In vitro storage of boar spermatozoa increases the demand of adenosine triphosphate for reactivation of motility

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Abstract
Background: Prolonging the shelf-life of liquid-preserved semen without compromising its fertilizing capacity may increase the efficiency of artificial insemination in pigs. Many fertilization-relevant processes are adenosine triphosphate dependent. The impact of semen storage and rewarming to body temperature on the energy status of spermatozoa is as yet unknown.

Objectives: To investigate the energy status of boar spermatozoa during storage and subsequent rewarming and to reveal the potential role of mitochondrial function for reactivation and maintenance of sperm motility.

Materials and methods: Extended semen samples (n = 7 boars) were used. Spermatozoa were challenged by storage at 17°C for 7 days and incubation at 38°C for 180 min. The adenosine triphosphate concentration and energy charge in semen samples and lactate concentration in the extracellular medium were assessed. Viability and mitochondrial activity were determined by flow cytometry, and clustered single-cell analysis of motility parameters was performed.

Results: The energy status was not affected by semen storage (p > 0.05). Rewarming resulted in a net reduction in adenosine triphosphate concentration, which increased with storage time (maximum Day 5: -24.2 ± 10.3%) but was not accompanied by a loss in viability, motility, or mitochondrial activity. Blocking glycolysis with 2-deoxy-D-glucose prevented the re-establishment of motility and mitochondrial activity after rewarming. Mitochondrial activity gradually subsided in virtually all spermatozoa during incubation at 38°C, while adenosine triphosphate and energy charge remained high. Concomitantly, extracellular lactate levels rose, and sperm populations with lower velocity, increased linearity, and low lateral head displacement grew larger. Size changes for major sperm subpopulations correlated with the percentage of viable spermatozoa with high mitochondrial activity (r = 0.44–0.70 for individual subpopulations, p < 0.01).

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Conclusion: Storage of boar spermatozoa increases the demand of adenosine triphosphate for reactivation of spermatozoa towards fast, non-linear, and hyperactivation-like motility patterns upon rewarming. Maintenance of glycolysis seems to be decisive for sperm function after long-term storage in vitro.

Keywords: adenylate energy charge, cluster analysis, mitochondria, motility, semen preservation

1 | INTRODUCTION

Artificial insemination (AI) is the most widely used and efficient assisted reproductive technique in pigs. Currently, AI is practiced in over 90% of sows bred in the major pork-producing countries except for China. Despite an already high reproductive performance, a further increase in AI efficiency is a main goal to accelerate genetic progress and to enhance economic benefit. On the level of semen production, attempts are made to reduce the number of spermatozoa per insemination dose and to prolong the shelf-life of liquid-preserved semen without compromising its fertilizing capacity.

The challenge in liquid semen preservation is to reduce sperm metabolism without comprising the sperm's integrity and function. Boar spermatozoa for AI are commonly preserved in the liquid state at temperatures between +15°C and +18°C. Cooling and storing semen doses are supposed to slow down all intracellular adenosine triphosphate (ATP) consuming and producing processes and concomitantly result in a complete immobilization of boar spermatozoa. Observations were made that ATP levels in stored semen samples declined, whereas after rewarming, the percentage of motile and viable spermatozoa reached a high level. This implies that liquid preservation of boar spermatozoa leads to profound imbalances in their energy status.

Fluctuations in ATP levels are typically interpreted as a mirror of the metabolic status of a cell or cell population. A more robust estimate of the actual energy status is the adenylate energy charge (EC or AEC), which considers not only the available ATP but also the ratio of the available ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) molecules in the adenylate pool. The EC is indicative of whether a majority of microbials, somatic cells, or spermatozoa in a sample can be considered vital. Manipulation of spermatozoa, such as a simple dilution with a semen extender, can already result in a pronounced decrease in the EC. The extent of such imbalances in ATP concentrations and EC during semen storage and whether the energy status can be partially or fully re-stored after warming the semen samples to body temperature have yet to be investigated.

Porcine spermatozoa are able to generate ATP through oxidative phosphorylation in the midpiece and through glycolysis at the fibrous sheath and in the sperm head. Although boar spermatozoa predominantly rely on glycolysis to utilize glucose and produce ATP, mitochondrial function and the related ATP production are indispensable for a range of ATP-dependent cell functions, such as calcium homeostasis, motility, or redox signaling, which in turn are involved in sperm capacitation, hyperactivation, or volume regulation. Sparks of elevated mitochondrial ATP production are especially required during specific fertility-relevant events, for example, capacitation and acrosome reaction.

The mixture of available extracellular substrates determines which pathway for ATP production is preferably used. For example, exposing boar semen to extenders with high glucose concentrations shifts the metabolism of boar spermatozoa predominantly toward glycolysis. In addition, natural energy substrates in seminal plasma, such as fructose, citrate, inositol, lactic acid (c.f. Table S1) might influence energy metabolism at storage time, even though their concentration is low and is further reduced by semen dilution. It is thus pivotal to gain knowledge on the extent to which deficits in mitochondrial function, ATP levels or EC are primed by liquid semen preservation and how they contribute to impaired sperm function under storage conditions.

The aim of our study was to determine the energy status of boar spermatozoa during storage and subsequent rewarming, and to reveal the potential role of mitochondrial function for the reactivation and maintenance of sperm motility. To this end, the mitochondrial transmembrane potential was monitored in viable spermatozoa, and cluster analysis was used to monitor changes in the movement patterns in subsets of motile cells. The implications of our findings for progress in semen preservation concepts are discussed.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Boar semen samples (n = 7) were diluted to 20 × 10^6 spermatozoa/ml in Beltsville Thawing Solution (BTS). Samples were evaluated on the day of semen collection, that is, Day 0, after 90 min of chilling at room temperature (21.3 ± 0.5°C, sample temperature: 23.7 ± 0.2°C), and after 1, 3, 5, and 7 days of storage at 17°C. ATP concentration, EC, and the amount of viable, acrosome-intact spermatozoa were assessed at storage temperature, that is, samples were kept at 17°C and immediately processed. Further measurements were performed after incubation at 38°C for 15, 30, 60, 120, and 180 min, which mimics the temperature and time period of the sperm’s journey through the porcine uterus to the female sperm reservoir in the lower oviductal isthmus. The percentage of viable spermatozoa with high mitochondrial transmembrane potential and motility parameters was only determined in
samples incubated at 38°C. Any procedures leading to sperm selection, for example, density gradient centrifugation, were not applied in the experiments.

In additional experiments, the putative accumulation of l-lactate in the extracellular medium over storage time and during incubation at 38°C was assessed. Furthermore, the effect of inhibiting glycolysis on sperm function was tested. To this end, the d-glucose in BTS was fully replaced with 2-deoxy-d-glucose while maintaining identical pH and osmolality.

