Cyclical changes in sperm volume during *in vitro* incubation under capacitating conditions: a novel boar semen characteristic

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The osmotic reactivity of boar spermatozoa during incubation *in vitro* was studied using a hypo-osmotic swelling test in conjunction with electronic measurement of cell volume. Sperm populations showed fluctuations in both iso-osmotic cell volume and hypo-osmotic volume response that fitted mathematical models for periodicity. Significant differences of frequency and amplitude were observed during sperm incubation under capacitating conditions as compared with those under non-capacitating conditions. In addition, different boars showed specific differences in their fluctuation characteristics under capacitating conditions. During incubation under capacitating conditions, a decrease in osmotic reactivity was observed that correlated with a decrease in motility, while the absolute value of the earliest maximum of the osmotic-induced response correlated with an increase in the proportion of discharged acrosomes. The time course of the cyclical behaviour of osmotic reactivity may be a useful parameter for assessing boar sperm response to capacitating conditions.

Introduction

It is now well established that during capacitation there are major changes in the functional state of the sperm plasma membrane (Yanagimachi, 1994; Töpfer-Petersen *et al.*, 1996). One aspect of these changes is an increasing destabilization which prepares the membrane for the acrosome reaction and fusion with the oocyte (see Harrison, 1996). An important likely consequence of the changes in sperm plasma membrane function is a change in ion transport regulation, resulting in a change in intracellular ionic concentrations. Since spermatozoa appear to behave as perfect osmometers (Drevius, 1972; Du *et al.*, 1994; Gilmore *et al.*, 1996), changes in cell osmole content, as well as the distribution of the osmotically active and inactive cells within a sperm population, might be revealed as volume changes of sperm cells under iso-osmotic environmental conditions. However, information about changes in cell osmole content might be more detectable under hypo-osmotic stress, since this would induce a rapid increase of cell volume due to the high water permeability of the spermatozoa (Gilmore *et al.*, 1996, and references therein). Thus, changes of cell volume measured under both iso-osmotic and hypo-osmotic conditions would be an expression of the dynamics of cell water balance as influenced by capacitation treatment. The objective of this study was the establishment and

preliminary testing of an analytical procedure for investigating cell volume dynamics during capacitation. The approach used was based on protocols developed for the application of electronic volume analysis to hypo-osmotic swelling tests (Petzoldt, 1988; Engel and Petzoldt, 1994; Petzoldt and Engel, 1994).

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals were obtained from Merck AG (Darmstadt).

Animals and sperm collection

Semen samples (whole ejaculates) were collected by the gloved-hand method from nine different mature boars (referred to later as boars A-I) of the German Landrace and Schwäbisch-Hällisch breeds housed at the Institute for Reproductive Medicine. Gel material was removed by filtration through sterile gauze and the spermatozoa were subsequently obtained by centrifugation of the sample for 10 min at 500 g. The pellet was resuspended in residual supernatant seminal plasma to a final concentration of about $1-2 \times 10^9$ cells ml⁻¹. During collection, transfer to the laboratory, and pre-manipulation incubation, the semen was

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Revised manuscript received 11 October 1999.

maintained at a temperature of at least 25°C to avoid cold shock. Samples were used for the experimental studies within 30 min of collection.

Incubation media

As a capacitation medium, the complete bicarbonate-containing Tyrode's-based medium of Harrison *et al.* (1993) was used (bT); this consisted of 96 mmol NaCl l⁻¹, 3.1 mmol KCl l⁻¹, 2 mmol CaCl₂ l⁻¹, 0.4 mmol MgSO₄ l⁻¹, 15 mmol NaHCO₃ l⁻¹, 0.3 mmol NaH₂PO₄ l⁻¹, 5 mmol glucose l⁻¹, 21.6 mmol sodium lactate l⁻¹ (Sigma-Aldrich Ltd, Steinheim), 1 mmol sodium pyruvate l⁻¹, 20 mmol Hepes l⁻¹ (Serva GmbH, Heidelberg), 3 mg BSA ml⁻¹ (final concentration; fraction V; Sigma product A-8022) and 10 µg phenol red ml⁻¹ and the medium was maintained in equilibrium with 5% CO₂. As a control, non-capacitating, medium a Hepes-buffered saline medium (HBS; Harrison *et al.*, 1993) was used; this consisted of 137 mmol NaCl l⁻¹, 10 mmol glucose l⁻¹, 20 mmol Hepes l⁻¹, 2.5 mmol KOH l⁻¹, 1 mg polyvinylalcohol ml⁻¹ (Sigma product P-8136) and 1 mg polyvinylpyrrolidone ml⁻¹ (Sigma product P-7443). Both media had an osmolality of 300 mOsm kg⁻¹, and a pH value of 7.4. Before use, both media were passed through a 0.2 µm filter.

Experimental protocol

Two investigations were made. Experiment 1 tested the general effect of incubation under capacitating conditions on three separate ejaculates from each of six boars and two separate ejaculates from a further three boars. Experiment 2 tested the specific effect of the capacitating medium on three separate ejaculates from each of three of the same nine boars.

In Expt 1, aliquants of the sperm suspensions were incubated in nine volumes of bT medium for 180 min under 5% CO₂; 95% air at 39°C. In Expt 2, sperm samples were incubated in nine volumes of HBS medium for 90 min at 39°C, after which the spermatozoa were collected by centrifugation at 500 g for 10 min (ambient temperature) and resuspended in a portion of the supernatant. One aliquot of the resulting sperm suspension was diluted in nine volumes of bT medium, another in nine volumes of HBS medium, and each was incubated for a further 90 min at 39°C (the bT suspensions were incubated under 5% CO₂).

