Energy metabolism during long-term storage and subsequent thermal stress in liquid preserved boar spermatozoa

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Presented by

Nguyen Thu Quynh

Born in Hanoi, Vietnam

Göttingen, September 2015

1. Name of supervisor: Prof. Dr. Dr. Matthias Gauly

2. Name of co-supervisor: Prof. PhD. Wolfgang Holtz

Prof.Dr. Dagmar Waberski

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Table of contents

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ALH	Amplitude of Lateral Head Displacement
ATP	Adenosine-5'-triphosphate
BCF	Beat Cross Frequency
BSA	Bovine Serum Albumin
°C	Grad Celsius
CASA	Computer Assisted Semen Analysis
DMSO	Dimethylsulfoxide
et al.	et alli
EC	Energy charge
EDTA	Ethylendiaminetetraacetate
FITC	Fluorescein isothiocyanate
Fig	Figure
FL 1-3	Filter Number 1 to 3
FSC	Forward scatter
g	Gravitational acceleration
h	Hour
HBS	Hepes buffered saline

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hz	Hertz
JC1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
KCl	Potassium Chloride
kg	Kilogram
KH_2PO_4	Monopotassium phosphate
KOH	Potassium hydroxide
L	Liter
LIN	Linearity
mg	Milligram
min	Minute
ml	Millilitre
mM	milimolar
mOsmol	milli -Osmol
µg	Microgram
µl	Microlitre
µm	Micrometre
Na^+	Sodium ion
NaCl	Sodium Chloride

NaOH	Sodium hydroxide
p	Significance level
PI	Propidium iodide
PNA	Peanut agglutinin
SSC	Side scatter
STR	Straightness
SD	Standard deviation
Tab	Table
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble

Introduction

In major pork producing countries, artificial insemination is used in more than 90 % of breeding sows. Maintenance of good sperm quality during semen preservation is mandatory to ensure high fertility. Boar spermatozoa are highly sensitive to cold shock, and therefore are stored in unfrozen stage. With currently marketed extenders, the ideal temperature to store liquid preserved boar semen for up to five days is between 16 and 18°C (Johnson et al. 2000, Riesenbeck 2011). Storage at lower temperature would be advantageous for prolonging semen shelf live, decreasing bacterial growth and slow- down of chemical reactions (Johnson et al. 2000, Schmid et al. 2013).

During hypothermic storage, sperm metabolism is suppressed to save energy for essential sperm functions on the route of fertilization. Spermatozoa specifically utilize ATP as the energy source for cellular activities such as motility, capacitation and acrosome reaction (du Plessis et al. 2015). Similarly to the situation in somatic cells, sperm function relies on a balanced level between ATP, ADP and AMP, which is expressed in the adenylate energy charge (Ford and Leach 1998). Spermatozoa are able to generate ATP through mitochondrial respiration and through glycolysis in the fibrous sheet and in the sperm head. The relevance of mitochondrial activity for ATP-production and sperm function is controversially debated and seems to vary between species (Rodriguez-Gil 2013). Depending on storage length and temperature, preservation of boar semen may cause deficits in energy metabolism and thereby affect sperm function. In the presence of metabolic substrates, activation of in hypothermically stored spermatozoa by re-warming to body temperature might partially or fully restore the energy metabolism. However, under the condition of both natural and artificial insemination, sperm need to survive in the female reproductive tract for up to 24 h until oocytes are released into the oviduct. It is well known that motility decreases when spermatozoa are exposed to prolonged thermal stress in vitro, particularly with a preceding longer preservation period. Whether in vitro storage and subsequent thermal stress leads to exhaustion of sperm energy metabolism and whether this is causative for the loss of motility is yet not clear.

The aim of the present study was to examine the effect of prolonged storage at different temperatures and subsequent thermal stress on the energy metabolism and sperm quality in liquid preserved boar spermatozoa. Therefore, protocols for ATP extraction were revised to ensure sensitive and precise measurements of adenine nucleotides by the luciferin-luciferase reaction. The role of mitochondrial membrane potential for ATP production and adenylate energy charge as well as the relation between motility and energy measures were elucidated.

Chapter 1

A revised protocol for efficient extraction of ATP from boar spermatozoa

Abstract

Mammalian spermatozoa utilize adenosine triphosphate (ATP) as energy source for key functions on the route to fertilization. ATP and its precursor nucleotides ADP and AMP are determined in many sperm physiology studies by the luciferin-luciferase reaction. Assay reliability strongly depends on the efficiency of ATP extraction and prevention of enzymatic degradation of adenine nucleotides. The aim of the present study was to develop a revised protocol for efficient ATP extraction and measurement of energy charge from diluted boar spermatozoa which yields consistently high ATP concentrations and energy charge in fresh and frozen samples. Boar semen samples diluted in BTS (n=6) were incubated with phosphatase inhibitor cocktail at room temperature or on ice. Boiling for ATP extraction with or without boiling buffer, centrifugation, and subsequent ATP assessment from the supernatant was done either directly after the inhibition step (fresh) or after freezing of subsamples at -20°C. Both inhibition on ice and the use of a boiling buffer increased the amount of extracted ATP ($p<0.05$). Combination of both steps resulted in the highest efficiency of ATP extraction. In fresh samples, measured ATP concentration was approximately tenfold higher when both steps were combined ($p<0.05$). Freezing had no impact on ATP concentration compared to freshly analyzed samples when phosphatase inhibition was done on ice and a boiling buffer was used ($p>0.05$). ATP content of fresh and frozen samples correlated best for this treatment ($r=0.83$; $p<0.05$). In conclusion, using a protocol with phosphatase inhibitor treatment on ice and a boiling buffer increases the efficiency of ATP extraction. Moreover, after treatment with inhibitors on ice samples can be frozen at -20°C for later assessment without affecting ATP content.

Keywords : ATP, energy charge, boar spermatozoa

1.1. Introduction

Adenosine triphosphate (ATP) is the energy source for key functions of spermatozoa on the route to fertilization. Spermatozoa specifically utilize ATP in energy dependent cellular activities such as motility (Mukai and Okumo 2004), capacitation (Travis et al. 2001), hyperactivation, and acrosome reaction (reviewed in Du Plessis et al., 2015). Due to the essential functions for maintenance and regulation of cellular function, the determination of ATP concentration is included in many sperm physiology studies. ATP content in spermatozoa is commonly determined by bioluminescence using the firefly luciferin-luciferase assay in a wide variety of species including fish (Perchec et al. 1995), boar (Long & Guthrie 2006), bull (Guminska 1997), domestic poultry (Wishart 1982), human (Blerkom et al. 1995). Protocols vary widely between reports and evidence for assay accuracy is often lacking. Long-term preservation experiments for several days require storing of semen samples in a frozen state for later assessment, ideally without changing the measured ATP content. In addition, the assay must allow maximum detection of intracellular ATP. Maximal detection of ATP depends on efficient phosphatase inhibition to prevent ATP degradation and an effective ATP extraction step. The immediate use of a phosphatase inhibitor cocktail containing acidic and alkaline phosphatases as well as tyrosine protein phosphatases in aqueous solution prior to ATP release was found to increase the amount of detectable ATP in sperm samples of turkey, rooster and boar (Long & Guthrie 2006). A simple method for extraction of ATP and other nucleotides is the boiling water method. In several studies, boiling buffers were used to support lysing the cell membrane for releasing intracellular ATP and to prevent the action of ATPase or other energy-expending enzymes without interfering with the luciferin-luciferase system (Ford & Leach 1998; Yang et al., 2002). Studies on fresh and stored boar semen using variants of the bioluminescence method report variable ATP contents ranging between 5 and 152 pmol/10^6 sperm (Long & Guthrie 2006; Dziekońska & Strzeżek 2011; Dziekońska et al. 2013) and only few studies consider the concentration of the ATP pre courser nucleotides ADP and AMP. The relative available concentrations of ATP, ADP and AMP allow the calculation of energy charge (EC) as first defined by Atkinson and Walter (1967). Surprisingly, only few information is available in literature about the EC of spermatozoa, although the EC is regarded as a more distinct indicator of the

metabolic energy status of living cells than ATP concentrations alone (Du Toit et al. 1993).

The objective of the present study was to develop a revised protocol for efficient ATP extraction and measurement of energy charge from diluted boar spermatozoa which yields consistently high ATP concentrations and energy charge in fresh and frozen samples. Protocol revision was based on the ATP and adenylate energy charge assay from Ford and Leach (1998) and key features of the ATP quantification assay for spermatozoa of Long and Guthrie (2006). The revised bioluminescence assay was then applied to determine ATP content and energy charge in chilled boar spermatozoa with consideration of the nucleotide concentration in semen extender media.

1. 2. Material and Method

1. 2. 1. Assay Development

Experiment 1

Experiment 1 was based on the protocol of Long and Guthrie (2006) and had the aim to repeat two methods which have been suggested as optimal processing steps to store samples in a frozen state for later ATP assessment without affecting the ATP content. In the control procedure, diluted semen samples were treated with a phosphatase inhibitor cocktail at room temperature (RT). Thereafter, ATP was extracted from the samples and subsequently analysed with a commercial firefly luciferin-luciferase assay. In the two test treatments, either the supernatant after ATP extraction was stored for three days at -20°C before thawing and use in the ATP assay; or the semen sample was frozen after treatment with phosphatase inhibitor cocktail and extraction of ATP was done post thaw. The overview on processing steps is as follows:

a. Fresh semen -> Inhibitor treatment at RT -> ATP extraction -> ATP assay (control)
b. Fresh semen -> Inhibitor treatment at RT -> ATP extraction -> - 20°C storage - Thawing at RT -> ATP assay

c. Fresh semen -> Inhibitor treatment at RT -> -20°C storage -> ATP extraction ->ATP assay

Experiment 2

Results from Experiment 1 indicated that a) ATP extraction from samples treated with inhibitor at room temperature seems to be suboptimal and that b) a small volume of the supernatant after ATP extraction is inconvenient for further processing. Therefore, the next experiment focussed on optimizing the ATP extraction procedure. A 2x2 factorial design was used to evaluate how the temperature during phosphatase inhibitor treatment (room temperature or on ice) and the use of a boiling buffer during ATP extraction a) would affect the amount of extractable ATP and b) whether the amount of extractable ATP from directly processed samples or frozen/thawed aliquots after three days storage at -20°C differ.