2.2 Chemicals and reagents

Propidium iodide (PI) was purchased from Sigma-Aldrich Productions GmbH (Steinheim, Germany), and peanut agglutinin conjugated to fluorescein-isothiocyanate (PNA-FITC) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyethylbenzimidazolylcarbocyanide iodide (JC-1) were purchased from Enzo Life Sciences GmbH (Lörrach, Germany). Hoechst 33342 (H342) was purchased from Life Technologies GmbH (Darmstadt, Germany).

2.3 Animals, semen collection, and dilution

Semen was obtained from seven mature, clinically healthy boars (Pietrain, German Large White, and crossbred animals) housed at the Unit for Reproductive Medicine, University of Veterinary Medicine Hannover, Hannover, Germany. Boars were kept and handled in accordance with the European Commission Directive for Pig Welfare (2008/120/EC). Ejaculates were collected, and the raw semen was processed for assessment of viability and acrosome integrity. The system was operated by SpermVision software (version 3.7, Minitüb GmbH). For each sample, 10 successive fields in the central axis of a chamber were recorded at 60 Hz (30 frames per 0.5 s).

The following parameters were considered: total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s), straight-line velocity (VSL; µm/s), linearity (LIN = VSL/VCL), wobble (WOB = VAP/VCL), amplitude of lateral head displacement (ALH; µm), and beat cross-frequency (BCF; Hz). Spermatozoa were defined by a head area between 23 and 120 µm². A spermatozoon was considered to be motile when its average head orientation change (AOC) was higher than 2.5° and considered to be progressively motile when the distance moved from A to B in a straight line (DSL) exceeded 4.5 µm.

2.5 Flow cytometer

All measurements were performed on a DAKO “Galaxy” flow cytometer (Dako Deutschland GmbH, Hamburg, Germany) controlled by FlowMax software (version 2.8, Sysmex Partec GmbH, Münster, Germany). It was equipped with an argon ion laser (488 nm, 20 mW) and an HBO mercury short arc lamp for excitation of the dyes. The HBO mercury short arc excitation spectrum was restricted with filters to wavelengths between 270 and 405 nm (main peak: 365 nm). Filters for detecting emitted fluorescent light (FL) were as follows: FL-1 (537.5/22.5 nm) for green, FL-2 (590/25 nm) for orange, FL-3 (630 nm longpass) for red, and FL-4 (465 nm bandpass) for blue FL. A 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline solution (HBS; 137 mM NaCl, 20 mM HEPES, 10 mM glucose, 2.5 mM KOH, 1 mg/ml BSA, pH 7.40 ± 0.05, 300 ± 5 mOsmol/kg) was used as sheath buffer.

2.6 Viability and acrosome integrity

In parallel to motility assessments, another aliquot of 2 ml diluted semen was incubated in a water bath at 38°C. After 10, 25, 55, 115, and 175 min, an aliquot of diluted semen (5 µl) was mixed with 980 µl pre-warmed HBS (38°C), 5 µl PI stock solution (1 mg/ml), 5 µl PNA-FITC stock solution (600 µg/ml), and 5 µl H342 stock solution (150 µg/ml) and incubated for an additional 5 min at 38°C before assessment on the flow cytometer. To assess stored samples, an aliquot of semen was directly transferred to pre-warmed HBS, stained, and analyzed after a 5-min incubation period at 38°C. Data from 10,000 events were collected for each sample. An overlap of the emission spectra from PI and PNA-FITC was compensated for post-acquisition. Data were evaluated as described elsewhere,23 and the percentage of viable, acrosome-intact (PI and PNA-FITC negative) spermatozoa was determined.
2.7 Mitochondrial transmembrane potential in viable spermatozoa

The principle of this measurement is based on the properties of the dye JC-1. Depending on the electrochemical gradient across the mitochondrial membrane, JC-1 reversibly transforms from a green fluorescent monomer (low transmembrane potential; emission peak approximately 529 nm) to an aggregated form (J-aggregates) emitting orange to red fluorescence (high transmembrane potential; emission peak approximately 590 nm). Combined staining with PI allowed differentiation of viable and dead spermatozoa.

Five subsamples of semen from each boar (1 ml each) were incubated at 38°C in a water bath, and one subsample was assessed after 15, 30, 60, 120, or 180 min of incubation. Fifteen minutes before assessment, 1 μl JC-1 (1.53 mM in dimethyl sulfoxide), 10 μl H342 (150 μg/ml in aqua dest.), and 20 μl PI (1 mg/ml in aqua dest.) were added to a subsample. For assessment on the flow cytometer, 5 μl (150 μg/ml in aqua dest.) and 20 μl PI (1 mg/ml in aqua dest.) were added to the stained sample. For assessment, 1 μl JC-1 (1.53 mM in dimethyl sulfoxide), 10 μl H342 (150 μg/ml in aqua dest.), and 20 μl PI (1 mg/ml in aqua dest.) were added to a subsample. For assessment on the flow cytometer, 5 μl of the stained sample was transferred to 995 μl of pre-warmed HBS (38°C). In each measurement, 10,000 events were collected.

Analogous to the gating strategy for viability and acrosome integrity, spermatozoa were defined as H342-positive events in the size range of a single spermatozoon. Analysis was further restricted to viable, that is, PI-negative spermatozoa based on the signal collected in channel FL-3 (red). The percentage of viable spermatozoa with high mitochondrial membrane potential (hMMP) was estimated by plotting signals from channel FL-1 (green; JC-1 monomers; low mitochondrial transmembrane potential) versus channel FL-2 (orange; J-aggregates; high mitochondrial transmembrane potential). The threshold between high and low mitochondrial membrane potential was defined for samples after a 15-min incubation period (Day 0) and kept constant throughout the experiment. The average fluorescence intensity for J-aggregates in all viable spermatozoa was estimated from data in the FL-2 channel. Compensations for spectral overlap of PI and JC-1 signals were set at the beginning of the experiment.

2.8 Inhibition of glycolysis in the presence and absence of seminal plasma

In an additional experiment, the effect of inhibiting glycolysis on sperm function was evaluated. To this end, the predominant component of BTS, that is, D-glucose, was fully replaced with 2-deoxy-D-glucose (product no. D8375, Sigma–Aldrich Production GmbH) while maintaining identical pH and osmolality. 2-Deoxy-D-glucose inhibits glycolysis because of the formation and intracellular accumulation of 2-deoxy-D-glucose-6-phosphate, thus inhibiting the function of hexokinase and glucose-6-phosphate isomerase.24 Seminal plasma contains glucose and other energy substrates,25,26 which may interfere with the experiment and therefore was removed. For this, aliquots of fresh semen samples (15 ml) were centrifuged (750 g, 10 min, room temperature). The sperm pellet was diluted to 20 × 10⁶ cells/ml in either variant of the BTS medium (seminal plasma-free samples). In addition, diluted samples with both BTS variants were supplemented with 10% (v/v) autologous seminal plasma (seminal plasma containing samples). Small aliquots (1.5 ml diluted semen in 1.5 ml Eppendorf cups) were kept for 90 min at room temperature and subsequently stored for 24 h at 17°C before being rewarmed to 38°C and assayed for motility, viability, and mitochondrial membrane potential.