In both experiments, 4 µl samples were taken from the incubation suspension at 15 min intervals and added to 10 ml of iso-osmotic (300 mOsm kg⁻¹ or hypo-osmotic (150 mOsm kg⁻¹) NaCl solution contained in snap-cap glass vials (ScharfGlas: Landgraf Laborgeräte, Langenhagen) and preincubated at 39°C (Engel and Petzoldt, 1994). The samples were then passed through a CASY 1 cell counter (Scharfe Systems GmbH, Reutlingen), which produced cell volume information on the basis of cell frequency distribution within 1024 electronic 'volume' channels. At each sampling time-point, such distributions were collected from a single iso-osmotic dilution and a single hypo-osmotic dilution using a CASY sample volume setting of 200 µl and a

size scale of 10 µm. Each sampling obtained data from > 8000 cells.

The volume distributions at all sampling points were measured 30 s after dilution in the iso-osmotic NaCl solution, or 3 min after dilution in the hypo-osmotic solution. These different times of exposure were adopted to allow parallel observations of motility as well as adequate rinsing of the CASY capillary tubing between each volumetric measurement. Thus, the incubation time in the measuring solution remained constant for all sampling points. The first sampling was performed immediately after the initial dilution in incubation medium.

At the start and end of each incubation, estimations were made of the sperm motility (at 39°C) and acrosomal integrity. Sperm morphology was examined in samples taken from the native ejaculates shortly after semen collection. The cells were fixed in formol citrate to assess their morphology and acrosomal state (Hancock 1956) and abnormal forms were classified according to Krause (1966).

Initial treatment of data

Analysis of the CASY measurements was carried out on the exported numerical data, that is, the count number in each of the 1024 effective particle size channels.

A mathematical approach (see Appendix) was adopted to 'normalize' each data set and allow proper statistical comparisons: (1) Data corresponding to events of small volume (subcellular debris and 'noise') and to events of large volume (probably due to agglutinated sperm) were discarded. This was done by ignoring data in channel numbers below 225 and above 630; these limits were determined from preliminary experiments and remained unaltered for all data sets in the entire study. After this elimination, the resultant data sets still contained > 7000 events and, in 90% of cases, > 10 000 events. (2) The area under the resultant distribution curve was calculated by integration, after which the cell number in each channel involved in the distribution curve was divided by this area. This procedure (approximately equivalent to dividing the cell number in each channel by the total number of cells in the selected data) enabled the volume distribution to be considered as a probability function of fixed value that was independent of the number of sperm cells involved in the actual measurement. This was an important consideration for valid application of the subsequent statistical tests of differences between curve shapes.

Because the two volume test solutions had different electrical conductivities (different concentrations of electrolyte), it was necessary to apply a correction factor (1.19 ± 0.07) to data recorded under hypo-osmotic conditions. This factor was obtained by comparison of volume measurements of standard latex beads (3.4 µm in diameter; Sigma) in the two solutions. A calibration factor for the CASY could be obtained from the same measurements, which related the modal peak value of the bead volume measurements on the nominal 10 µm scale of the machine to the given diameter of the beads; this factor (*F*) was used to calculate the sperm volume equivalents in physiological terms (see Appendix equations A6 and A7).

Analysis of the volume distribution curves

Ejaculate variation within boars. The Kolmogorov–Smirnov two-sample test (Young, 1977; Müller, 1991) was used to compare volume distributions obtained from replicate ejaculates during incubation under capacitating conditions; pair-wise comparisons were made within sampling time-points (that is, both hypo-osmotic and iso-osmotic distributions from ejaculate 1 from boar A at sampling point 1 were compared with parallel distributions from ejaculate 2 from boar A at sampling point 1; ejaculate 1 was compared with ejaculate 3, and ejaculate 2 with ejaculate 3, also at sampling point 1; the whole procedure was then repeated for sampling point 2, and so on). Three ejaculates were considered from each of five boars (A–E).

Building of average volume distributions. Two sets of ‘average volume distributions’ were obtained for each incubation system (one for iso-osmotic response and one for hypo-osmotic response) to investigate the effects of incubation under capacitating and non-capacitating conditions. These average volume distributions were constructed by averaging for each ‘volume’ channel the normalized frequencies from the trials of three different ejaculates from the same boar. Thus, average distributions were obtained for each of the sampling time points involved in the two incubation systems.

Boar response to capacitating conditions. Differences among boars with respect to the shapes of their average volume distributions during incubation under capacitating conditions were examined by means of the Kolmogorov–Smirnov test. Five boars were compared in a pairwise fashion; at each sampling point, the average hypo-osmotic distribution for boar A was compared with that for boar B, likewise the average iso-osmotic distribution, and so on.

Analysis of derived volumetric parameters

Definition of volumetric parameters. The volumetric parameters used are described (Fig. 1).

The relative volume shift V_r was used as a measure of the effect of hypo-osmotic stress and was defined on the basis of the iso-osmotic and hypo-osmotic volume distributions (Petzoldt and Engel, 1994):

$$V_r = V_{\text{hypo}} / V_{\text{iso}}$$

where V_{hypo} is the modal value of the hypo-osmotic volume distribution, and V_{iso} is the modal value of the iso-osmotic volume distribution. In a situation in which several sperm subpopulations contributed to a distribution, the values pertaining to the dominant osmotically active subpopulation were used. A cell subpopulation was considered as osmotically active if its $V_r > 1$.