1. 2. 2. Assay validation

Intra-day and Inter-day variation for ATP assay and Energy charge assay

After determining optimum conditions for effective ATP extraction from boar spermatozoa, the repeatability (intra-day variation) for ATP assessments and energy charge assessments was determined. Intra-day variation was assessed by calculating the coefficient of variation for 6 different assay runs from aliquots of the same diluted semen samples. Inter-day variation was determined by comparing results of aliquots from frozen/thawed samples at different days.

1. 2. 3. Assay Application

ATP and Energy charge in spermatozoa of hypothermic stored semen samples

Boar spermatozoa are usually preserved in the liquid state at 17°C. During processing and storage, molecules from membrane-damaged spermatozoa may diffuse from the intracellular compartment to the medium. The presence of nucleotides in the liquid phase may be a confounding factor for the determination of ATP content and Energy charge in diluted spermatozoa. Diluted semen samples (n=6) were stored at 17°C and 5°C for 24 h and 72 h. At each time of storage,

extender and spermatozoa were separated by centrifugation of samples through a discontinuous Percoll® gradient with layers of 70% and 35% Percoll® working solution. While spermatozoa are enriched in the pellet of the tube, semen extender stays on top of the 35% Percoll layer. The concentration of nucleotides for calculation of the adenylate energy charge (ATP, ADP and AMP) determined from pelleted spermatozoa and from the extender. In addition, the amount of sperm with intact plasma and acrosome membrane was determined from the pellet.

After ejaculation, spermatozoa are mixed with the secretion of the accessory sex glands. For three out of six boars, also the nucleotide concentrations in semen and seminal plasma after isothermic (36°C) separation on a discontinuous Percoll® were determined.

1. 2. 4. Animals and semen collection

Semen was collected from a total of six healthy, mature boars (Pietrain, Germany Large White and crossbred animals) housed at Unit for Reproductive Medicine of Clinics, University of Veterinary Medicine Hannover. The ejaculates were collected by gloved hand method into disposable semen collection bags with integrated filter (Minitube, Tiefenbach, Germany), which were enclosed in insulated plastic thermos cups pre-heated to 38°C. Immediately after collection, semen was transferred to the laboratory and isothermically (33°C) diluted with Beltsville Thawing solution (BTS; Minitube). Sperm concentration was assessed by the “Thoma neu” counting chamber with phase-contrast microscope (Zeiss, Jena, Germany) at 400x magnification (ocular 10x, objective 40x, phase 2). Only normospermic ejaculates were used for the experiments i.e. ejaculates with ≥ 100 ml volume, ≥ $160x10^6$ sperm/ml concentration, ≥ 70 % motile spermatozoa, ≤ 25 % morphological abnormal sperm. Diluted semen with a final concentration of 20×10^6 sperm/ml was kept at room temperature and used at the day of collection to develop and validate the ATP and EC assay. In case diluted semen was stored, samples (100 ml) were kept for 90 min at room temperature. Then, samples were transferred to a storage unit (17°C). Cooling to 5°C was achieved by holding semen for 60 min at 17°C, followed by 60 min at 10°C before samples were stored at 5°C.

1. 2. 5. Reagents

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), and Roth (Karlsruhe, Germany)

1. 2. 6. ATP assay

The ATP assay can be divided into the following steps:

1) Preparation of an ATP standard curve
2) Sample preparation
3) ATP extraction
4) ATP detection with luciferin/luciferase reaction

Variations of the single steps are described in the respective sub-chapters.

1. 2. 6. 1. ATP standard curve preparation

An ATP standard solution was prepared by dissolving the content (1mg) of one vial (2×10^6 µmol) of ATP standard (FLAAS, Sigma Aldrich, St.Louis, MO, USA) with 1 ml AMPUWA water (Fresenius Kabi, Bad Homburg, Germany). From this stock solution a serial dilution with concentrations of 62.5, 125, 250, 500, 1000, 2000 pmol/ml was prepared. A standard dilution series was prepared for each day. Aliquots of the stock solution were stored at -20°C until use.

A volume of 25 µl of ATP standard concentrations (62.5, 125, 250, 500, 1000, and 2000 pmol ATP/ml) and a blank sample (AMPUWA water) were added to the wells of a 96 wells microliter plate with white wall and clear bottom, (Greiner Bio-One, Frickenhausen, Germany). Then, the ATP assay mix solution (FLAAM, Sigma-Aldrich, St.Louis, MO, USA) was diluted 1:25 with dilution buffer (FLAAB, Sigma-Aldrich, St.Louis, MO, USA). A volume of 100 µl of the diluted ATP assay mix were added to each well by using an automatic pipette (Biohit eLine E 1000, Biohit, Rosbach, Germany). The plate was briskly swirled and the amount of produced light immediately measured with a Tecan GENios Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) controlled by "Magellan" software (Version V5.03, Tecan

Group Ltd., Männedorf, Switzerland). All standards and blank samples were prepared and measured in duplicate.

The relative light units (RLU) measured for each ATP standard concentration were subtracted from the light units obtained from the blank sample. The corrected values for the RLU are proportional to the amount of ATP in the standard samples. A linear regression between RLU and ATP concentrations was established (c.f. Figure 1 A).

1. 2. 6. 2. Sample preparation

The effectiveness of phosphatase inhibitor cocktail in preventing ATP degradation was evaluated using whole semen (Long and Guthrie 2006). 100 µl of diluted boar semen were incubated with 1 µl phosphatase inhibitor cocktail (P5726, Sigma-Aldrich, Steinheim, Germany) at room temperature or on ice for 30 minutes. Inhibitor treatment was followed by ATP extraction, and ATP and energy charge assessment or freezing of subsamples at -20°C.

1. 2. 6. 3. Extraction of ATP

After inhibitor treatment, fresh or frozen samples were boiled either with or without a boiling buffer solution (50 mM Tricine, 10 mM $MgSO_4$, 2 mM EDTA, pH =7.8) for nucleotide extraction. Samples without addition of boiling buffer were directly heated for 10 min in a Thermomixer 5436 (Eppendorf, Hamburg, Germany). When a boiling was used, tubes containing 900 µl boiling buffer were heated for 5 min at 95°C before the samples were added. After addition of samples, the mixture was heated for 10 min to reach 95 – 96°C. After that, the tubes were chilled on ice for 10 min and then centrifuging at 5000 x g for 30 min at 4°C (Universal 30 RF, Hettich, Tuttlingen, Germany). The supernatant was used for determination of ATP and EC.

1. 2. 6. 4. ATP measurement

A volume of 25 µl of each sample was added to a well of a 96 well microliter plate. Then, 100 µl of the diluted ATP reaction mix were added by an automatic pipette. The plate was swirled briskly and the amount of light produced was immediately measured. Each run of samples was accompanied by a blank sample and a standard ATP sample. All samples were prepared and analysed in duplicate. After

correction of all RLU values for background light as assessed by the blank sample, the standard sample was used to calculate a correction factor for each run. Light production in samples and ATP standards increases over time and any delay between start of the reaction and reading the emitted light may lead to a bias in the data (Figure 1B and 1C). The correction factor for each run was calculated by dividing the light units for the standard sample in a given run by the light units obtained during preparation of the standard curve for a given ATP concentration. The correction factor was applied to calculate the corrected value of relative light units for each sample. Relative light units were averaged for each sample and the ATP concentration determined by using the linear equation of the ATP standard curve (y = a*x + b), where relative light units are “y” and the ATP concentration is “x”.

1. 2. 7. Energy charge measurement

The energy charge was determined by modification of procedures described by Ford and Leach (1998). The modifications are described below. Three aliquots (each 100 µl) of the samples to be analyzed for nucleotides were incubated each with 25 µl of one of three different buffers (buffer A, buffer B, buffer C). Reaction buffer A was used for determination of ATP and contained 75 mM Tricine, 5 mM $MgCl_2$ and 0.0125 mM KCl, pH 7.5. Reaction buffer B was used for determining the combined amount of ATP and ADP. In addition to reaction buffer A, buffer B contained 0.1 mM phosphoenolpyruvate (P7002, Sigma-Aldrich) and 0.08 µg/µl of pyruvate kinase (Sigma P1506). Phosphoenolpyruvate and pyruvate kinase stock solutions were centrifuged for 5 min at 10000 x g, the pellet diluted 1: 3 in 20 Mm Tris and 0.1% bovine serum albumin (Sigma A2153 pH = 7.5) before addition to buffer B

Phosphoenolpyruvat (PEP) reacts with ADP to form ATP. The reaction is catalysed by pyruvate kinase:

$$\mathrm{ADP} + \mathrm{PEP} \xrightarrow{\text{Pyruvate kinase}} \mathrm{ATP} + \mathrm{Pyruvate}$$

Tubes containing reaction mix A and B were incubated at 30°C for 30 min.

Reaction buffer C was used for determining the combined amount of ATP, ADP, and AMP. In addition to reaction buffer B, buffer C contained 0.1 µg/µl of adenylate (myo)

kinase (Sigma M 3003). The adenylate (myo) kinase stock solution was centrifuged for 5 min at 10000 x g, the pellet diluted 1: 12 in 20 Mm Tris and 0.1% bovine serum albumin, pH = 7.5) before addition to buffer C. The adenylate kinase converts AMP in the sample to ADP and subsequently phosphoenolpyruvate and pyruvate kinase convert ADP to ATP.

$$\mathrm{AMP} + \mathrm{ATP} \xrightarrow{\text{adenylate kinase}} 2\,\mathrm{ADP}$$

Tubes containing reaction mix C were incubated at 30°C for 90 min. All three tubes were boiled at 95°C for 3 min to stop reactions and then chilled on ice until assayed for ATP content. Measurement and calculation of the ATP content was similar to the procedure described for the ATP assay. Concentrations of ADP and AMP were obtained by subtracting the results from the appropriate measurements. The energy charge was calculated as described by Ball and Atkinson (1975) by the following formula:

$$\text{Adenylate energy charge (EC)} = \frac{[\mathrm{ATP}] + 0.5\,[\mathrm{ADP}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}]}$$

1. 2. 8. Percoll centrifugation

Aliquots of 4 mL extended semen stored at 5 and 17°C or after iso-thermic at 38°C for 30 min were carefully layered over the two step Percoll® gradient (35 and 70%), and tubes were centrifuged at 300 x *g* for 10 min followed by 15 min at 750 x *g* without stopping the centrifuge. After centrifugation, the supernatant were checked on microscope and collected for ATP and energy charge measurement. The pellet spermatozoa were gently re-suspended in BTS extender to final concentration 20 x 10^6 sperm/ml for determination of intact plasma and acrosome membrane, and measurement of ATP/ energy charge.