2.9 ATP and energy charge assay

2.9.1 Sample preparation and nucleotide extraction

ATP content and EC in spermatozoa were determined by recently described methods.10 The protocols were based on modifications of previously published assays.27,28 In short, 100 μl of a diluted boar semen sample (stored at 17°C or incubated for 15, 30, 60, 120, and 180 min at 38°C) was incubated with 1 μl phosphatase inhibitor cocktail (P5726, Sigma–Aldrich Production GmbH) on ice for 30 min. After inhibitor treatment, samples were stored at -20°C for later ATP and EC assessment.

Extraction of sperm adenylate nucleotides (ATP, ADP, AMP) was achieved by treating the frozen samples with 900 μl pre-heated boiling buffer solution (50 mM Tricine, 10 mM MgSO₄, and 2 mM EDTA, pH 7.80) and boiling for 10 min at 95°C. Subsequently, the samples were chilled on ice for 10 min and centrifuged at 5000 g for 30 min at 4°C. The supernatant was used to determine ATP, ADP, and AMP.

2.9.2 ATP assay

ATP was determined using a luciferase reaction kit in accordance with the manufacturer’s protocol (FL-AA kit, Sigma–Aldrich Productions GmbH), Bioluminescence was measured with a Tecan GENios Pro plate reader (Tecan Group Ltd.) controlled by ‘Magellan’ software (Version V5.03, Tecan Group Ltd.). The ATP content of the spermatozoa was calculated from an ATP standard curve.

2.9.3 Energy charge assay

Three aliquots (100 μl each) of the samples to be analyzed for nucleotides were each incubated with 25 μl of one of three different buffers (Buffers A, B, and C). Buffer A contained 75 mM Tricine, 5 mM MgCl₂, and 0.0125 mM KCl (pH 7.5) for determining ATP. Buffer B contained 0.1 mM phosphoenolpyruvate (P7002, Sigma–Aldrich Productions GmbH) and 0.08 μg/μl pyruvate kinase in addition to Buffer A. Buffer B was used to convert ADP to ATP. Tubes containing Buffers A and B were incubated at 30°C for 30 min. Buffer C contained, in addition to Buffer B, 0.1 μg/μl adenylic (myo) kinase (M3003, Sigma–Aldrich Productions GmbH). Adenylic (myo) kinase converts AMP to ADP. Samples incubated with Buffer C were used for the combined assessment of ATP, ADP, and AMP. Tubes containing Buffer C were incubated at 30°C for 90 min. Pre-heated boiling buffer was added
to all tubes at 95°C for 3 min to stop the enzymatic reactions. Samples were then chilled on ice and processed for assessment of the total amount of ATP. The AEC was calculated as described by Ball and Atkinson:

\[
\text{AEC} = \frac{[\text{ATP}] + 0.5 [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \quad (1)
\]

### 2.9.4 L-Lactate assessment

Semen samples were centrifuged (3360 g, 10 min at room temperature or 38°C) to obtain a sperm-free supernatant. The supernatant (0.1 ml) was mixed with 0.5 ml 0.36 N HClO₄ for deproteinization. After 10 min of incubation, the samples were centrifuged (10,000 g, 10 min at room temperature). The supernatant (50 µl) was mixed at room temperature with assay buffer (404 mM Tris-(hydroxymethyl)-aminomethane, 125 mM L-glutamic acid [stock: 0.771M in 74% (v/v) 1 N NaOH in aqua dest.], 24 mM NaOH, 4.1 mg glutamate-pyruvate transaminase [GPT, product no. 10737127001, Roche Diagnostics GmbH, Mannheim, Germany], and 404.9 mg nicotinamide adenine dinucleotide [NAD, product no. 1012793001, Roche Diagnostics GmbH] in 100 ml aqua dest.). After 1 min, extinction at 334 nm was read in a photometer for background assessment. Subsequently, 5 µl L-lactate dehydrogenase solution (stock: 100 mg in 10 ml aqua dest.; product no. 10107085001, Roche Diagnostics GmbH) was added to each sample, and extinction was read after 30, 40, and 50 min to confirm stable readings of plateau values. Samples with known L-lactate concentrations served as calibration standards.

### 2.9.5 Statistical analysis

Data were analyzed using Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA, USA) and Statistical Analysis Software (SAS Institute, Inc., version 9.3, Cary, NC, USA). Data from all parameters were assumed to be normally distributed. Data after incubating the samples for a period of 15–180 min at 38°C were analyzed with a two-factorial analysis of variance (ANOVA) for repeated measurements to estimate the influence of storage time at 17°C and incubation time at 38°C (PROC GLM) or glucose variant and incubation time at 38°C (PROC GLM). Pairwise comparisons were performed with Student’s t-test for paired samples (PROC UNIVARIATE). Dunnett’s two-tailed test was applied to evaluate whether the drop in ATP content upon rewarming of samples from 17°C to 38°C differed between stored samples and the samples at the day of semen collection.

Cluster analysis was performed as described previously. Data of motility descriptors for every single, motile spermatozoon after incubation periods of 15, 30, and 180 min at 38°C from all storage time points (Day 0, Day 1, Day 3, Day 5, and Day 7) were combined in one data set. Eight motility descriptors were available from CASA analysis (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF). Parameters were correlated after testing for normality (PROC UNIVARIATE and PROC CORR). Whenever two or more parameters correlated ≥0.90, only one was chosen to enter the final clustering procedure to reduce the number of variables. Chosen variables were standardized to a mean of 0 and a standard deviation of 1 to avoid any bias in the clustering procedure because of different parameter scales.

Hierarchical clustering was performed using squared Euclidian distance as a distance measurement and the “centroid” algorithm for cluster fusion (PROC CLUSTER). The choice of a suitable solution from the clustering procedure was guided by the cubic clustering criterion, pseudo-F statistics, and pseudo-\(t^2\) values. The aim was to obtain a solution that explained as much of the variance in the data set and contained as few as possible major clusters. To test whether the distribution of spermatozoa between the revealed cluster differed between the incubation periods (pooled data set) or between days at a given incubation time, a chi-square test for homogeneity was performed (PROC FREQ). It was tested whether the storage period had a confounding effect on the results from the pooled data set using the Cochran–Mantel–Haenszel test (PROC FREQ). Cramer’s V (ranging from 0 to 1) was used to measure the effect size that storage or incubation had on the distribution of spermatozoa to the different clusters, that is, the size of sperm subpopulations with distinct motility patterns. For interpretation, the following guidelines were followed as suggested by Cohen: \(V < 0.10\) = no effect, \(0.10 < V \leq 0.30\) = slight effect, \(0.30 < V \leq 0.50\) = moderate effect, and \(V > 0.50\) = strong effect. Spearman’s correlation coefficients were calculated for selected parameters (PROC CORR). The significance level was set at \(p < 0.05\).