Time series analysis. Because the osmotic volume responses V_r and V_{iso} appeared to show periodic fluctuations, their changes with time were investigated using time series analysis, a model of periodic fluctuations (see Appendix). Application of this procedure provided amplitude and

Fig. 1. Diagrammatic definition of volumetric parameters of boar sperm populations. (a) Effect of incubation (from sampling time point n to sampling time point m) on iso-osmotic volume distribution. V_{iso} = modal value of the iso-osmotic volume distribution. ■: Distribution at time-point n ; □: distribution at time-point m . (b) Effect of hypo-osmotic stress at sampling time-point n . V_{hypo} = modal value of the hypo-osmotic volume distribution. ■: Iso-osmotic volume distribution; ○: hypo-osmotic volume distribution. (c) Time-based cyclical fluctuations in osmotic response. V_r , the relative volume shift = $V_{\text{hypo}} / V_{\text{iso}}$; $V_r(n) = V_r(0), V_r(1), \dots, V_r(N)$, that is, the relative volume shift changes at the different sampling time points, where $n = 1 \dots N$, 1 being the first sampling point (at $t = 0$ min) and N = the last sampling point (at the end of the incubation period). The points are the actual individual V_r values for the particular incubation series (‘averaged’ data from ejaculate replicates for a given boar). The curve represents the derived time analysis model from the sequence of V_r values (see Appendix, equations (A3), (A4) and (A5)); T_i = i -th cycle duration of V_r (or V_{iso}) fluctuations (T_1 is the first, etc.) $V_r(\text{max})$ = the earliest maximum of V_r (actual value for a given boar); $\Delta V_r = V_r(\text{max}) - V_r(N)$.

frequency values, the main characteristic parameters of rhythmic biological fluctuations.

Normalization of the model functions. The oscillations of V_r and V_{iso} fitted to the model of periodical fluctuations were normalized using a similar procedure to that described for the volume distribution data, so that the time-based functions of osmotic response could be considered as a probability function of fixed value. In this way, generalized

comparisons of the time response could be carried out using a Kolmogorov–Smirnov test as described above.

Other analytical procedures

The influence of animals and incubation time on conventional sperm parameters and on derived volumetric parameters during incubation under capacitating conditions were examined by analysis of variance using a general linear procedure of the SAS-Program (cross-classification with repetitions, Müller, 1991; SAS, 1989). Relationships among different spermatological parameters (including the volumetric parameters) were tested by Pearson correlation analysis.

Miscellaneous

Calculations were performed by the use of CASY, Excel, and SAS software; SAS-language-based programmes were written to enable data standardization and time series analysis.

Results

Conventional sperm parameters

Descriptive statistics of the conventional sperm parameters for native ejaculates and for sperm samples at the start and end of incubation in capacitation medium for the nine boars involved in the study are listed (Table 1). The motility characteristics of samples during incubation in either capacitating or control medium after preincubation in a non-capacitating medium are also shown (Table 2). The

values indicate that, in general and despite differences between the various samples, the ejaculates from the nine boars were of good quality as judged by conventional parameters.

Individual boar differences in volume response

Within-boar comparisons of volumetric distribution curves were made on groups of three ejaculates from each of five boars (A–E) using the Kolmogorov–Smirnov two-sample test to test whether valid ‘average’ data could be constructed for the individual boars. No significant differences in curve shape were seen between the three ejaculates in such groups in 56 of 65 trials of iso-osmotic distribution tests and in 55 of 65 trials of hypo-osmotic distribution tests (within-triplet trials of data for each of 13 sampling time points for each of five boars = 65).

When inter-boar comparisons were made on the same five boars (using average values for their three ejaculates), two of the five boars (B and D) showed significant differences ($P < 0.05$) from the others in their iso-osmotic cell volume distributions at the start of incubation in the capacitation medium. Three of the five boars (B, D and E) showed significant differences ($P < 0.05$) from each other and from the other two in their hypo-osmotic response at the start of incubation. These same boars also showed significant differences in either iso-osmotic or hypo-osmotic responses during such incubation ($P < 0.05$). Two types of individual cell volume distribution response to hypo-osmotic conditions in boars having similar types of iso-osmotic volume distribution curve (boars A and E) are shown (Fig. 2). The shapes of the hypo-osmotic volume distributions were significantly different ($P < 0.05$).

These analyses validated the construction of ‘average’ volumetric data sets and indicated that the volumetric tests used in the present study were sufficiently sensitive to reveal differences among boars.

Table 1. Descriptive statistics of conventional parameters of the boar sperm samples used in Expt 1

	Mean* $\pm$ SD ($n = 9$)	Range ^a	Number of ejaculates
Percentage of spermatozoa motile in native semen	89.2 $\pm$ 5.7	78–95	24
Percentage of spermatozoa motile at start of incubation ^b	83.9 $\pm$ 5.9	73–90	24
Percentage of spermatozoa motile at end of incubation ^b	59.6 $\pm$ 22.4	15–80	24
Percentage of normal forms in native semen	84.6 $\pm$ 11.3	59–96	24
Decrease in percentage of normal acrosomes during incubation	14.9 $\pm$ 2.0	12–18	19 ^c

*Overall mean $\pm$ SD for all nine boars.

^aValues are means for individual boars.

^bIncubation under capacitating conditions.

^cFive missing values.

Table 2. Descriptive statistics of the boar sperm motility changes observed during Expt 2 (incubation in control and capacitating medium after preincubation in control medium)

	Control medium	Capacitating medium
Percentage of spermatozoa motile at start of second incubation	82.7 $\pm$ 2.5	82.9 $\pm$ 2.6
Percentage of spermatozoa motile at end of second incubation	78.9 $\pm$ 1.0	70.0 $\pm$ 1.6*

Values are mean $\pm$ SD for nine ejaculates.

*Significantly lower than in control medium ($P < 0.05$).

Fig. 2. Two types of boar sperm cell volume distribution response to hypo-osmotic stress. 'Average' distributions (obtained at start of incubation) are presented for two boars that showed similar responses to iso-osmotic dilution. —■—: iso-osmotic dilution; —●—: hypo-osmotic dilution. (a) Boar A: after hypo-osmotic dilution, the spermatozoa showed a discrete population distribution with increased volume. (b) Boar E: after hypo-osmotic dilution, the spermatozoa showed a more broadly dispersed population with increased volume. The Kolmogorov-Smirnov test showed no significant difference between the iso-osmotic volume distributions of these two boars, whereas the hypo-osmotic volume distributions were significantly different ($P < 0.05$). V_r was 2.11 in (a) and 3.14 in (b).