1. 2. 9. Assessment of plasma and acrosome membrane integrity

Integrity of plasma and acrosomal membranes was assessed using a mix propidium iodide (PI)/FITC-PNA/ Hoechst staining. Briefly, aliquots of 5 µl sample of diluted semen after storage at 5 an 17°C or from pellet after Percoll centrifugation was mixed with 980 µl HBS pre-incubated at 38°C in the incubator (137 mM NaCl, 20

mM HEPES, 10 mM glucose, 2.5 mM KOH, 1mg/ml BSA, pH 7.4 at 20°C, 300 ± 5 mOsmol/kg), 5 µl PI stock solution (1 mg/ml), 5 µl FITC-PNA stock solution (600 µg/ml) and 5 µl Hoechst 33342 stock solution (150 µg/ml) and incubated for 5 minutes at 38°C in an incubator. Flow cytometric analysis of stained samples was performed on a DAKO Galaxy flow cytometer (Dako Cytomation GmbH, Hamburg, Germany), equipped with a 488-nm blue argon laser and BP 537.5/22.5, BP 590/25, LP 630-nm and BP465- nm filters for green, orange, red and blue fluorescence, respectively. The sperm population was identified by characteristic forward and side scatter distribution patterns, and fluorescence intensities (in logarithmic mode) were collected for 10,000 events per sample, at a rate of 400 to 800 events/s. Data were analyzed using FloMax software (Partec GmbH, Münster, Germany). Correction of data for non-DNA particles was performed as proposed by Petrunkina et al. (2010).

1. 2. 10. Statistic analysis

Analysis of data was performed using Excel (Microsoft Office 2007, Microsoft Corporation, Washington, United States) and the Statistic Analysis Software (SAS, Version 9.2, Cary, NC, USA). Data were tested for normal distribution with a Shapiro-Wilk test. In case of normal distribution data from different treatments were compared with Student's t-test. If no normal distribution could be achieved by data transformation, comparisons were done with Wilcoxon's signed-rank test. Data were correlated with Pearson correlation coefficient. All data in this study are reported as mean ± standard deviation (SD). The significance level was set at $P<0.05$.

1. 3. Results

1. 3. 1. Assays development

Experiment 1.

Levels of ATP in boar sperm were similar when ATP was measured of fresh samples (treatment a, 2240 pmol ATP/ 10^6 spermatozoa) was the frozen-stored supernatant after ATP (treatment b, 2035 pmol ATP/ 10^6 spermatozoa; Figure 2). Surprisingly, when ATP was extracted from samples that were only treated with phosphatase inhibitor before freezing (treatment c, 3185 pmol ATP/ 10^6 spermatozoa), a sharp increase in ATP levels was noted ($P< 0.05$).

Experiment 2.

Both, inhibition on ice and the use of a boiling buffer increased the amount of extracted ATP from fresh samples ($p<0.05$, Figure 3). A higher ATP content in both fresh and frozen samples was detected when inhibitor treatment took place on ice ($P<0.05$). No difference in fresh analysed and frozen samples was detected when either inhibitor treatment on ice or a boiling buffer or both were used for samples preparation and ATP extraction ($P>0.05$). The combined use of phosphatase inhibitor treatment on ice and use of a boiling buffer resulted in the highest values of detectable ATP ($P< 0.05$). ATP content of fresh and frozen samples correlated significantly ($r= 0.94$, $P< 0.01$; Figure 4).

1. 3. 2. Assay validation

Intra-day and Inter-day variation for ATP assay and Energy charge assay

After determining optimum conditions for effective ATP extraction from boar spermatozoa, the procedure was tested for repeatability. Table 1 shows the repeatability (intra-day variation) for ATP assessments and energy charge assessments. The coefficients of variation for intra-day variation were below 10% for both, ATP assay (average: 5.7 %; range: 3.0 – 9.1 %; Table 1A) and adenylate energy charge assay (average: 4.5 %; range: 2.4 – 7.0%; Table 1B). The inter-day variation for the ATP assay (average: 8.0 %; range: 3.6 – 12.3 %;Table 2A) and adenylate energy charge assay (average: 3.4 %; range: 1.7 – 4.9 %;Table 2B) Freezing of the samples had no impact on ATP content and results of the adenylate energy charge assay (Table 2A and 2B).

1. 3. 3. Assay Application

ATP and Energy charge in spermatozoa of hypothermic stored semen samples

The ATP concentration was higher in sperm stored at 17°C compared to 5°C ($P<0.05$; Figure 5A). Storage had no impact on the ATP and ADP concentration. The AMP concentration increased from 24 h until 72 h storage in samples held at 5°C. The ATP concentration and energy charge, but not ADP and AMP concentration, in

sperm were highly correlated with the percentage of sperm with intact plasma and acrosomal membrane (Table 3).

The ATP content of the isolated semen extender was negligible irrespective of storage time and temperature (Figure 5B). Almost no ADP and AMP were detectable in the supernatant of samples stored at 17°C. On the contrary, a considerable amount of ADP (58% of amount found in spermatozoa) was detected in the supernatant for samples stored for 24 h at 5°C. The ADP concentration decreased until 72 h storage. The AMP concentration in the supernatant of samples at 5°C was more than threefold higher than the AMP concentration in sperm at 24 h and more than twofold higher at 72 h storage. The concentration of AMP in the supernatant was inversely correlated with the percentage of sperm with intact plasma and acrosomal membrane (Table 3).

There was no ATP and almost no ADP and AMP found in seminal plasma directly after collection (Table 4). The energy charge of spermatozoa ranged between 0.85 and 0.92.

1. 4. Discussion

A reliable method for extraction of adenine nucleotide from cells is determined by complete release of intracellular adenine nucleotides from intact cells, complete and irreversible inactivation of all adenine nucleotide converting enzymes, and no interference with enzymes used in the luciferin-luciferase assay (Lundin & Thore 1975). In this study, a revised protocol for efficient extraction of ATP for subsequent measurement of cellular ATP and energy charge using the luciferin-luciferase reagent is presented. Modifications were based on assay protocols described by Long & Guthrie (2006) and Ford & Leach (1998). Key feature of the revised assay was an improved method for ATP extraction by phosphatase inhibition on ice and use of a boiling buffer. Efficient and consistent ATP extraction is considered as the most critical step for intracellular ATP measure. With the method reported here 10 to 20-fold higher ATP concentrations in boar spermatozoa were measured compared to previous reports (Long & Guthrie 2006, Dziekońska & Strzeżek 2011, Dziekońska et al. 2013). The improved method therefore enhanced sensitivity and could be used for samples where only smaller numbers of spermatozoa are available. Attempts to

modify ATP extraction were triggered by the observation that freezing of samples before ATP extraction revealed higher ATP concentrations compared to fresh semen or samples frozen after ATP extraction (Exp. 1). It was assumed that freezing induced membrane disruption and thereby gave access to previously not released ATP residues. We then found that phosphate inhibition on ice as described by Guthrie et al. (2011) in striped bass spermatozoa and the use of a Tricine boiling buffer (Ford & Leach 1998) yielded highest ATP concentration without detectable difference between fresh and frozen-stored boar semen samples (Exp. 2). Therefore, ice conditions effectively prevented ATP degradation during phosphatase inhibitor treatment. In absence of ice, degradation processes lead to at a loss of 40 % detectable ATP within 15 min (Long & Guthrie 2006). After the inhibition step, heating the sample in presence of a boiling buffer was performed to extract ATP. Previously, the tricine buffer as used in the present study was found to be the most effective buffer among the ten tested for ATP extraction and does not interfere with the luciferin-luciferase system (Webster et al. 1980). Since then the tricine buffer was used in many sperm ATP-assays of different species (Leach 1988; Ford & Leach 1998; Ho &Suarez 2003; Yi et al. 2008).

The present assay revealed a linear relationship between ATP standard concentration and RLU from 31 to 2000 pmol ATP (Fig 1). This contrasts with the report of Long and Guthrie (2006) that values higher than 160 pmol ATP required a log/ log transformation to achieve linearity. It is important to note that sensitivity and precision of the luciferin-luciferase assay is high (Holm-Hansen, 1978, Long & Guthrie 2006). Consequently, sources of variation rather lay in the efficiency of ATP extraction and prevention of ATP degradation than in the luciferin-luciferase reaction. High correlations between ATP concentration of fresh and frozen-stored samples together with low intra-assay variation for both ATP and EC values (Exp. 2) demonstrate that the present method is suitable for routine assessment of cellular energy metabolism in stored samples. Application of this assay for measurement of ATP concentrations and energy charge in fresh and frozen-thawed boar semen was highly repeatable with low intra- and inter-assay variation. EC in fresh boar semen was on high level (0.9) and corresponds to EC measured in freshly ejaculated boar spermatozoa after determination of adenine nucleotides by a fluorometric enzymatic assay (Kamp et al. 2003). Similarly high EC levels (0.8-0.9) are considered as

physiological in freshly ejaculated human spermatozoa (Chulavatnatol et al. 1977). It is important to note, that the presence of membrane defect sperm may influence ATP and EC values in semen samples. Leakage of adenine nucleotide through disrupted membranes in the surrounding medium may result in low ATP/ADP/AMP concentration in a membrane-defect subpopulation of cells. In fact, the present study revealed a high positive correlation between the proportion of membrane intact sperm and ATP and EC levels, respectively, whereas AMP in the supernatant was negatively correlated to the energy measures. Consequently, in samples with distinct amounts of membrane damaged cells obtained ATP and EC values will rather reflect the proportion of viable (membrane intact) cells in the sample rather than the energy status of living cells. Any determination of energy status of cells therefore should include information on the integrity of the plasma membranes. In accordance with observations of Long &Guthrie (2006), the ATP content of seminal plasma was negligible in fresh semen samples. In the present study membrane disruption was induced by lowering the semen storage to temperature to 5°C (Exp.3). Under these conditions when boar spermatozoa were cooled below lipid phase transistion temperature (30° to 10°, Drobnis et al., 1993, Schmid et al., 2013), increased ADP and AMP concentration were found in the extender medium regardless of the storage period. The hypothesis that cooling-induced rearrangement of lipids domains increases membrane permeability (Drobnis et al. 1993) and thus leads to leakage of intracellular nucleotids through disintegrated membranes was confirmed. The lower intracellular ATP concentrations in samples stored at 5°C may partially result from loss of precursor nucleotides AMP and ADP and partially from impaired activity of ATP generating enzymes. Noteworthy, cooled semen samples were incubated at 38° for 15 min to reactivate temperature-dependent enzyme activity before samples for adenine nucleotides were taken. Thus additionally other, temperature-independent anabolic energy metabolic pathways may got affected by cooling-induced rearrangement of lipids domains.