### 3 RESULTS

#### 3.1 Sperm viability, ATP levels, and energy charge in stored semen samples

The viable, acrosome-intact sperm population at Day 5 (90.7 ± 2.4%) and Day 7 (89.0 ± 3.1%) was marginally, but significantly, lower than that at Day 0 (93.7 ± 0.5%) or Day 1 (92.1 ± 2.1%; \(p < 0.05\)). This demonstrates that sperm viability was maintained at a high level throughout storage time.

ATP levels and EC were higher at the day of semen collection, that is, at 23.7 ± 0.2°C (Day 0; ATP: 438.5 ± 21.4 pmol/10⁵ spermatozoa; EC: 0.78 ± 0.05) than at Day 1 (384.52 ± 14.0 pmol/10⁵ spermatozoa; EC: 0.70 ± 0.07; \(p < 0.05\)) when spermatozoa had been fully cooled to 17°C. ATP concentrations and EC at Days 3 and 5 were again similar to Day 0 (Figure 1A). The data indicate that the energy status of spermatozoa is balanced during semen collection.

#### 3.2 ATP levels of stored semen samples after rewarming to 38°C

The ATP level of semen samples at 17°C was higher than that of the same samples incubated at 38°C (\(p < 0.05\)). Only on the day of dilution...
FIGURE 1  Adenosine triphosphate (ATP) levels and energy charge (EC) during storage of semen at 17°C and after rewarming to 38°C. (A) ATP concentration of semen samples (n = 7) during storage at 17°C and after 15 min of incubation at 38°C. Different lowercase letters (a–c) within a column indicate significant differences (p < 0.05) between days of storage. (B) Drop in ATP levels after a 15-min incubation period at 38°C expressed as percent deviation from starting values (i.e., ATP concentration at 17°C). A hash indicates a significant difference compared with the day of semen collection (Day 0; p < 0.05). All values are means and standard deviations.

Table 1

| Storage | ATP (pmol/10^6 sperm) | EC | ATP (pmol/10^6 sperm) | EC |
|---------|-----------------------|----|-----------------------|----|
| Day 0   | 438.5 ± 21.4^ab       | 0.78 ± 0.05^a | 421.3 ± 54.6^a       | 0.74 ± 0.05^a |
| Day 1   | 384.5 ± 14.0^c        | 0.70 ± 0.07^b | 326.5 ± 12.6^c       | 0.67 ± 0.03^b |
| Day 3   | 484.4 ± 44.0^b        | 0.73 ± 0.12^a,b | 387.9 ± 43.6^b       | 0.66 ± 0.09^a,b,c |
| Day 5   | 451.8 ± 56.8^a        | 0.77 ± 0.08^a,b | 338.9 ± 33.1^a,b,c   | 0.72 ± 0.06^a,b |
| Day 7   | 397.9 ± 67.1^b,c      | 0.71 ± 0.07^a | 325.1 ± 60.5^a       | 0.62 ± 0.08^a |

*samples on day 0 were assessed 90 minutes after dilution and subsequent holding at room temperature (21.3 ± 0.5°C, sample temperature: 23.7 ± 2.0°C.

The difference (delta) in ATP levels between stored samples and those incubated for 15 min at 38°C increased with storage time (p < 0.05). At Day 0, the difference was lowest (32.3 ± 49.3 pmol), while at Day 5, it reached its maximum (112.9 ± 57.5 pmol). For a standardized comparison, the relative decrease in the ATP concentration for a sample at 17°C and after a 15-min incubation period at 38°C was calculated (Figure 1B). Significantly more of the available ATP was converted during rewarming at 38°C when samples had been stored for 3, 5, or 7 days than at the day of semen collection (Day 0; p < 0.05). These results suggest that ongoing semen storage increases the expense of ATP for reactivating sperm’s metabolism.

3.3 Sperm motility, mitochondrial membrane potential, ATP levels, and energy charge in stored semen samples during incubation at 38°C

The absolute values for PM, the percentage viable spermatozoa with high mitochondrial transmembrane potential, ATP concentrations, and EC are illustrated in Figure 2, while the relative changes are displayed in Figure S1. Values after a 15-min incubation period were chosen as reference points. A comparison of values for individual incubation times between days of storage is given in Tables S2–S4.

At Days 0 and 1, the percentage of progressive motile spermatozoa remained stable during a 180-min incubation period at 38°C (Figures 2A and S1A,B). A gradual decrease in PM was noted at Days 3, 5, and 7, respectively, when samples were incubated for 60 min or more (Figure 2A and S1C–E; p < 0.05). The average percentage of viable, acrosome-intact spermatozoa never dropped below 70% throughout the experiment (Table S2).

In contrast to PM, the percentage of viable spermatozoa with hMMP steeply declined with increasing incubation time irrespective of storage time (Figure 2B). After a 60-min incubation period, fewer than 40% of the spermatozoa were viable with a high mitochondrial transmembrane potential (max: 38.9% at Day 1; min: 13.5% at Day 7). After a 180-min incubation period, viable spermatozoa with a high mitochondrial transmembrane potential were virtually absent in the samples (Figure 2B, Table S3). The absence of this sperm population was not because of an increased permeability of the spermatozoa for PI but because of a consistently decreasing fluorescence intensity of the J-aggregates (Figure S2).

The decline in the amount of viable spermatozoa with hMMP during incubation at 38°C was barely reflected in changes in the ATP concentration. At all storage days, the ATP concentration remained at a stable level for at least 60 min during incubation at 38°C (Figure 2C). A step-wise, significant decrease in the ATP concentration was detected at Days 0 and 5 and started between 60 and 120 min of incubation. On all other days, the average ATP concentration gradually decreased but remained statistically comparable to values after a 15-min incubation period (Figure 2C).

Changes in EC showed the same pattern as for ATP concentration (Figure 2D). In addition to significant gradual changes in ATP content at Days 0 and 5, the EC indicated a significant stepwise decrease in ATP and/or ADP during incubation at Day 1 (Figure 2D). Only at Day 0, when samples had still not been cooled below 20°C prior to rewarming...
FIGURE 2 Adenosine triphosphate (ATP) levels, energy charge, sperm motility, and mitochondrial function during storage of semen at 17°C and subsequent incubation at 38°C. (A) Percentage of progressive motile spermatozoa, (B) amount of viable spermatozoa with a high mitochondrial transmembrane potential (hMMP), (C) ATP concentration, and (D) energy charge of boar semen samples following incubation at 38°C for up to 180 min after different days of storage (Day 0 = day of semen collection). Values are expressed as the means and standard deviations (n = 7). Different letters (a–d) on a given day indicate significant differences between incubation times (p < 0.05). The relative change for each parameter on a given day of storage is depicted in Figure S1.