Temporal dynamics of iso-osmotic volume and hypo-osmotic response

Inspection of average volumetric data obtained for nine boars revealed that the modal values V_{iso} and V_{hypo} fluctuated during incubation. An example of the temporal dynamics of V_{iso} during incubation in capacitation medium is shown (Fig. 3). Therefore, time series analysis was applied to V_{iso} and to the derived osmotic response parameter V_r , to test whether their changes could be fitted to a periodic fluctuation model (see Appendix). Comparing the experimental data with the calculated model trigonometric functions (see Appendix) by stepwise multiple regression analysis, the fit was found to be significant ($P < 0.05$ for the null hypothesis that there was no evidence of fit) for all nine boars, both for V_{iso} and for V_r . Therefore, the time course of cellular osmotic reactivity appeared to represent a multimodal but cyclic event and could be approximated by a linear combination of trigonometric functions.

Fig. 3. Typical example of cyclical variation in iso-osmotic volume during incubation of boar spermatozoa under capacitating conditions. The data shown are for boar C (obtained from 'average' distributions). V_{iso} , the modal value of the iso-osmotic volume distribution, has been expressed in femtolitre (fl) equivalents (calculated by comparison with measurements on standard latex beads), and the time-base has been expressed as minutes of incubation to relate the data to more 'physiological' parameters.

Grouping of boars with respect to characteristics of cyclical volumetric response

The numerical values for the volumetric parameters obtained during incubation in capacitating medium were compared among individual boars. Applying equations A4 and A5 of the Appendix, two significantly different ($P < 0.05$) V_r time-course shapes were seen: six of the nine boars (A, B, E, F, G, and H) showed one type, the other three boars (C, D and I) showed another type. The volumetric parameters and associated conventional spermatological parameters for the two groups of boars are listed (Table 3). The groups showed no difference in either mean V_{iso} itself or the periodicity of V_{iso} cyclicity. (The calculation of V_{iso} as a volume equivalent (Table 3; Fig. 3) yielded values in close agreement with other estimates of boar sperm volume, for example those of Du *et al.* (1994)). However, there were significant differences in V_{rmax} and in the cyclical frequency of V_r changes, as well as a significant difference with respect to decrease of motility during the incubation period. The correlation matrix of volumetric versus conventional sperm parameters is shown (Table 4). V_{rmax} correlated significantly with the duration of the first V_r cycle and also with acrosomal deterioration, while the percentage decrease of V_r correlated both with the duration of the first cycle and with the decrease in motility. The duration of the first cycle correlated both with decrease in motility and with acrosomal deterioration.

Media effects

The comparative effects of capacitating (bT) and non-capacitating (HBS) medium on volumetric parameters of three boars are shown (Table 5). Two types of comparison were made for the two media. In the first, the behaviour of spermatozoa during the first 90 min of incubation in bT during Expt 1 was compared with behaviour of spermatozoa (from the same animals) during the initial incubation in HBS during Expt 2. All three boars involved showed significant

Table 3. Comparison of derived volumetric and conventional sperm parameters for two groups of boars with respect to the response to capacitating conditions

	Group 1 (<i>n</i> = 6) ^a	Group 2 (<i>n</i> = 3) ^b
V_r max	2.8 ± 0.2	3.2 ± 0.3*
Relative decrease in V_r during incubation ($100 \times \Delta V_r / V_r$ max)	10.6 ± 6.3	21.9 ± 4.6*
Interval lengths (min) of V_r cycles (see Fig. 1c)		
First cycle (T_1)	41 ± 12	62 ± 3*
Second cycle (T_2)	36.5 ± 9	44 ± 9
Third cycle (T_3) ^c	38.5 ± 11	32 ± 5
V_{iso} (femtolitre equivalents) ^d	22.2 ± 2.5	21.3 ± 2.1
Mean interval length (min) for V_{iso} cycles ^e	47 ± 9	46 ± 7
Motility (%)		
in native semen	90.3 ± 4.8	87.2 ± 7.9
at start of incubation (M_0)	84.2 ± 4.9	83.3 ± 8.8
at end of incubation (M_N)	72.0 ± 7.4	53.8 ± 22.0
Relative decrease in motility during incubation ($100 \times [M_0 - M_N] / M_0$)	15.3 ± 7.0	36.8 ± 20.5*
Morphology		
Percentage of normal forms in original ejaculate	85.1 ± 13.4	83.5 ± 7.6
Decrease in percentage of normal acrosomes during incubation	14.2 ± 1.9	16.6 ± 1.6

Values are means ± SD.

V_r , relative volume shift; V_{iso} , modal value of the iso-osmotic volume distribution.

^aGroup 1: boars A, B, E, F, G and H; ^bGroup 2: boars C, D and I

^cFor boars D and E, values of double the half-cycle duration, that is $2 \times [T_3 (1/2)]$, were used because the full cycle was not completed by the end of incubation.

^dFor each boar, the V_{iso} value was for the same time-point at which its V_r max was detected.

^eAnalysis of V_{iso} fluctuations showed that variations between successive cycle durations were much less than fluctuations for V_r . These values were obtained by linear regression calculation using pooled data (see Appendix, paragraph after equation A5).

*Group 2 significantly different from Group 1 ($P \leq 0.05$).