In conclusion, a revised protocol for efficient and highly repeatable ATP extraction in boar spermatozoa is presented which allows freezing of samples at -20°C prior to the luciferin-luciferase reaction without affecting the ATP content and energy charge. The application of the revised ATP assay is recommended to study energy metabolism in boar spermatozoa, particularly when only low sperm number are

available or samples need to be stored for later assessment. In any case cell membrane integrity of the original semen sample should be considered to avoid misleading data interpretation.

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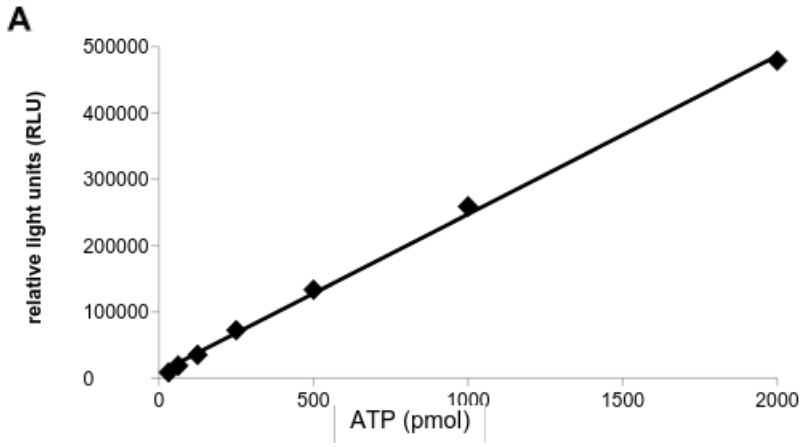
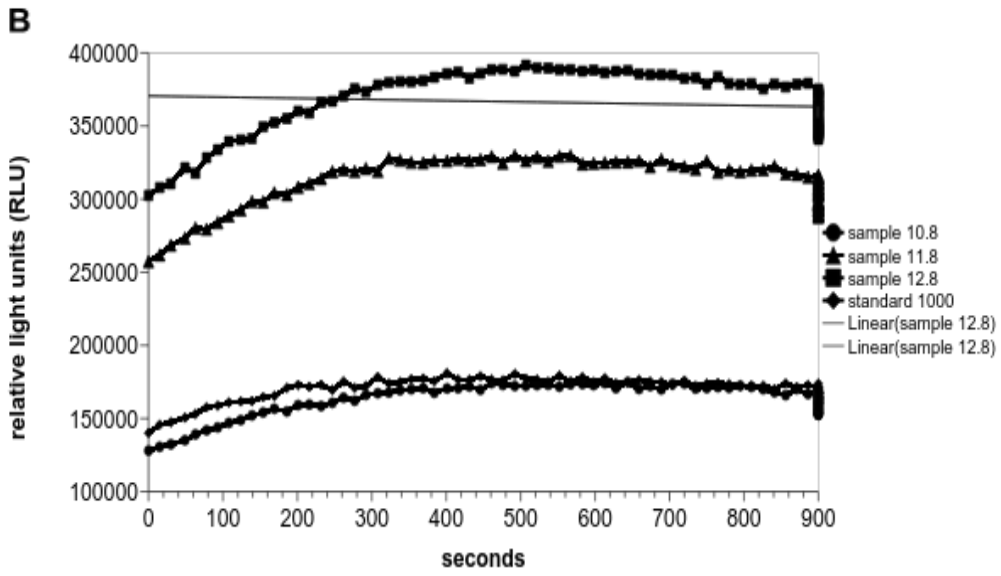
Figures and Tables





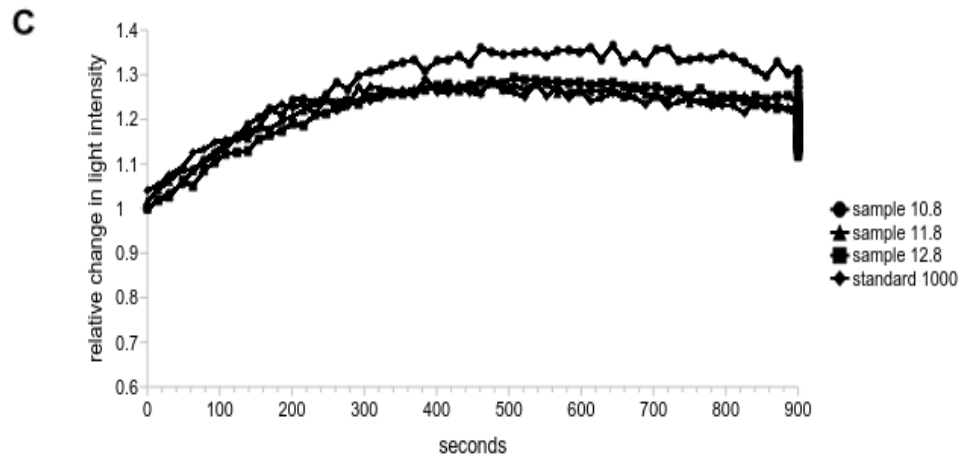


Figure 1. Example of linearity of standard curves with the linear range from 31.25 pmol to 2000 pmol ATP (A). Relative light emission of samples or standard solutions increases over a time irrespective of the initial starting values (B). Relative changes in light intensity over time are similar for samples (10.8, 11.8, 12.8) or standard solutions (C; e.g. 1000 pmol ATP).

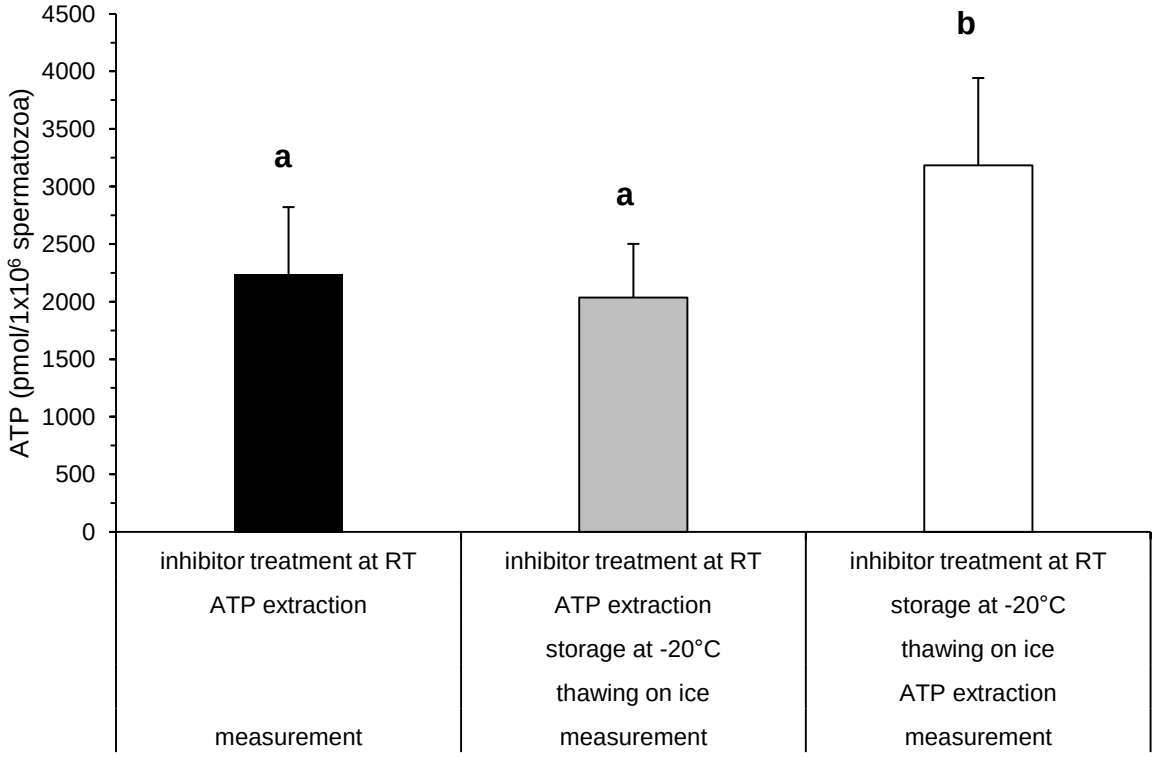


Figure 2 . Evaluation of processing variants for storing samples frozen at -20°C prior to the ATP assay. Fresh semen samples was diluted in BTS extender (20 x 10^6 sperm/ml). Immediate processing and ATP determination (control) was compared with two different ways of processing:

(a) Inhibitor treatment at room temperature - ATP extraction - measurement (control)

(b) Inhibitor treatment at room temperature - ATP extraction – storage at -20 °C – thawing on ice - measurement

(c) Inhibitor treatment at room temperature – storage at -20 °C – thawing on ice – ATP extraction - measurement

All values are mean ± SD (n= 11). Different letters indicate significant differences between processing variants ($p< 0.05$, Wilcoxon signed-rank test).