At 38°C, there was a steep decline in the percentage of viable spermatozoa with high mitochondria accompanied by a gradual decline in the EC and a delayed drop in ATP concentration. In addition to the aforementioned parameters, the AMP to ATP ratio was calculated (Figure S3) because the AMP/ATP ratio serves as an additional indicator of energy stress. The AMP/ATP ratio was significantly increased after 120 and 180 min of incubation on all days of the experiment (Figure S3). There were significant correlations between ATP content at 17°C and TM after incubation at 38°C for 30 min (r = 0.35, p < 0.05) and EC at 17°C and STR of the motility trajectory after a 180-min incubation period at 38°C (r = 0.55, p < 0.001). No further correlations between the ATP content or EC in samples (n = 35) stored at 17°C with either motility parameters or the percentage of viable spermatozoa with high mitochondrial transmembrane potential during incubation were found.

3.4 Sperm motility, viability, and hMMP after inhibition of glycolysis in the absence and presence of seminal plasma

In seminal plasma-free samples, inhibition of glycolysis by replacement of D-glucose with 2-deoxy-D-glucose in the semen extender BTS prevented the reactivation of motility and the establishment of a hMMP in viable spermatozoa during a 30-min incubation period at 38°C (p < 0.001). Viability and acrosome integrity were not affected by inhibited glycolysis (Figure 3A; p > 0.05). In the presence of seminal plasma, the inhibition of glycolysis reduced the activating effect of incubation on motility (p < 0.05) and hMMP (p < 0.01; Figure 3B). These data suggest that firstly, reactivation of sperm motility after storage in a glucose-rich extender relies on glycolysis, and secondly, that seminal plasma contributes to sperm reactivation by providing energy substrates for mitochondrial ATP production.

3.5 L-Lactate concentration in the extracellular medium in stored semen samples during incubation at 38°C

The L-lactate concentrations in the extracellular medium, that is, semen extender plus seminal plasma, did not differ between Days 1 and 7 (p > 0.05; Figure 4A). The L-lactate concentration in the extracellular medium of Day 1 semen increased after 120 and 180 min of incubation at 38°C (p < 0.05). Data are presented as absolute concentrations in mmol/L (Figure 4B) and as normalized values...
Impact of D-glucose (G) and 2-deoxy-D-glucose (2-DG) on re-establishing motility after boar semen storage at 17°C. Semen samples were stored as 1.5 ml aliquots either in Beltsville Thawing Solution (BTS) with 205 mM glucose or in a modified BTS where glucose was replaced with an equal amount of 2-DG to inhibit glycolysis. After being stored for 24 h at 17°C, samples were incubated for up to 30 min at 38°C prior to analysis for total motility, the percentage of viable spermatozoa with intact acrosomes (propidium iodide and peanut agglutinin [PNA] negative), and the percentage of viable spermatozoa with high mitochondrial membrane potential (hMMP) as assessed with propidium iodide and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). (A) Parameters for samples preserved in the absence of seminal plasma (n = 3 boars). (B) Parameters for samples preserved in the presence of seminal plasma (n = 3 boars). Different letters indicate a significant difference between samples with G and 2-DG analyzed in combined data sets for a 15- and 30-min incubation period (a, b: p < 0.05; c, d: p < 0.01; e, f: p < 0.001; n = 6 samples).

L-Lactate concentrations in extender medium after different storage times at 17°C or incubation at 38°C. (A) Basic L-lactate concentration in the extracellular medium, that is, extender plus seminal plasma, did not differ between Day 1 (24 h) and Day 7 (168 h) for samples stored at 17°C (n = 6 boars; p > 0.05). (B) The L-lactate concentration in the extracellular medium increased after 120 and 180 min of incubation at 38°C. Data were collected from samples previously stored for 24 h at 17°C (n = 6 boars). Values for 0 min incubation were taken from samples at 17°C. (C) Normalized L-lactate concentration for the samples depicted in (B). Different letters (a–c) indicate significant differences (p < 0.05). This indicates that thermic stress induces excess L-lactate accumulation, which is no longer counterbalanced by the mitochondria and thus leads to externalization of L-lactate.

Changes in sperm motility patterns in stored semen samples and during subsequent incubation at 38°C

The solution that was chosen from the clustering procedure consisted of eight major clusters and explained 67.6% of the variation in the data set (Figure 5A). The eight major clusters accounted for 94.9% of the single spermatozoa (50,422 cells), while the remaining 5.1% of the spermatozoa (2716 cells) were distributed in 61 further clusters. Sperm in the major clusters were characterized by significantly different motility characteristics (p < 0.05; Figure 5A, Table S5), which were reflected in the corresponding sperm trajectories (Figure 5B). The chi-square test indicated a significant relation between storage time and the distribution of spermatozoa to the different clusters (p < 0.0001). However, the linear effect of storage time on sperm distribution was low, as indicated by Cramer’s V after 15 min ($\chi^2$ [32; N = 20,016] = 926.7, p < 0.0001, V = 0.11), 30 min ($\chi^2$ [32;
Figure 5 Cluster analysis of sperm motility characteristics. (A) The analysis was based on 53138 sperm tracks from 105 samples recorded after 15, 30, and 180 min incubation times at Days 0, 1, 3, 5, and 7 of semen storage. Single sperm data from different days of storage were combined. Only clusters containing more than 5% spermatozoa at least one of the incubation times are listed. Motility descriptors (mean ± SD) are given for each cluster as well as the percentage of spermatozoa assigned to each cluster. (B) Representative overlay images from computer-assisted semen analysis (CASA) to depict the main sperm subpopulations (clusters) with respect to movement patterns. Each overlay consists of thirty consecutive images taken at 60 Hz. The pink line indicates the curvilinear distance. (C) Distribution of spermatozoa to different clusters based on motility parameters after 15, 30, and 180-min incubation time at 38°C. Sperm distribution in the different clusters changed significantly depending on incubation time (p < 0.05)

\[ N = 17,330 = 333.7, p < 0.0001, V = 0.07 \], or 180 min incubation \( \chi^2 \) [32; N = 15,792] = 560.6, p < 0.0001, V = 0.09). Therefore, no further comparisons between storage times are described.

The distribution of spermatozoa in the different clusters changed with incubation time at 38°C \( \chi^2 \) [16; N = 53,138] = 5653.8, p < 0.0001: Figure 5C). The cell chi-square indicated that deviations in the expected sperm distribution for Clusters 3 and 5 after a 15-min incubation period and for Clusters 4 and 5 after a 180-min incubation period had the strongest influence on the results of the chi-square test. Thus, changes in the size of these three clusters can be considered the most important changes when incubating boar spermatozoa in glucose-rich semen extenders at 38°C. The number of spermatozoa with moderate speed, wide ALH, and moderate LIN (Cluster 4) or low LIN (Cluster 5) declined from 32% (15 min) to 27% (30 min) and 14% (180 min) for Cluster 4, and from 14% to 7% and 5% for Cluster 5, respectively. On the other hand, the presence of spermatozoa with low velocity, low ALH, and moderate to high LIN increased over time (Clusters 1–3). The predominant change was reflected in an increase in Cluster 3 from 13% (15 min) to 28% (30 min) and 33% (180 min), respectively. The general shift in many spermatozoa from motility patterns with relatively high velocity, low LIN, and wide ALH to a slower swimming speed with higher LIN and lower head displacement was also reflected in the decrease in the average values for VCL and ALH associated with a concomitant increase in LIN in progressively motile spermatozoa (Table S4).