Table 4. Correlation matrix for conventional and derived volumetric parameters for boar sperm cell suspensions incubated under capacitating conditions (Expt 1)

	dV_r	T_1	M_0	dM	dAcr ^a
V_r max	0.53	0.78*	-0.09	0.51	0.97**
Percentage decrease in V_r (dV_r)	1.0	0.67*	-0.35	0.87**	0.70
Duration of first V_r cycle (T_1)		1.0	-0.3	0.75*	0.86*
Motility at start (M_0)			1.0	-0.55	-0.46
Percentage decrease in motility (dM)				1.0	0.88*

V_r , relative volume shift

^aDecrease in percentage of normal acrosomes

* $P \leq 0.05$, ** $P \leq 0.001$.

differences between the two media with respect to periods of oscillation of V_r (section (a) in Table 5). Moreover, periods of oscillation for V_{iso} could be calculated by applying the equations $t_m = -1.74 + 1.56m$ for incubation in bT and $t_m = -1.35 + 1.85m$ for incubation in HBS (where m is the order number of a V_{iso} maximum or minimum, and t_m is the time point of appearance of this particular extreme; details relating to the derivation of these equations are given in the Appendix). This calculation had to be performed using pooled data from the three boars and could be made only for V_{iso} fluctuations, for which variation in cycle duration between successive cycles and inter-boar differences between the three particular boars were not significant. It

yielded $T = 47$ min in capacitating medium and $T = 56$ min in control medium (significantly different, $P < 0.05$).

The second type of comparison considered behaviour in Expt 2, in which direct comparisons could be made on split samples from the same ejaculate during 90 min incubation either in bT medium or in HBS medium, after preincubation in HBS. All three boars showed a significant overall decline in V_r during incubation in capacitating medium after 90 min preincubation in control medium, as compared with their behaviour during incubation in the control medium (see Table 5, section (b), where the mean ratio of V_r in HBS to V_r in bT is given). A lower (or decreasing) V_r in bT is detected as a significant deviation of the ratio from 1. Although the

Table 5. Comparison between capacitating (bT) and non-capacitating (HBS) media with respect to volumetric parameters of boar spermatozoa

	Boar A	Boar B	Boar C
(a) Frequency solutions to periodic model equation A3 ^a			
HBS	{3, 4, 9}	{3, 5, 6, 7}	{3, 6, 7, 8}
bT	{3, 4, 5, 6, 7, 8}*	{4, 6, 7, 9}*	{3, 4, 5, 6}*
(b) V_r in HBS / V_r in bT ^b	1.22 ± 0.14**	1.44 ± 0.42**	1.31 ± 0.52

^aComparison of the initial 90 min incubation in Hepes-buffered saline medium (HBS) during Expt 2 with first 90 min incubation in bicarbonate-containing Tyrode's-based medium (bT) during Expt 1. From each incubation, a time series of experimental values of V_r (relative volume shift) was obtained for which a set of solutions of equations A3 and thence A4 in the Appendix was obtained; each frequency λ_k fits the equation $\lambda_k = 2\pi n\lambda$, where n is a natural number (2, 3, 4 etc.) and λ is the base frequency. The set of numbers given are the values of n satisfying the fit of V_r cyclic frequency to the periodic model.

^bDirect comparison of V_r in HBS with V_r in bT for each sampling time point during the 90 min of second incubation in Expt 2 (that is, on split samples) (Means ± SD; $n = 7$).

*Significantly different from HBS ($P < 0.05$ by Kolmogorov-Smirnoff comparison of the time series of V_r changes in the two media).

**Significantly different ($P < 0.05$) from 1 (that is, from the null hypothesis that there was no difference between bT and HBS).

deviation overall was not significant for boar C, the deviation became significant at later sampling time points. The decrease in motility during incubation in bT was also significantly greater than it was in HBS (see Table 2).

Response of boar E to capacitating conditions

Although only minor differences were seen among semen samples in terms of conventional sperm parameters, and none were of poor initial quality, one of the boars (E) showed especially rapid changes in volumetric response to capacitating conditions. In boar E, two modes (that is, two subpopulations) could be distinguished within the hypo-osmotic volume distributions after 75 min of incubation for all three ejaculates tested, one with $V_r > 1$, the other with $V_r < 1$; in one of the ejaculates, two peaks became visible immediately after the start of incubation. Only the values of the osmotically active cell fraction ($V_r > 1$) were used for temporal modelling. The V_r time course for both subpopulations is shown (Fig. 4) and, whereas the osmotically inactive subpopulation showed no volume fluctuations, the osmotically active subpopulation continued to exhibit regular cyclicity throughout, although its numbers decreased in proportion to the whole from about 90 to about 65% during the incubation.

Discussion

This study used a CASY cell counter to collect electronic volume data for several thousand individual cells within samples from boar sperm suspensions to monitor changes in cell volume during incubation under capacitating and non-capacitating conditions. All other things being equal, cell volume essentially reflects the water balance between the inside and outside of the cell, which in turn reflects the ionic distribution inside and outside the cell (for review, see Hoffmann and Dunham, 1995). Such distribution depends not only on the controlled passage of ions through plasma membrane ion channels, but also on the activity of energy-dependent ion pumps working against natural ion

Fig. 4. Osmotic response of two visible boar sperm subpopulations within the same cell suspension incubated under capacitating conditions. The data relate to boar E, in which the ejaculates exhibited two modal values in their hypo-osmotic volume distributions. —●—: V_r , the relative volume shift, of the osmotically active cell subpopulation; —○—: V_r of the osmotically inactive cell subpopulation.

concentration differences between the cell interior and the surrounding environment. Many studies have provided evidence that during capacitation, which prepares the sperm for interaction with the egg, major changes in plasma membrane function occur (for reviews, see Yanagimachi, 1994; Harrison, 1996; Töpfer-Petersen *et al.*, 1996). Because sperm cells are 'perfect osmometers' (Drevius, 1972; Du *et al.*, 1994; Gilmore *et al.*, 1996), the present study was undertaken to test the hypothesis that such changes in plasma membrane function are monitored through the resulting cell volume changes. It was further hypothesized that changes in plasma membrane characteristics are also monitored more sensitively via cell volume response to hypo-osmotic stress. Therefore, two tests were applied: measurement of cell volume under iso-osmotic conditions and measurement of cell volume after controlled exposure to hypo-osmotic conditions.