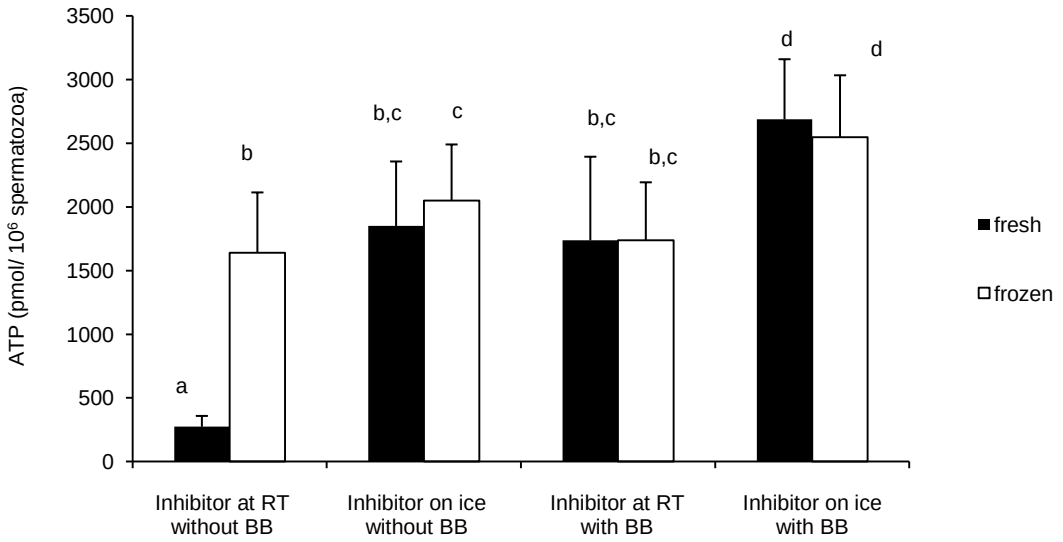


Figure 3. Comparison of different processing conditions for determination of ATP concentration in diluted boar semen samples (n=6). Inhibitor treatment was either performed at room temperature or on ice. An aliquot of each sample was stored at -20°C (frozen) while the other part was further processed and analysed (fresh). ATP extraction in samples was performed with or without a boiling buffer. Different superscripts indicate significant differences ($p<0.05$; Student´s t-test for paired observations).

BB = boiling buffer, RT = room temperature

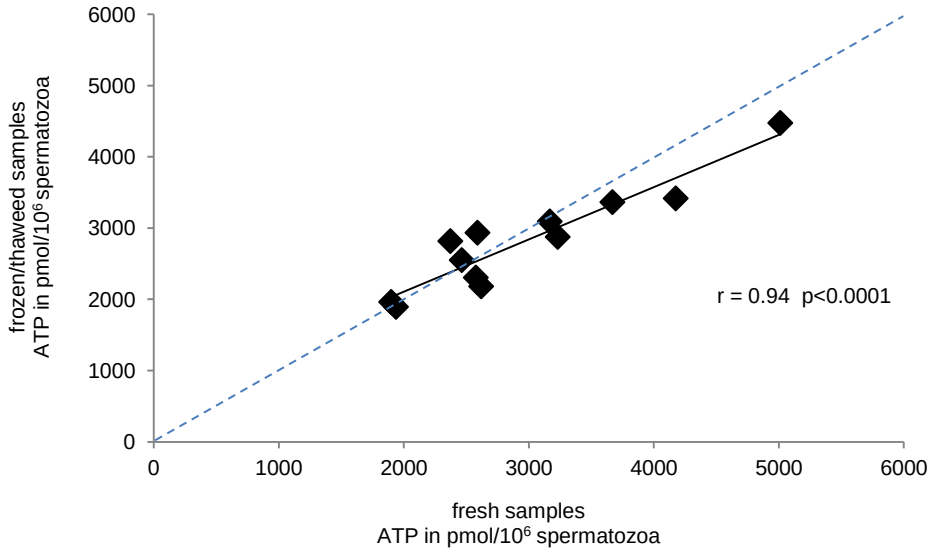


Figure 4. Correlation between the ATP concentration in directly analysed subsamples (fresh) and subsamples assessed after storage at -20°C (frozen) using both inhibition on ice and boiling buffer treatment (n=12; Pearson´s correlation coefficient). The dashed lined indicates the equation where x=y.

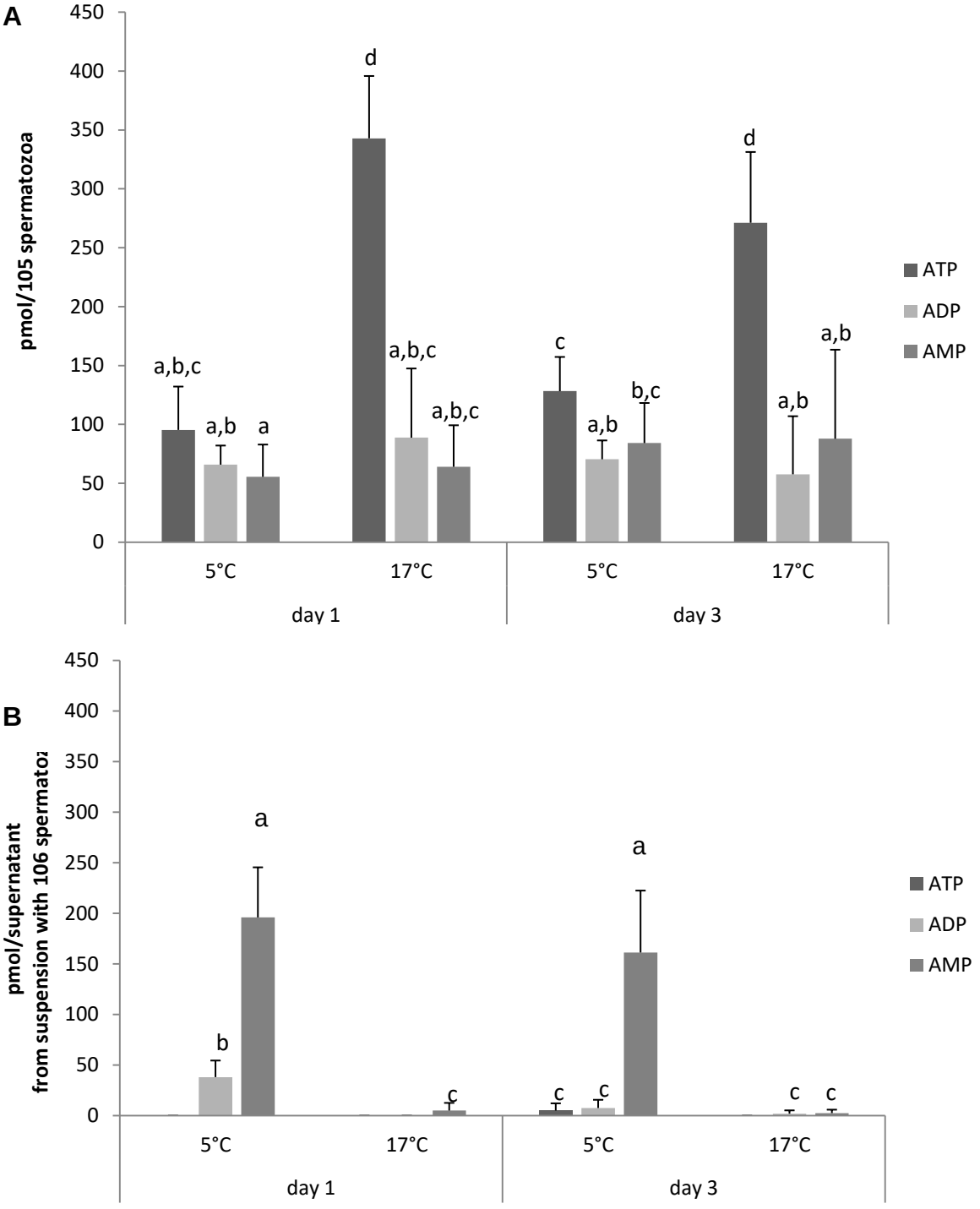


Figure 5. ATP, ADP and AMP content of boar spermatozoa (A) and medium (B) after storage at 5°C and 17°C (n=6). Boar semen was diluted in BTS extender to 20x10^6 sperm/ml (day of dilution =day 0). Supernatant and spermatozoa were separated by centrifugation through a discontinuous Percoll® gradient before assessment of nucleotides. All values are mean ± SD ($p<0.05$).

Table 1 Intra-Assay variation for assessment of ATP and EC in boar semen samples.

A ATP concentration (pMol/10^6 spermatozoa)

	Measurement								
Sample	1	2	3	4	5	6	Mean	SD	CV
1	3630	3907	3983	3865	3931	4071	3898	149	3.8
2	3211	2885	3118	2786	3082	3151	3039	166	5.5
3	3058	2974	3143	3118	2902	3026	3037	90	3.0
4	2476	2643	2669	2743	3060	2630	2704	195	7.2
5	3983	3211	3316	3365	3230	3143	3375	308	9.1
							Mean CV:		5.7

B Energy charge (EC)

	Measurement								
Sample	1	2	3	4	5	6	Mean	SD	CV
1	0.74	0.68	0.72	0.67	0.70	0.66	0.70	0.03	4.4
2	0.65	0.66	0.71	0.65	0.72	0.77	0.69	0.05	7.0
3	0.65	0.68	0.68	0.71	0.69	0.66	0.68	0.02	3.2
4	0.68	0.65	0.69	0.67	0.66	0.69	0.67	0.02	2.4
5	0.74	0.69	0.66	0.67	0.63	0.68	0.68	0.04	5.4
							Mean CV:		4.5

Table 2 Inter-Assay Variation in assessment of ATP and EC in fresh processed and frozen thawed boar semen samples.

ATP concentration (pMol/10^6 spermatozoa)

		Frozen/thawed				
Sample	fresh	same day	3 days later	mean	SD	CV
1	3988	3676	3219	3628	387	10.7
2	3038	2894	2833	2922	105	3.6
3	3037	3275	3566	3293	265	8.0
4	2704	3171	2947	2941	234	8.0
5	3375	2923	2651	2983	366	12.3
6	3143	2872	3152	3056	159	5.2
					Mean CV:	8.0

Energy charge (EC)

		Frozen/thawed				
Sample	fresh	same day	3 days later	mean	SD	CV
1	0.70	0.77	0.72	0.73	0.04	4.9
2	0.69	0.65	0.64	0.66	0.03	4.0
3	0.68	0.64	0.68	0.67	0.02	3.5
4	0.67	0.67	0.65	0.66	0.01	1.7
5	0.68	0.64	0.66	0.66	0.02	3.0
6	0.68	0.69	0.65	0.67	0.02	3.1
					Mean CV:	3.4

Table 3 Pearson correlation coefficients between the percentage of sperm with intact plasma and acrosomal membrane and the amount of ATP, ADP and AMP per 10^6 sperm. Values were determined from the sperm pellet after Percoll centrifugation. Data are combined values of samples after storage at 5°C and 17°C for 24 h and 72 h, respectively (n=24).