Two groups of spermatozoa were present at consistent levels at all time points. These were a group of fast spermatozoa with moderate ALH and high LIN (Cluster 6; 6%–9%) and a group of very fast spermatozoa with high ALH and moderate LIN (Cluster 7; 9%–14%; Figure 5C). This indicates that some spermatozoa did not rely at all on active mitochondria to (transiently) show these highly active motility patterns. In contrast, very fast spermatozoa with highly erratic movement (wide ALH and low LIN; Cluster 13) were almost exclusively present after a 15-min incubation period (4% of all spermatozoa) and reduced to 1% thereafter (Figure 5C). The cell chi-square indicated that deviations in sperm distribution for Clusters 3 and 7 after a 15-min incubation
Changes in sperm motility patterns depending on incubation time after a different number of storage days. Distribution of spermatozoa to different subpopulations (clusters) based on motility parameters after 15, 30, and 180-min incubation time at 38°C. Data are displayed for the different numbers of storage days. The sperm distribution in the different clusters changed significantly depending on the incubation time ($p < 0.05$). Cramer’s $V$ (ranging from 0 to 1) indicates the effect size that incubation had on the distribution of spermatozoa to the different clusters, that is, the size of sperm subpopulations with distinct motility patterns. For interpretation, the following definitions were used: $V < 0.10 = \text{no effect}$, $0.10 < V \leq 0.30 = \text{slight effect}$, $0.30 < V \leq 0.50 = \text{moderate effect}$, and $V > 0.50 = \text{strong effect}$.

When controlling for storage length, the Cochran–Mantel–Haenszel test still indicated a significant relation between incubation time and distribution of spermatozoa to the different clusters ($p < 0.0001$). A stratified view of the data set is presented in Figure 6. Significant changes in sperm distribution between all incubation times were indicated on any given day by Pearson’s chi-square test. The effect size of incubation time as estimated by Cramer’s $V$ (ranging from 0 to 1) was highest when comparing distributions after 15 and 180 min, followed by comparisons between incubation periods of 15 and 30 min. Only at Day 0 was the effect of the incubation time higher between incubation periods of 30 and 180 min than between those of 15 and 30 min (Figure 6). The data suggest that chilling and storage advance changes in motility patterns during incubation at 38°C when compared with fresh semen.

The effect of incubation time on sperm distribution to the different motility clusters was lowest at Days 5 and 7 (Figure 6). Cluster 4 was the only cluster that obviously changed with prolonged incubation time at Days 5 and 7.
**TABLE 1** Pearson correlation coefficients between selected parameters for samples incubated for up to 180 min at 38°C

| ATP (pmol/10^5 spermatozoa) | EC     | hMMP (%) |
|-----------------------------|--------|----------|
| PI and PNA-FITC negative (%)| 0.29***| 0.39***  |
| Total motility (%)          | 0.48***| 0.26***  |
| Progressive motility (%)    | 0.45***| 0.23**   |
| VAP (µm/s)                  | 0.08   | 0.25**   |
| VCL (µm/s)                  | 0.10   | 0.31***  |
| VSL (µm/s)                  | −0.03  | 0.09     |
| STR                         | −0.15  | −0.23**  |
| LIN                         | −0.13  | −0.26*** |
| WOB                         | −0.11  | −0.28*** |
| ALH (µm)                    | 0.16*  | 0.22**   |
| BCF (Hz)                    | 0.16*  | 0.37***  |
| hMMP (%)                    | 0.39***| 0.48***  |

Abbreviations: ALH, amplitude of lateral head displacement; ATP, adenosine triphosphate; BCF, beat cross-frequency; EC, energy charge; hMMP, viable spermatozoa with high mitochondrial transmembrane potential; LIN, linearity; PI, propidium iodide; PNA-FITC, peanut agglutinin conjugated to fluorescein-isothiocyanate; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.

Note: Data from all incubation times and days of storage were combined (n = 175 samples).

*p < 0.05.

**p < 0.01.

***p < 0.001.

### 3.7 Relation of ATP levels and energy charge with sperm motility parameters and mitochondrial activity

Based on all semen samples (n = 175), the ATP content was positively correlated with TM (r = 0.56), PM (r = 0.48), the percentage of spermatozoa with high MMP (r = 0.58), and the percentage of viable, acrosome-intact spermatozoa (r = 0.51; all p < 0.001). Sperm velocity and other motility descriptors were not correlated or weakly correlated with the ATP concentration (Table 1). Instead, the ATP concentration in a sample was positively correlated with the amount of spermatozoa with low LIN and moderate VCL, that is, Cluster 4 (r = 0.53, p < 0.01; n = 105; Table 2). At the same time, high ATP concentrations inversely correlated with the number of spermatozoa with high LIN and low to moderate VCL, that is, Clusters 1–3, respectively (Table 2). Together, the data indicate that a high MMP, ATP supply and sperm motility are only moderately correlated with each other.

EC correlated highest with the percentage of spermatozoa with high MMP (r = 0.51), the percentage of viable, acrosome-intact spermatozoa (r = 0.48; both p < 0.001) and, to a lesser extent, with TM (r = 0.28) and PM (r = 0.23; both p < 0.01). Correlations with the average motility descriptors for progressively motile spermatozoa were weak (Table 1).

The EC of a given sample showed a comparable or stronger positive correlation than the ATP concentration with the amount of moderately active spermatozoa from Cluster 4 (r = 0.49), Cluster 5 (r = 0.48), or hyperactivation-like moving cells from Cluster 13 (r = 0.34; all p < 0.01; Table 2). At the same time, a high EC in a sample inversely correlated with the number of spermatozoa with high LIN and low to moderate VCL, especially with the size of Cluster 1 characterized by relatively slow-moving spermatozoa (r = −0.51, p < 0.01; Table 2).

The percentage of viable spermatozoa with high MMP showed a positive correlation with the TM (r = 0.43), PM (r = 0.40), and the percentage of viable, acrosome-intact spermatozoa (r = 0.49; all p < 0.001; Table 1). The amount of spermatozoa with low to moderate LIN and moderate to high VCL, that is, Cluster 4 (r = 0.70), Cluster 5 (r = 0.63), and Cluster 13 (r = 0.44; all p < 0.001; Table 2), were positively correlated with the percentage of viable spermatozoa with high mitochondrial transmembrane potential. In contrast, the relative amount of spermatozoa in clusters with high LIN and low to moderate VCL, that is, Cluster 1 (r = −0.59), Cluster 3 (r = −0.55), and Cluster 5 (r = −0.42; all p < 0.001), were inversely correlated with the percentage of viable spermatozoa with high mitochondrial transmembrane potential. Correlations between the percentage of spermatozoa with high MMP and Cluster 2 or 7 were not evident. These data suggest that mitochondrial activity and oxidative phosphorylation modulate the directionality of boar sperm motility trajectories toward less linear sperm movement.