The sperm samples provided a broad distribution of

volume 'events' across the 1024 electronic channels of the CASY cell counter. Events at the lower extreme were undoubtedly due to non-cellular particulate matter, while events at the higher extreme were most likely due to cellular aggregates; therefore, these extremes were excluded from further analysis. A spread of individual cell volumes was detected in between these extremes. Although this spread may have been due in part to the highly asymmetric shape of the spermatozoa, it was clear that it reflected other, more specific, characteristics. Using the Kolmogorov–Smirnov test, distributions could be compared sensitively: replicate ejaculates within boars showed consistent distributions whereas there were significant differences among boars. Therefore, 'average' distributions for the individual boars could be constructed, which made it possible to follow the volume changes of their spermatozoa during incubation.

Despite the spread of detectable volumes for individual cells within sperm samples, the distributions always exhibited a mode that reflected the 'most likely' cell volume value within the sample. Therefore, further analysis was based on these modal values. The modal volume under iso-osmotic conditions (V_{iso}) was used to track cell volume behaviour itself, while a derived function (V_r , the relative increase in modal volume following hypo-osmotic stress) was used to track overall changes in plasma membrane characteristics. At the start of investigations, sperm cell volume changes of a gradual and progressive nature were expected as the sperm plasma membrane underwent the slow overall change of capacitation. However, while there was indeed an overall gradual change in volume characteristics, both parameters revealed unexpectedly cyclical changes. The periodicity and amplitude of these cyclical changes was enhanced by capacitating conditions. Moreover, there was significant inter-boar variation.

Attempts were made to validate these unexpected findings further. Sperm volume distributions might have been affected by the use of K^+ -free NaCl solutions as the osmotic testing media, since Bredderman and Foote (1971a) concluded that the presence of external K^+ was necessary for volume stabilization in bull spermatozoa. However, boar sperm volume distributions obtained in NaCl solutions were found to be statistically indistinguishable (by the Kolmogorov–Smirnov test) from those obtained in HBS (which contained $2.5 \text{ mmol } K^+ \text{ l}^{-1}$, close to the optimum value reported by Bredderman and Foote, 1971a), regardless of whether the solutions were iso- or hypo-osmotic (this finding was valid only for the short exposure times of the volumetric test protocols used).

Spermatozoa adhere to surfaces (Harrison *et al.*, 1978, 1982). Therefore, it seemed possible that, in the present study, cell samples were being measured that were enriched in a moribund sperm subpopulation that did not adhere to the glass surface of the diluting chambers. However, volume distribution curves in hypo-osmotic NaCl were indistinguishable from distributions in HBS, which contains polyvinyl alcohol and polyvinylpyrrolidone as a means of preventing adhesion to surfaces (see Harrison, 1988). Moreover, the numbers of cells contributing to these curves did not differ significantly between NaCl solutions and HBS, whether samples were from spermatozoa suspended in HBS

or in bT, which contains BSA, itself a preventative of adhesion to surfaces (Harrison *et al.*, 1982).

An alternative source of artefactual bias might have been the tendency of live boar spermatozoa to agglutinate, especially in capacitating media (see Harrison, 1997). If increasing numbers of a specific subpopulation of spermatozoa had tended to agglutinate as a consequence of capacitation, the discarding of large events from the analyses would have left data pertaining to an increasingly unrepresentative sperm population. Although there were inevitable variations in the number of cellular events that provided the volume distribution curves for analysis, these variations neither fitted the periodic models derived for the volume changes nor showed as great variation as did the volume changes. Therefore, no evidence was found to suggest that the observations reported here were due to artefacts of experimentation or analysis.

Although there have been numerous estimations of sperm volume and studies of sperm response to osmotic shock, to our knowledge only one other study of time-related sperm volume changes during incubation *in vitro* has been carried out (Bredderman and Foote, 1971a,b). In this work, evidence was provided that increases in bull sperm volume were associated with cell deterioration; sperm volume and long-term survival was maintained through energy-dependent mechanisms, apparently via the activity of the plasma membrane Na–K exchanger. However, Bredderman and Foote (1971a,b) sampled at widely spaced intervals during incubation, and noted no cyclical changes.

Changes in V_{iso} could be taken as indicating changes in osmotic conditions within the cell, that is, changes in intracellular ionic composition. Ionic conditions within the cell are maintained via a balance between flow (usually inward) through ion channels (specific or non-specific) and the energy-dependent activity of ion transport systems (usually outward). An exception to the inward–outward concept is K^+ , which tends to exit the cell via K^+ channels and is recovered through inward transport by the Na–K exchanger. The Na–K balance in cells is particularly important since it regulates the steady-state plasma membrane potential, which in turn can affect the degree to which voltage-dependent ion channels are open or closed. A range of ion channels and exchange mechanisms have been identified in spermatozoa, hence changes in the activity or efficacy of these could affect the osmotic conditions within the cell.

The volume of the cell, as well as reflecting intracellular ionic conditions and membrane ion transport function, also reflects the resistance of the plasma membrane to stretching or deformation. This resistance may be due to membrane potential, to the state (or structural arrangement) of the lipid within the plasma membrane bilayer, or to interaction between the plasma membrane and the underlying actin cytoskeleton ('membrane skeleton'; see Fox and Boyles, 1988). In spermatozoa, the resting membrane potential is relatively small and is not strongly K^+ -dependent (see Rink, 1977); however, Zeng *et al.* (1995) showed that the membrane potential in mouse spermatozoa becomes more negative during capacitation, as a result of which activation of voltage-sensitive ion channels may become more likely (see