Storage temperature		ATP pmol/10^6 sperm	ADP pmol/10^6 sperm	AMP pmol/10^6 sperm	Energy charge	AMP** pmol/10^6 sperm
% PI & PNA-FITC negative		0.90*	0.02	-0.05	0.70*	-0.82*

*p<0.001

**AMP in supernatant

Table 4 Nucleotide content of sperm and seminal plasma directly after collection.

		ATP pmol/10^6 sperm	ADP pmol/10^6 sperm	AMP pmol/10^6 sperm	Total nucleotide content pmol/10^6 sperm	Energy charge
Seminal plasma	Boar 1	0	0	20.9	20.9	
	Boar 2	0	0	0.0	0	
	Boar 3	0	4.3	37.2	41.5	
Sperm	Boar 1	938.5	112.6	34.8	1086.0	0.92
	Boar 2	952.7	180.4	45.7	1178.9	0.88
	Boar 3	625.2	20.9	98.1	744.3	0.85

Chapter 2

The energy status of thermically stressed boar spermatozoa after long-term storage in vitro is not dependent on high mitochondrial membrane potential

Abstract

A variety of ATP dependent processes are required to maintain the functional integrity of spermatozoa. The aim of the present study was to investigate the impact of a long-term storage and subsequent thermal stress on the cellular ATP level and adenylate energy charge (AEC) of extended boar spermatozoa. Metabolic energy data were related to mitochondrial membrane potential (MMP) of live sperm as assessed by flow cytometry using the JC-1/PI assay and to sperm kinematics evaluated by computer-assisted semen analysis (CASA). Ejaculates of seven boars were diluted in Beltsville Thawing Solution, cooled to 17°C and stored for 24, 72, 120, and 168 h. At each time point, samples were analyzed before and after 15, 30, 60, 120, and 180 min incubation at 38°C. High levels of motile and membrane intact sperm, ATP content and EC were maintained throughout semen storage. ATP levels and AEC of spermatozoa during incubation at 38°C were significantly affected by storage length and incubation time ($P<0.05$). ATP content and total motility of sperm declined moderately and earliest after 60 min incubation ($P<0.05$), while the percentage of live sperm with high MMP decreased dramatically already after 30 min of incubation at 38°C ($P < 0.05$). Concomitantly with the decrease of MMP, motility patterns changed from an activated to a steady state pattern. ATP values of samples stored at 17°C decreased during incubation for 15 min at 38°C ($P<0.05$). With ongoing storage time the relative difference between ATP levels before and after incubation increased (day 0: 7.1, day 5: 24.2 pmol/10^5 sperm, $P<0.05$) indicating an increasing relative expense of ATP to regain a functional (motile) state during re-warming. Total motility correlated moderately with the ATP content ($r = 0.48$, $P<0.05$) and low to moderate to % live sperm with high MMP ($r = 0.38$, $P< 0.05$). In conclusion, energy reserves in liquid preserved boar spermatozoa remain stable during prolonged storage at 17°C, though the contribution of oxidative phosphorylation in mitochondria seems to be rapid and only short-term compared to

glycolysis. The transitory increase in ATP levels yield by mitochondrial activity is associated with motility activation. Moreover, the storage-associated increase of the ATP expense of energy for reactivation of motility indicates that ATP dependent cell functions become increasingly vulnerable during semen storage.

Keywords: energy metabolism, boar spermatozoa, semen storage

2.1. Introduction

The storage of boar spermatozoa in the liquid state at temperatures between +15°C and +18°C is the most common way to preserve the male gametes from this species (Johnson et al. 2000). Semen samples can be stored more than three days with none or only a minor reduction of motility and plasma membrane integrity (Henning et al 2012; Schmid et al. 2013). However, data from insemination trials in vivo suggest that the fertilizing ability is already reduced within 48 of storage (Waberski et al. 1994, Haugan et al. 2005). Elucidating the underlying cause for a reduced fertility already in the first days of storage although standard parameters are indicating high quality has been as consistent challenge. The assessment of dynamic regulatory processes such as calcium influx under capacitating conditions has indicated that the cellular function is gradually impaired the longer samples are stored (Henning et al. 2012). Many complex regulated cell functions, such as calcium homeostasis, capacitation, hyper-activation or volume regulation, are partly dependent ATP processes (reviewed by Miki 2007, Suarez 2008). Therefore, deficits in ATP levels or energy charge may at least be co-factors that contribute to an impaired sperm function.

Somatic cells and spermatozoa rely on balanced levels of ATP, ADP and AMP which is expressed in the adenylate energy charge (Ford and Leach 1998). Chilling of cells or spermatozoa below body temperature reduces all metabolic activities, and consequently, also the generation and consumption of ATP decline. Whether this leads to imbalances in the energy charge of the spermatozoa is not clear. Earlier studies report conflicting results on ATP levels in stored boar semen. While ATP levels of density gradient selected spermatozoa did not change during storage over five days (Long & Guthrie 2006), other studies found that storage at 17°C leads to a decrease in ATP values over time (Fraser et al. 2002, Yi et al. 2008, Gogol et al. 2009, Dziekonska et al. 2013). It is yet unknown to which extent re-warming to body temperature and ongoing thermal stress affect energy metabolism of sperm following long-term storage. The relevance of mitochondrial activity for ATP levels and sperm function is controversially debated. Spermatozoa are able to generate ATP through oxidative phosphorylation in the mitochondria and through glycolysis at the fibrous sheath and in the sperm head (Rodriguez-Gil 2013). Positive

correlations between motility, mitochondrial function and ATP levels in fresh boar spermatozoa suggest a certain dependency on mitochondrial activity (Fraser et al. 2002). On the other hand, results from mouse models suggest that glycolysis and not oxidative phosphorylation is the major source of ATP production and indispensable for sperm motility (Miki et al. 2004).

By employing recently refined protocols for assessment of ATP levels and EC in pig spermatozoa (Nguyen et al., unpublished) we sought to shed new light on the relation of mitochondrial activity, energy balance and sperm function, i.e. motility, during liquid preservation and subsequent thermal stress. The aim was to test whether prolonged storage of liquid preserved semen leads to deficits in energy metabolism of boar spermatozoa already at storage temperature or after subsequent rewarming to body temperature for up to three hours. Effects of storage at 17°C and subsequent incubation at 38°C on mitochondrial membrane potential, ATP levels and energy charge were evaluated. Changes in overall motility and sperm movement patterns were assessed as these parameters may be impaired by deficits in available ATP or a decrease in energy charge.

2. 2. Materials and Methods

2. 2. 1. Experimental design

Boar semen samples (n=7) were diluted to a sperm concentration of $20x10^6$ sperm/ml in Beltsville Thawing Solution (BTS, Minitube, Tiefenbach, Germany) and stored at 17°C. Day of semen collection and dilution was designated day 0. Samples were evaluated on day 0, 1, 3, 5, and 7 of storage, respectively. ATP concentration, adenylate energy charge (EC), and the amount of sperm with intact plasma and acrosome membrane were assessed in stored samples and after 15, 30, 60, 120, and 180 min incubation at 38°C. The percentage of live sperm with high mitochondrial transmembrane potential and motility parameters as assessed by computer assisted semen analysis (CASA) were determined only in samples incubated at 38°C.

2. 2. 2. Chemicals and reagents

All chemicals were of analytical grade. Propidium iodide (PI) was from Sigma Aldrich (Steinheim, Germany), peanut agglutinin conjugated to fluorescein-isothiocyanate (PNA-FITC) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Enzo Life Sciences (Lörrach, Germany). Hoechst 33342 (H342) was purchased from life technologies (Darmstadt, Germany).

2. 2. 3. Animals, semen collection and dilution

Semen was obtained from seven mature, clinically healthy boars (Pietrain, German Large White and crossbred animal) housed at the Unit for Reproductive Medicine of Clinics, University of Veterinary Medicine Hannover.

One full ejaculate from each boar was collected by the 'gloved hand' method into disposable semen collection bags with an integrated filter (Minitube, Tiefenbach, Germany) to remove the gel fraction. Collection bags were enclosed in insulated plastic thermos cups pre-heated to 38°C. Immediately after collection, semen was transported to the laboratory in Styrofoam boxes. Sperm concentration was assessed with a hemocytometer chamber ('Thoma neu'). Motility of raw semen was estimated with a phase-contrast microscope (Zeiss, Jena, Germany) at 160x magnification (ocular 10x, objective 16x, phase 1). The morphology of 200 spermatozoa was assessed after liquid fixation of 50 µl raw semen in 300 µl fixation buffer (10 mM citric acid in aqua dest. with 4 % formalin (v/v)) using phase contrast microscopy (x1000 , oil immersion). Spermatozoa were classified according to a simplified scheme based on morphology classifications proposed by Waberski et al 1990. In case of multiple defects per cell only the most severe abnormality was considered. The hierarchy for severity of sperm defects was: 1) Duplicate sperm parts (e.g. two tails), 2) loose heads, 3) acrosome abnormalities (e.g. detached acrosome), 4) head abnormalities (e.g. tapered head), 5) abnormalities in the neck, mid-piece, principal and end piece (e.g. bent tails), and 6) cytoplasmic droplets. Only spermatozoa whose entire outline was visible were considered in the assessment.

Only normospermic ejaculates were used for the experiments, i.e. ejaculates with ≥ 100 ml volume, ≥ $160x10^6$ sperm/ml concentration, ≥ 70 % motile spermatozoa, ≤ 25

% morphological abnormal sperm. Semen was diluted isothermically (33°C) in a one-step procedure with Beltsville Thawing Solution (BTS; Minitube, Germany) to a final concentration of $20x10^6$ cells per mL. After dilution, semen samples were kept at room temperature for 1.5 h and subsequently stored at 17°C.

2. 2. 4. Motility assessment

An aliquot of 2 ml diluted semen was transferred into a 10 ml tube and incubated in a water bath at 38°C. After 15, 30, 60, 120 and 180 min, respectively, an aliquot (3 µl) was loaded into one chamber of a pre-warmed (38°C) four-chamber slide (Leja, Nieuw Vennep, The Netherlands) with a chamber depth of 20 µm for analysis. At the same time, an aliquot of each semen sample (100 µl) was processed for assessment of ATP concentration and energy charge.