### 4 DISCUSSION

The present study demonstrates that long-term preservation of boar spermatozoa in a liquid state does not affect ATP levels and EC, but with ongoing storage time, the cells spend an increasing degree of ATP to reactivate motility during rewarming.

Consistent with the minor fluctuations in ATP and EC levels during storage at 17°C, TM, and sperm viability remained at a high level throughout the experiment. Although our results seem to be conflicting when compared with those of earlier studies reporting decreasing ATP levels during storage time in chilled and liquid-preserved samples, it is important to note that in the latter studies, the lowered ATP levels coincided with markedly reduced sperm motility and viability. Our results strengthen observations from preliminary data that viable spermatozoa can maintain their energy metabolism in balance for several days at 17°C. A biasing effect of nucleotides in the extender/seminal plasma mixture on our results can be excluded because only negligible traces of nucleotides are present in stored boar semen samples with high amounts of viable spermatozoa.

One of the most striking observations from our study was that ATP concentrations in the semen samples declined by up to 25% upon rewarming to body temperature. The relative drop was all the more pronounced, the longer the samples had been stored and was not related to cell death because motility and viability remained at a high level. Thus, rewarming spermatozoa can be viewed as a “switch on” situation that forces the cell to regain balance between energy consumption and production. Whether long-term storage at 17°C causes a higher demand for ATP or a diminished ability to generate ATP or a mixture of both to reactivate sperm metabolism is not yet clear. In this context, the potential involvement of subtle oxidative stress leading...
to inactivation of enzymes in the pathways of glycolysis and/or oxidative phosphorylation should be considered. Interestingly, the present study shows that boar spermatozoa in a glucose-rich semen extender apparently rely heavily on functional glycolysis, not only for reactivating motility after chilling and storage but also for re-establishing a high mitochondrial transmembrane potential. However, the micromolar levels of energy substrates from 10% seminal plasma were sufficient to outperform the general motility-inhibiting effect of a glycolysis block by 2-deoxy-D-glucose in a subset of spermatozoa. Citrate was present in extender variants with 2-deoxy-D-glucose either combined with or without seminal plasma. Therefore, citrate may be excluded as a “rescuing” molecule. The available energy substrates in seminal plasma, fructose, glucose, and sorbitol can probably also be excluded as key molecules that rescued motility and mitochondrial membrane potential because they would require the functional availability of the putatively inhibited hexokinase to become phosphorylated and enter glycolysis. A fructokinase activity for direct phosphorylation of fructose has not yet been detected at a significant level in boar spermatozoa.37 Thus, the most likely candidate seems to be L-lactate from seminal plasma, which, independent from glycolysis, can efficiently be metabolized by spermatozoa in the mitochondria.37

One possible factor influencing the expense of ATP for reactivating stored spermatozoa might include the activation of ATP-dependent transporters that are involved in intracellular ion homeostasis, particularly in the regulation of cytosolic calcium levels.38,39 It has been demonstrated that sperm reactivation after chilling-induced silencing coincides with a release of calcium from intracellular stores, resulting in a hyperactivation-like motility pattern in a subset of spermatozoa 5 min after starting the warming process.40 Thereafter, the population of hyperactivated spermatozoa declined, which indicates a time-delayed regulation of the free intracellular calcium levels.40 Notably, in the present study, changes in sperm kinematic patterns during rewarming remained largely unaffected by storage length. This observation demonstrates the robustness of the sperm’s energy metabolism to ensure vital sperm function on the route of fertilization even under prolonged preservation conditions.

As stated above, ATP and EC remained consistently high during storage from 24 h up to 7 days, but there was a drop in ATP levels and EC within the first 24 h of storage. Kamp et al.11 demonstrated that the cooling of boar semen to 15°C reduced oxygen consumption by 98.6%, which is interpreted as a downregulation of oxidative phosphorylation in the mitochondria. However, energy generation from glycolysis is maintained under anaerobic conditions at 17°C, as deduced from the accumulation of glycerol 3-phosphate in the spermatozoa and lactate in the extracellular medium. Hence, within the first 20 h of chilling and storage, excess lactate in the spermatozoa contributes to a gradual intracellular acidification (pH 6.2).31 The significantly reduced ATP concentration and the reduced EC after 24 h of storage that we observed suggest that the net production of ATP is slightly negative until a temporary equilibrium is reached. A combination of factors, that is, a low intracellular pH, the presence of ATP, and probably the intracellular presence of citrate (from the semen extender), are finally all inhibitory to the phosphofructokinase activity and thus glycolysis.41,42 In line with these assumptions, no increase in the glycolysis product L-lactate in the extracellular medium was observed between Days 1 and 7 of storage. Consequently, the “resting” level for the EC between Days 1 and 7 (0.70–0.77) at 17°C was always lower than for freshly diluted semen at Day 0 or when compared with raw semen10,11 or freshly isolated epididymal spermatozoa.43 The concept of lactate/pH-mediated silencing of sperm motility is comparable to the physiological situation in sperm storage tubules in birds, where these factors lead to pH-dependent reduction in ATPase activity in the spermatozoa.43 By this, motility is stopped, while ATP levels remain stable. A slight rise in extracellular pH during storage in a BTS extender44 may even be favorable to lower the inhibitory effect of pH on phosphofructokinase. This may have contributed to the observation that at Days 3 and 5 of storage, ATP levels and EC at 17°C were slightly increased and again similar to levels at the day of dilution (Day 0, sample temperature: 23.7 ± 0.2°C).

The ATP levels in this study were assessed at the level of each individual sample. Extrapolating ATP levels from a whole sample to a single spermatozoon is a delicate process. This would require a sample with 100% viable spermatozoa and no ATP in the medium. These conditions were virtually fulfilled at Day 0 after a 15-min incubation period at 38°C, with 92.2% motile and 92.4% viable spermatozoa with high mitochondrial transmembrane potential. At that time, each

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**TABLE 2** Correlation between sperm presence in major clusters, adenosine triphosphate (ATP), energy charge (EC), and mitochondrial function in stored semen samples during incubation at 38°C

| ATP (pmol/10⁵ spermatozoa) | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 13  |
|---------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Sperm presence (%) in cluster… | −0.24* | −0.45** | −0.21* | 0.53** | −0.19 | −0.04 | 0.27** | 0.18 |
| EC                        | −0.20* | −0.51** | −0.35** | 0.49** | −0.12 | 0.12  | 0.48** | 0.34** |
| hMMP (%)                  | −0.16 | −0.59** | −0.55** | 0.70** | −0.42** | 0.14  | 0.63** | 0.44** |

Abbreviation: hMMP, viable spermatozoa with high mitochondrial transmembrane potential.

Note: Data after incubation for 15, 30, and 180 min from all days of storage were combined, and Spearman correlation coefficients were calculated (n = 105). Each cluster represents spermatozoa with a distinct motility pattern (c.f. Figure 3).