Florman *et al.*, 1999). Studies by Harrison *et al.* (1996) have shown that, in boar spermatozoa, the structural arrangement of lipid also changes when the spermatozoa are exposed to capacitating conditions. Knowledge of cytoskeletal activity in spermatozoa is less clear. A range of cytoskeletal elements has been detected in spermatozoa, but membrane skeletal structures have not been identified; indeed, there is much variation with respect to reports of actin distribution within spermatozoa (see Moreno-Fierros *et al.*, 1992). Direct evidence of interaction between an actin cytoskeleton and the plasma membrane is sparse, but measurements by Spungin *et al.* (1995) showed an increase in membrane-associated filamentous actin during capacitation. Castellani-Ceresa *et al.* (1992) have presented evidence that filamentous actin is associated with sub-membranous structures in boar spermatozoa, and that induction of the acrosome reaction with a Ca^{2+} -ionophore induces actin polymerization and attachment of the resultant filamentous actin to detergent-insoluble sperm structures. Moreno-Fiennes *et al.* (1992) presented data from guinea-pig spermatozoa indicating that rates of polymerization and depolymerization of actin varied considerably in different cellular regions. Therefore, cyclical changes in V_{iso} observed in the present study may reflect changes in the membrane skeleton resulting from changes in the state of actin, which either affect membrane mechanical resistance directly or affect ion transport mechanisms (see Mills and Mandel, 1994).

The large increase in modal volume caused by exposure to hypo-osmotic stress (expressed by the derived function V_r) was a sensitive sperm parameter, showing large cyclical changes during incubation and large differences among boars. Although changes in V_r may have been due directly to changes in intracellular osmotic conditions (leading to greater or lesser water influx during hypo-osmotic stress), the magnitude of the changes indicates changes either in ion permeability or in membrane deformability. (As V_{hypo} was measured 3 min after dilution in hypo-osmotic medium, the changes in V_r are unlikely to be due to changes in actual water permeability through the membrane since water permeability in spermatozoa is very rapid (see Gilmore *et al.*, 1996 and references therein). Kulkarni *et al.* (1997) demonstrated that the bull sperm swelling response to hypo-osmotic stress is dependent on the activity of a quinine-sensitive channel through which K^+ may exit so as to reduce and equilibrate intracellular osmolality. The activity of this channel may be, at least in part, under the control of the actin cytoskeleton (see Mills and Mandel, 1994). Noiles *et al.* (1997) found that treating mouse spermatozoa with cytochalasin D not only decreased F-actin concentrations within cells but also greatly affected the parameters of their volume response to hypo-osmotic stress. Therefore, the cyclical changes in V_r detected in the present study, like the changes in V_{iso} , reflect changes in the dynamics of actin rearrangement and microfilament formation.

At present, as with the underlying basis for the volumetric changes themselves, an explanation for the enhanced cyclical behaviour of volume changes in spermatozoa in capacitating medium as compared with non-capacitating medium can only be guessed at. The cyclical changes may stem directly from the rapid changes in plasma membrane lipid

architecture that occur in capacitating medium (Harrison *et al.*, 1996). Alternatively, they may be due to the enhancement of cAMP concentrations in capacitating medium (Harrison, 1997) bringing about modifications in the actin cytoskeleton and, thence, on the quinine-sensitive K^+ channels (see Mills and Mandel, 1994).

The novel discovery of the potential for cyclical changes in sperm behaviour reported here has been based on analyses of the modal volume values in overall sperm populations. A problem when investigating behaviour of sperm populations is the heterogeneity of such populations and the difficulty of associating behavioural changes with particular subpopulations. The question arises as to whether the cyclical changes detected relate to a single subpopulation or are simply the net result of unilateral changes in successive cohorts of sperm undergoing capacitation (Bedford, 1970). Although even dead cells could not be distinguished within distributions obtained from iso-osmotic dilution, they could be largely distinguished and eliminated from analysis within the distributions resulting from hypo-osmotic dilution, because such damaged cells showed little or no swelling response and, therefore, contributed very little to the modal volume V_{hypo} (this can be demonstrated by comparing the volume distributions of normal samples with samples that had been cold-shocked or permeabilized with digitonin; after hypo-osmotic dilution the untreated samples showed $V_{\text{hypo}} \gg V_{\text{iso}}$ whereas the damaged samples showed $V_{\text{hypo}} \leq V_{\text{iso}}$). That the cyclical changes related to live cells was demonstrated by the observation that a diminishing subpopulation continued to express cyclical characteristics while increasing numbers of cells were becoming osmotically inactive. With respect to the possible contribution of succeeding cohorts, the number of cells within the analysed portion of the distribution remained essentially constant. Moreover, in samples from boars other than boar E, there was only occasional evidence of build-up of a subsidiary, osmotically inactive modal peak. Thus, while the counter-hypothesis that successions of subpopulations were responsible for cyclical characteristics within the sperm samples cannot be eliminated entirely, the present observations do not support this. Natural cyclical behaviour of cells is not unknown, and is considered to be brought about by subtle self-stabilizing feedback interactions between parallel control systems.

Petzoldt and Engel (1994) reported that differences in cell volume distribution among human sperm ejaculates could be related to conventional sperm parameters, and Petzoldt *et al.* (1994) suggested that volume measurements after hypo-osmotic stress might be used to study the response of sperm membranes to preservation procedures. The present study has revealed clear differences between individual boars in terms of the amplitude and periodic parameters of the cyclical variation in the osmotic response of their spermatozoa. It was possible to divide the nine boars involved in the present study into two groups on this basis and these groups also differed significantly with respect to the decrease in motility during the *in vitro* incubation period. Although insufficient fertility data were available for any correlation to be made with the volumetric parameters, $V_{r\text{max}}$ was correlated with acrosomal loss during