The sperm motility parameters were determined by a computer-assisted semen analysis (CASA) system (SpermVision®, Minitube, Tiefenbach, Germany). The microscope (BX41TF, Olympus, Hamburg, Germany) was equipped with a 20 fold objective, a camera adapter (U-TV0,63XC, Olympus, Hamburg, Germany), and a camera with a resolution of 648 x 484 pixels (Accu Pixel TM 6760CL, JAI A/S Glostrup, Denmark). The system was operated by Sperm Vision® software (Version 3.7, Minitube, Tiefenbach, Germany). For each sample, 10 successive fields in the central axis of a chamber were recorded at a rate of 30 pictures per 0.5 second per field.

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), straightness (STR = VSL/VAP), 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 μm^2 and 120 μm^2. 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 (VSL) exceeded 4.5 µm.

2. 2. 5. Flow cytometer

All measurements were performed on a DAKO "Galaxy" flow cytometer (DAKO, Hamburg, Germany) controlled by "FloMax®" software (version 2.8, Partec, Münster, Germany). It was equipped with an argon ion laser (488 nm, 20 mW) and an HBO-lamp for excitation of the dyes. HBO excitation spectrum was restricted with filters to wavelengths between 270 nm and 405 nm (main peak: 365 nm). Filters for detection of emitted fluorescent light were FL-1 (537.5/22.5 nm) for green, FL-2 (590/25 nm) for orange, FL-3 (630 nm LP) for red and FL-4 (465 nm BP) for blue fluorescent light. A 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.

Integrity of the plasma and acrosome membrane

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), 5 µl Hoechst 33342 stock solution (150 µg/ml), and incubated for further 5 minutes at 38°C before assessment on the flow cytometer. For assessment of stored samples, an aliquot of semen was directly transferred to the pre-warmed HBS, stained, and analysed after 5 min incubation at 38°C. Data from 10,000 events were collected for each samples. The overlap of the emission spectra from PI and PNA-FITC was mathematical compensated post acquisition.

Hoechst 33342 was used to the stain the chromatin of all DNA-containing particles in the samples. This enabled the distinction between cellular debris (H342 negative) and the DNA-containing cells (H342 positive), i.e. predominantly spermatozoa as verified by microscopy in channel FL-4. For data evaluation, a logical gate defined the DNA containing events (H342 positive) with a forward scatter signal in the size range of a single spermatozoon. Events fitting in this gate were considered for further evaluation. Propidium iodide, detected in FL-3, was used to differentiate between spermatozoa with intact plasma membrane (PI negative) and damaged plasma membrane (PI positive). Peanut agglutinin conjugated to fluorescein

isothiocyanate (PNA-FITC) is a peanut lectin which has high specificity for binding to Gal-β(1-3)-GalNAc structures. In boar spermatozoa, it binds to the inner leaflet of the outer acrosomal membrane (Flesch et al. 1998). The emitted green fluorescence (channel FL-1) allowed to distinguish between sperm with intact (PNA-FITC negative) and defective outer acrosomal membrane (PNA-FITC positive). For statistical evaluation, the percentage of spermatozoa with intact plasma and acrosome membrane was determined (PI & PNA-FITC negative).

2. 2. 6. Assessment of mitochondrial transmembrane potential in live spermatozoa

The principle of this measurement is based on the properties of the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Depending on the electrochemical gradient of the mitochondrial membrane, JC-1 reversibly transforms from a green fluorescent monomer (low transmembrane potential; emission peak approx. 529 nm) to an aggregated form emitting orange to red fluorescence (high transmembrane potential; emission peak approx. 590 nm). An additional staining with PI allowed differentiation of live and dead spermatozoa.

Five sub-samples of semen from each boar (1 ml each) were incubated at 38°C in a water bath for 15, 30, 60, 120, and 180 min, respectively. Fifteen minutes before assessment, 1 µl JC-1 stock solution (1.53 mM), 10 µl Hoechst 33342 stock solution (150 µg/ml), and 20 µl PI stock solution (1 mg/ml) were added to 1 ml diluted semen. For assessment, 5 µl of the stained sample were transferred to 995 µl pre-warmed HBS (38°C). In each measurement, 10,000 events were collected.

Spermatozoa were defined as Hoechst 33342 positive in the size range of single spermatozoa. Analysis was further restricted to live, i.e. PI-negative, spermatozoa. The percentage of live spermatozoa with high mitochondrial membrane potential was estimated by plotting signals from channel FL-1 (green) vs channel FL-2 (orange). The threshold between high and low mitochondrial membrane potential was defined for samples after 15 min incubation time (day 0) and kept constant throughout the experiment. The average fluorescence intensity for JC-1 aggregates (arithmetic mean) in live sperm was calculated from data in the FL-2 channel. Compensation

between PI and JC-1 spectral overlap was adjusted at the beginning of the experiment.

2. 2. 7. ATP and energy charge assay

2. 2. 7. 1. Sample preparation and nucleotide extraction

ATP content and adenylate energy charge (EC) in spermatozoa were determined by recently described methods from Nguyen et al. (unpublished). The protocols are based on modifications of assays described by Ford and Leach (1998) and Long and Guthrie (2006). 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) were incubated with 1 µl phosphatase inhibitor cocktail (P5726, Sigma-Aldrich, Steinheim, Germany) on ice for 30 minutes. 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 $MgSO_4$ and 2 mM EDTA, pH 7.80) and boiling for 10 minutes at 95°C. Subsequently, samples were chilled on ice for 10 minutes and centrifuged at 5,000 g for 30 minutes at 4°C. The supernatant was used for determination of ATP, ADP, and AMP.

2. 2. 7. 2. ATP assay

ATP was determined using a luciferase reaction kit according to the manufacturer's protocol (FL-AA kit, Sigma-Aldrich, Steinheim, Germany). Bioluminescence was measured with Tecan GENios Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) controlled by "Magellan" software (Version V5.03, Tecan Group Ltd., Männedorf, Switzerland). Light emission was calibrated using standard solution of ATP and ATP content in spermatozoa was calculated from an ATP standard curve (Nguyen et al., unpublished).

2. 2. 7. 3. Energy charge assay

Aliquots 100 µl of the samples to be analyzed or nucleotides was incubated with 25 µl of three different buffers. Buffer A contained 75 mM Tricine, 5 mM $MgCl_2$ and 0.0125 mM KCl (pH 7.5) for determination of ATP. Buffer B contained in addition to buffer A, 0.1 mM phosphoenolpyruvate (P7002, Sigma- Aldrich, Steinheim, Germany) and 0.08 µg/µl of pyruvate kinase. Buffer B was used for conversion of ADP to ATP. Tubes containing buffer A and B were incubated at 30°C for 30 min. Buffer C, contained in addition to buffer B, 0.1 µg/µl of adenylate (myo) kinase (M3003, Sigma-Aldrich, Steinheim, Germany). Adenylate (myo) kinase converts AMP to ADP. Samples incubated with buffer C were used for combined assessment of ATP, ADP, and AMP, respectively. 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 energy charge was calculated as describe by Ball and Atkinson (1975); c.f. Nguyen et al. (unpublished).

2. 2. 8. Statistical analysis

Data were analyzed with using Excel (Microsoft Office 2010, Microsoft Corporation, Washington, USA) and Statistical Analysis Software (SAS, version 9.3, Cary, NC, USA). Data from all parameters were assumed to be normal distributed. Data after incubation of samples for 15 min to 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. Pair-wise comparisons were done with Student's t-test for paired samples. Data that were directly obtained from samples stored at 17°C (ATP content, EC and amount of live, acrosome intact sperm) were compared between different days of storage with Student's t-test for paired samples. Pearson correlation coefficients were calculated for selected parameters. The significance level was set at $P<0.05$.

2. 3. Results

2. 3. 1. Membrane integrity, ATP content and energy charge of samples stored at 17°C

The amount of sperm with intact plasma and acrosomal membrane was at day 5 (90.7 ± 2.35 %) and day 7 (89.0 ± 3.11 %) marginally, but significantly lower when compared to day 0 or day 1 (Table 1; $P<0.05$). A drop in ATP levels and EC was detectable between day of dilution (day 0; 438.5 ± 21.4 pMol/105 sperm) and day 1 (384.52 ± 14.0 pMol/10^5 sperm; $P<0.05$). However, the ATP concentration on day 3 to day 7 was similar to day 0 (Table 1). Concomitant with the ATP concentration, the EC was also lower on day 1 (0.70 ± 0.07) when compared to day 0 (0.78 ± 0.05). The EC remained on the same level as on day 1 throughout the storage period (Table 1).

In general, ATP levels in semen samples held at 17°C were higher when compared to ATP levels after incubation at 38°C ($P<0.05$). Only exceptions were at the day of dilution (day 0) after 15 min incubation and at day 7 after 30 min incubation. At both times, ATP concentrations in semen at 17°C and at 38°C did not differ ($P>0.05$).

The difference (delta) in ATP levels between stored samples and those incubated for 15 min at 38°C increased with storage length (Supplemental Figure 2A). On day 0, the average delta was lowest (32.3 ± 49.3 pMol) while on day 5 it reached its maximum (112.9 ± 57.5 pMol; $P<0.05$). For a standardized comparison, the decrease in ATP within 15 min incubation was expressed as relative change with respect to ATP concentrations in samples at 17°C (Supplemental Table 2B). The relative decrease in ATP demonstrates that the with prolonged storage an increasing amount of the initially available ATP is converted within the first 15 min of incubation at 38°C (day 0: 7.1 ± 10.96 %; day 5: 24.2 ± 10.31 %; $P<0.05$; Supplemental Figure 2B).

Comparable to the situation for ATP levels, a higher EC was present on day 0, day 3, and day 7 in samples at 17°C than in samples after incubation at 38°C. On day 1 and day 5, EC at 38°C was similar to that of samples at 17°C until 60 min and 30 min

incubation, respectively (p>0.05). Thereafter, EC in samples at 38°C was again lower when compared to samples at storage temperature (P<0.05).