*p < 0.05.
**p < 0.01.
***p < 0.001.
spermatozoon would have had an average ATP content of 4.21 fmol (value not adjusted for sperm viability). Nonetheless, it would be rash to assume that all viable spermatozoa have an identical ATP content or EC. The presence of sperm subpopulations with distinctly differing motility patterns after rewarming suggests that spermatozoa with a diverse set of energy levels are present in each semen sample. Moreover, ATP and EC are highly dynamic parameters that oscillate within certain limits according to the functional requirements of the cell. This reduces any observation only to a snapshot in time. Given that dynamic monitoring of ATP levels at the single-cell level is currently becoming available for somatic cells, it is probably only a matter of time before such reporter probes can also be expressed in spermatozoa.

The energy metabolism of spermatozoa is under the control of sensor molecules such as AMP-activated protein kinase (AMPK). The enzyme AMPK is activated when AMP/ATP ratios shift in favor of AMP as observed in the present study, which indicates energy deficits. An increasing amount of phosphorylated (activated) AMPK was detected in boar semen samples during storage at 17°C, which coincides with a clearly reduced motility, although viability was maintained. Phosphorylation of AMPK also occurs during prolonged incubation of boar spermatozoa in defined media at 38°C without a change in TM. It is thus tempting to speculate that increases in the AMP/ATP ratio, as observed after long-term incubation at 38°C in the present study, may have triggered AMPK signaling, which then promoted glycolysis and consequently lactate accumulation in the extracellular medium, as observed in our experiments. However, studies on the expression level of (activated) AMPK in viable, single spermatozoa with different AMP/ATP ratios would be required to prove this assumption.

Differences in the energy state between spermatozoa could translate into different kinematic patterns visualized by cluster analysis. The modulating role of mitochondria on sperm motility patterns becomes apparent from our observation that the percentage of spermatozoa with high MMP is equally or more strongly correlated with descriptors of the sperm motility pattern and the size of sperm subsets (cluster) with distinct movement patterns than with the general percentage of total and progressive motile spermatozoa. In particular, the decrease in the major sperm cluster, that is, Cluster 4, consisting of a sperm subpopulation with moderate speed, wide ALH, and moderate LIN, was highly positively correlated with the decline in mitochondrial activity (r = 0.70), while the increase in spermatozoa with low motility (Cluster 3) was inversely correlated (r = -0.55).

Despite the general notion that boar spermatozoa generate energy mainly by glycolysis, the role of mitochondrial ATP production in modulating the LIN of sperm movement and velocity is emerging. However, the degree to which either of the two pathways contributes to the sperm’s energy balance is highly dependent on medium composition, especially the provided energy substrates. In the present study using a typical glucose-rich semen extender, sperm subpopulations with moderate speed, wide ALH, and moderate LIN (Cluster 4) or hyperactivation-like motility patterns (Cluster 3) after 15 min of incubation gradually declined during thermic incubation in favor of subpopulations with slower VCL, a straighter swimming path, and less ALH (Cluster 3). At the same time, mitochondrial activity rapidly declined without affecting the percentage of progressive motile spermatozoa. This indicates that during rewarming in a semen storage medium, a transition from a punctual sperm motility activation supported by oxidative phosphorylation in the mitochondria (and probably elevated free intracellular calcium concentrations) to a steady-state maintenance of motility predominantly achieved by glycolysis took place. This hypothesis is supported by the fact that extracellular lactate levels rose in the absence of mitochondrial activity at 38°C, while high levels (>70%) of motile and viable spermatozoa were maintained. It seems unlikely that such increased extracellular lactate levels were solely a result of glycolytic enzymes or lactate leaking from non-viable spermatozoa. Instead, lactate may have accumulated in the cytoplasm of viable cells because further metabolism in the mitochondria did not occur. In general, excess intracellular lactate is transported out of cells through proton-linked monocarboxylate transporters (MCT) in order to balance the intracellular lactate level and intracellular pH. Lactate-specific MCT are expressed in spermatozoa of men, mice, rats, hamsters, and probably pigs.

As stated above, our experiments were performed in a classical glucose-rich boar semen extender to mimic the environment of preserved semen during rewarming at insemination. Compared to seminal plasma, the classical semen extender BTS used here raised glucose levels approximately 1000-fold. Recently, it was shown that mitochondrial activity and concomitant induction of high-speed linear boar spermatozoa are influenced by the glucose level in the sperm suspension during an incubation period of up to 3 h at 37°C. Levels as low as 30.6 mM resulted in a higher average mitochondrial transmembrane potential and a higher average VSL but no change in ATP levels when compared with cells incubated in the presence of a high glucose level (153 mM). Whether similar effects on motile sperm subpopulations can be reproduced with a glucose-reduced variant of the BTS extender (original glucose concentration: 205 mM) and whether such an extender would be suited for semen storage at 17°C remain to be shown. Earlier data show a higher viability for spermatozoa preserved in glucose-reduced variants (100–150 mM) of the BTS extender from which some were supplemented with lactate, but data on mitochondrial function and sperm motility are lacking. However, a minimum amount of glucose or another energy substrate may be required in a semen extender to allow reactivation of motility and mitochondrial function in preserved semen. In any case, our current data suggest that liquid semen preservation in a simple BTS extender for 3 days or longer primes spermatozoa for depletion of the ATP pool during rewarming. Future directions of semen preservation might aim at designing semen extenders, which support the replenishing of the ATP pool during the rewarming phase. In stallion spermatozoa, supplementation with pyruvate, which can efficiently be metabolized by mitochondria with a high ATP yield, has already been successfully evaluated to increase the longevity of the spermatozoa. An alternative strategy would be to not cure the symptom of increased ATP depletion but to prevent the increased investment of ATP for re-establishing sperm functions. Semen extenders with drastically lowered glucose levels may be a
step forward in reducing putative glycolytic stress in liquid-preserved semen.\footnote{21}

In conclusion, long-term storage of liquid-preserved boar spermatozoa increases the demand of ATP for reactivation of spermatozoa toward fast, non-linear, and hyperactivation-like motility patterns upon rewarming. The energy status is not affected by the storage length. Initiation of glycolysis and re-establishment of full mitochondrial activity, particularly in the initial phase of rewarming of spermatozoa in the female genital tract, might be decisive for sperm function after long-term storage in vitro. To this end, future research focused on improving semen preservation should be directed at the pathophysiological ATP-consuming mechanisms that are induced by rewarming after sperm storage in vitro.

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CONFLICT OF INTEREST
The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS
Heiko Henning designed the study with contribution from Dagmar Waberski. Quynh Thu Nguyen, Ulrike Wallner, Anne-Marie Luther, and Heiko Henning performed the experiments. Heiko Henning and Quynh Thu Nguyen analyzed the data with contributions from Martin Beyerbach. The manuscript was written by Heiko Henning, Quynh Thu Nguyen, and Dagmar Waberski. All authors reviewed and approved the final version of the manuscript.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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