incubation, the tendency for V_r to decrease was correlated with the decrease in motility, and the duration of the first V_r cycle was correlated both with the decrease in motility and with acrosomal loss. Acrosomal loss and decrease in motility are both indicative of a tendency towards destabilization within the sperm population. Capacitation is considered a destabilizing process (see Harrison, 1996), and so it may be possible to use volumetric parameter values to indicate the destabilizing response of the sperm population to *in vitro* capacitating conditions. Therefore, the boars in group 2 would appear to have produced semen that undergoes capacitational destabilization more rapidly, or to a greater degree, than the semen from boars in group 1; indeed, the high V_r max shown by the spermatozoa from group 2 boars may indicate a less stable disposition. However, at present, the implications of such a diagnosis are not clear. High response to *in vitro* capacitating conditions may be undesirable if the boar semen is to be used for conventional artificial insemination. *In vivo*, spermatozoa are usually inseminated ≥ 12 h before ovulation and must then survive in the female tract until the eggs arrive; a high tendency to destabilization in the environment of the female reproductive tract would lessen the likelihood of sufficient spermatozoa remaining fully active to fertilize the eggs once these are ovulated. Despite this uncertainty, parameters by which spermatozoa response to physiological conditions can be monitored *in vitro* are valuable potential tests of sperm fitness. Therefore, the discovery of the cyclical changes in the osmotic response of boar spermatozoa indicates a hitherto undiscovered physiological property and a potential means by which boar reproductive quality might be judged.

This study was supported by the Karl-Eibl Foundation, Neustadt a.d. Aisch. The authors are most grateful to D. Waberski for much help in preparing the initial text of the manuscript.

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Appendix

Normalization of the volumetric data

The volume distributions were normalized to ensure that volumetric data were independent of the total cell number detected in the particular sampling. First, the original histogram $V(x)$ (where x is the ongoing number of the effective particle size channel) was 'cut' at cursor positions x_1 and x_2 , thus $V(x) = 0$ for $x < x_1$, and $x > x_2$. Next, the area under the distribution curve was calculated between the intervals x_1 and x_2 . Finally, the normalized histogram $V_{\text{norm}}(x)$ was built up from

$$V_{\text{norm}}(x) = \frac{V(x)}{\int_{x_1}^{x_2} V(x) dx} \quad (\text{A1})$$

Integration of equation A1 yields the value 1, that is, for every normalized volume distribution:

$$\int_{x_1}^{x_2} V_{\text{norm}}(x) dx = 1 \quad (\text{A2})$$

The integral of the volume distribution function $V(x)$ (that is, the divisor in (A1)) is a mathematical equivalent to the total number of cells involved in the actual measurement. For the normalized volume distribution function $V_{\text{norm}}(x)$, its integral (A2) is a constant (that is, 1) independent of the number of cells.

Time series analysis

Construction of model functions. The changes $V_r(t)$ and $V_{\text{iso}}(t)$ (that is, the time behaviours of the relative volume shift V_r and iso-osmotic volume V_{iso}) can be calculated on the basis of a periodic fluctuation model described by a finite Fourier-series (Anderson, 1971; Müller 1991):

$$V(t) = \sum_{k=1}^K a_k \cdot \sin(\lambda_k t) + b_k \cdot \cos(\lambda_k t) + M(t) \quad (\text{A3})$$

Given a time series of experimental values V_n of the relative volume shift or of the iso-osmotic cell volume at the sampling points t_n (for example, t_1 at 0 min, t_2 at 15 min), the frequencies λ_k valid for this model can be determined from the periodogram $I(\lambda)$ (Müller 1991):

$$I(\lambda) = \frac{1}{2\pi N} \left| \sum_{n=1}^N x_n e^{-i\lambda n} \right|^2 \quad (\text{A4})$$

The frequencies λ_k granting $I(\lambda_k) \geq I(\lambda)$ for all λ_k can be calculated numerically from equation (A4) by varying λ_k with respect to the scanning theorem (scanning rate $1/15 \text{ min}^{-1}$), K being the number of solutions for λ_k . Given the frequencies λ_k , the coefficients a_k, b_k in equation (A3) can be calculated by regression analysis of experimental data V_n and trigonometric functions $\sin(\lambda_k t)$ and $\cos(\lambda_k t)$. $M(t)$ is a non-periodic trend (usually polynomial or exponential). In the present study, the equations (A3) and (A4) were applied at the integer values $n = 1, 2, \dots, N$ (where N was the last sampling point).

Calculation of cycle durations. The maxima and minima of the model functions were used to determine the cycle durations of the volumetric oscillations. Time points t_m yielding the local extreme values $V(t_m)$ (that is, the maximum or minimum value) of the model function $V(t)$ are given by solutions of the equation:

$$\frac{dV_{\text{iso}}(t)}{dt} = 0 \quad (\text{A5})$$

The solutions t_m were calculated numerically by substituting the model function $V(t)$ according to equation (A3) into equation (A5) and varying t . Given all solutions of this equation over the time interval under consideration, the intervals between successive extreme values t_m and t_{m+1} (half-cycle durations) could be calculated as $T_m(1/2) = t_{m+1} - t_m$. With respect to the relative volume shift V_r , the periods T_m for each individual boar were obtained by the sums of two successive half-cycle durations $T_{m-1}(1/2) + T_m(1/2)$. With respect to the iso-osmotic cell volume in suspensions during different incubation treatments, the mean period of $V(t)$ was determined by linear regression of t_m and by number m of the extreme value: from the regression model $t_m = am + b$, it follows that $T_m(1/2) = t_{m+1} - t_m = a$ and $T_m = 2a$.

Calculation of volume equivalent

The relationship between particle size channel x , effective diameter D (in μm) and estimated volume equivalent V (in femtolitres) is given by equations (A6) and (A7):

$$D = 10 \cdot x / 1024 \quad (\text{A6})$$

$$V = \frac{\pi}{6} \cdot D^3 \cdot F \quad (\text{A7})$$

where F is a calibration factor obtained by measuring V_{iso} for latex spheres of known size (Sigma; see Materials and Methods); $F = V_{\text{iso given}} / V_{\text{iso measured}}$.