate present

Figure 3

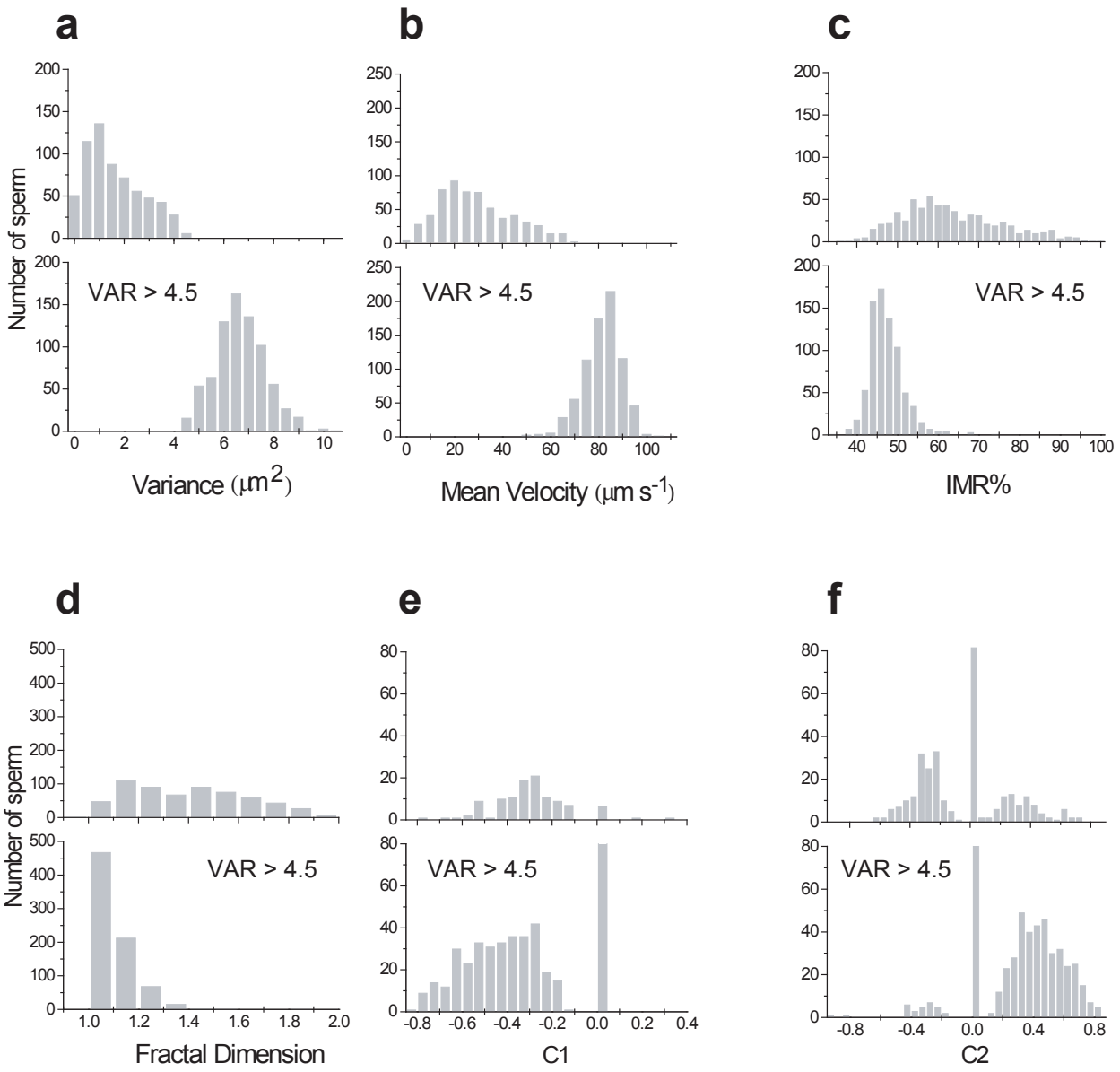
a
b
c
d
e
f
Number of sperm
VAR > 4.5
Variance (μm2)
Mean Velocity (μm s-1)
IMR%
Fractal Dimension
C1
C2

Figure 4