2. 3. 2. Motility parameters, membrane integrity and mitochondrial membrane potential of samples incubated at 38°C

Total motility and progressive motility were both significantly influenced by storage length and incubation time at 38°C (Figure 1, Table 2). On day 0 and day 1, incubation at 38°C had no significant effect on progressive motility when compared to values after 15 min incubation. A gradual decrease in progressive motility was noted on day 3, day 5, and day 7, respectively, when samples were incubated for 60 to 180 minutes (Figure 1A; P<0.05). Average progressive motility was above 70% until samples were incubated on day 5 for 180 minutes. The most pronounced decrease in progressive motility occurred on day 7. Motility significantly decreased from 80.4 ± 3.6 (15 min) to 74.0 ± 4.0 (60 min) and finally 54.2 ± 20.0 (180 min; figure 1A; all P<0.05). Notably, average total motility and percentage of sperm with intact plasma and acrosome membrane (PI & PNA-FITC negative) never dropped below 70 % throughout the experiment (Table 2 and Table 3).

The average motility parameters of progressive motile spermatozoa were influenced by incubation time (except VSL) or an interaction of incubation time and storage (except VSL and BCF). Only VSL and STR were influenced by storage length. Velocity curved line (VCL), linearity (LIN) and amplitude of lateral head-displacement (ALH) were chosen as representative parameters to illustrate the influence of prolonged incubation at body temperature on sperm motility. An overview on all parameters is given in Table 1. Changes of motility parameters after prolonged storage for 5 to 7 days became for most parameters only evident after 60 to 180 min incubation at 38°C (Table 1).

On each day of storage VCL was highest after 15 min incubation (Figure 2A). On day 0, day 1 and day 3, VCL was lower between 30 min to 180 min incubation when compared to 15 min incubated samples (P<0.05; Figure 2A). Values between 30 min and 180 min showed no consistent differences. On day 5 and day 7, as similar drop in VCL was present between 15 min and 30 min incubated samples (P<0.05), but it was less prominent. Linearity (VSL/VCL) of the sperm track increased between 15

min and 30 min incubation irrespective of previous storage length ($P<0.05$; Figure 2B). On day 1, day 3, and day 5, a second rise in linearity was present between 30 min and 60 min of incubation (Figure 2B). Thereafter, linearity remained constant. The amplitude of lateral head-displacement (ALH) decreased step-wise during incubation on day 0 (Figure 2C). A similar decrease in ALH between 15 min (3.1 ± 0.4 µm), 30 min (2.4 ± 0.4 µm) and 60 min incubation (2.1 ± 0.3 µm) was present at day 1. Values remained constant between 60 min and 180 min incubation. On day 3, day 5, and day 7, ALH decreased significantly between 15 and 30 min incubation ($P<0.05$, Figure 2C). Values remained constant during further incubation.

In contrast to the moderate changes observed for CASA parameters, the percentage of live sperm with high mitochondrial transmembrane potential (hMMP) declined dramatically during incubation at each day of storage (Figure 1B). After 15 min incubation, the percentage of live spermatozoa with hMMP ranged between 92.5 % (day 0) and 82.5 % (day 7). These values stepwise decreased to less than 40 % after 60 min incubation (max: 38.9 % at day 1; min: 13.5 % at day 7). After 120 min incubation, the amount of live spermatozoa with hMMP was at all days of storage below 10 % (max: 9.3 % at day 1; min: 2.6 % at day 5). After 180 min incubation, live sperm with hMMP were virtually absent in the samples (Figure 1B; Supplemental Table 1). The decline in live spermatozoa with hMMP was consistent with a general decline in fluorescence intensity for all live spermatozoa over incubation time, independent of the days of storage (Supplemental Figure 1).

2. 3. 3. ATP content and energy charge of samples incubated at 38°C

Both, storage time prior to incubation and duration of incubation at 38°C, had a significant influence on the ATP content and energy charge of diluted boar spermatozoa (Figure 3A). At all days of storage, ATP levels remained at a comparable level for at least 60 min during incubation at 38°C. A step-wise decrease was noted on day 0 and day 5, starting between 60 min and 120 min of incubation. On all other days, ATP values fluctuated, but remained comparable to those after 15 min incubation.

Values for energy charge showed a different pattern. With ongoing storage, values remained more and more stable during incubation at 38°C (Figure 3B). On day 0, a

step-wise decrease occurred between 15 min and 30 min as well as between 30 min and 180 min incubation (P<0.05). On day 1, values decreased between 30 and 60 min incubation and remained constant thereafter. On day 3 and day 5, EC was stable for at least 60 min, before values decreased after 180 min (day 3) or 120 min (day 5), respectively. On day 7, values for EC fluctuated during incubation, but never were lower than the value after 15 min incubation.

ATP concentrations and EC values were compared after incubation at 38°C between days of storage (Supplemental Table 1). On day 1, day 5, and day 7, ATP levels were lower than on day 0 within the first 60 min at 38°C (P<0.05; Supplemental Table 1). Thereafter, no systematic pattern was visible. No systematic difference between samples on day 0 and the following days of storage were detectable for EC (Supplemental Table 1)

2. 3. 4. Relation of ATP content and energy charge in samples stored at 17°C with mitochondrial activity and sperm motility after incubation at 38°C

There was no significant correlation of ATP content or energy charge in samples stored at 17°C with motility parameters or percentage of sperm with high mitochondrial transmembrane potential after incubation at 38°C for 15, 30, 60, 120 or 180 minutes, respectively (n = 35; data not shown). Only exceptions were a significant correlation between ATP content at 17°C and total motility after incubation at 38°C for 30 minutes (r = 0.35, P < 0.05) and energy charge at 17°C and straightness of the motility trajectory after 180 minutes incubation at 38°C (r = 0.55 , P<0.001).

2. 3. 5. Relation of ATP content and energy charge in samples incubated at 38°C with mitochondrial activity and sperm motility

The ATP content was positively related to total motility (r = 0.48), progressive motility (r = 0.45), the percentage of sperm with high MMP (r = 0.38) and the percentage of live, acrosome intact sperm (r = 0.29; all P < 0.001; all n=175). Sperm velocity and other motility descriptors were not or only weakly (ALH, BCF) related to the ATP content (Table 4).

Energy charge correlated highest with the percentage of sperm with high MMP (r = 0.47), the percentage of live, acrosome intact sperm (r = 0.39; both P < 0.001) and, to a lesser extent, with total motility (r = 0.26) and progressive motility (r = 0.23; both P < 0.01). Weak to moderate positive correlations existed with sperm velocity (VAP and VCL), ALH and BCF. Consequently, a weak negative correlation between EC and descriptors of the sperm trajectory (STR, LIN, WOB) were present (Table 4).

The percentage of live sperm with high MMP showed positive correlation with total motility (r = 0.43), progressive motility (r = 0.40), and the percentage of live, acrosome intact sperm (r = 0.49; all P < 0.001). A high percentage of live sperm with high MMP also positively correlated with the average curvilinear velocity (r = 0.41), amplitude of lateral head-displacement (r = 0.46), and beat cross frequency in progressive motile spermatozoa (r = 0.34; all P < 0.001). At the same time, a high percentage of live sperm with high MMP was negatively correlated with the linearity and straightness of the sperm trajectory, (r = - 0.59 and r = -0.60; both P < 0.001). Similar, but weaker correlations were also observed for energy charge and motility parameters (c.f. Table 4).

2. 4. Discussion

In the present study, boar spermatozoa were challenged in two ways. They were first held for prolonged time at 17°C in commonly used BTS extender medium. After hypothermic in vitro-storage, they were subjected to a prolonged incubation at 38°C mimicking the temperature situation after insemination. In the female reproductive tract, spermatozoa have to sustain such thermal conditions for several hours until ovulation occurs. Therefore, it is wise to include thermal stress challenges when studying metabolic activity of in vitro stored boar semen. Consistent with previous observations (Guthrie & Welch 2005; Long & Guthrie 2006, Henning et al. 2012. 2015), a high degree of motile and viable sperm, ATP content and EC was maintained throughout semen storage. However, sperm parameters and energy measures of stored semen changed throughout incubation at 38°C under aerobic conditions in presence of glucose (188 mM in BTS extender). Independent of storage time, a high degree of viable spermatozoa re-established a high mitochondrial transmembrane potential within 15 min of incubation at 38°C. Thereafter, with ongoing incubation the proportion of these cells declined rapidly.

Noteworthy, neither viability and overall motility, nor ATP levels and energy charge were immediately affected. Significant decreases in ATP content and energy charge, if any, were only observed after a prolonged incubation period. This observation supports the view that the contribution of oxidative phosphorylation to the total ATP pool is relatively low compared to glycolysis. Indeed, other researchers suggest that the dominant source of ATP production in boar spermatozoa in the presence of glucose is glycolysis (Marin et al. 2003). Notably, the establishment of a high mitochondrial transmembrane potential within 15 minutes of incubation was accompanied by a decrease in ATP content and (in part) EC at each storage day. This indicates that ATP consuming processes are slowed down at 17°C and regain effect at body temperature. Thus, re-warming of spermatozoa can be viewed as a "switch on" situation that forces the cell to regain balance between energy consumption and production. ATP levels in stored semen samples did not vary considerably during long-term storage. However, the relative expense in ATP that the cells spent to regain a functional, i.e. motile state, during rewarming increased considerably with storage length. This observation indicates that ATP-dependent cell functions get more and more vulnerable with increasing storage length due to a depletion of the ATP pool during rewarming.

The high mitochondrial transmembrane potential in plenty of spermatozoa shortly after rewarming may be indicative for a pivotal role of mitochondria in regaining motility and sperm functionality. However, mitochondria seem to have only a short-term effect as with ongoing incubation the transmembrane potential decreases. This decrease may indicate a transition in energy metabolism from high punctual activation to steady state maintenance in vitro. Activated sperm motility, e.g. after ejaculation is characterised by a symmetrical wave pattern of the flagellum and relatively linear sperm trajectories. In contrast, hyper-activated motility is characterised by an asymmetrical beating of the sperm tail, increased curvilinear velocity, and more circular movement (Suarez et al. 1992; Kojima et al. 2015). Similar to the transition in energy metabolism, a transition in motility patterns was found in the current experiment. Spermatozoa showed a hyper-activation-like motility pattern with high curvilinear velocity, low linearity and high amplitude of lateral head-displacement after 15 minutes incubation and switched to a slower curvilinear velocity, with a more straight swimming path less lateral head-displacement

thereafter. As mentioned earlier, the steady state motility might be maintained predominantly be glycolysis. This hypothesis is indirectly supported by the observation that the percentage of sperm with high MMP is stronger correlated with descriptors of the sperm motility pattern than ATP levels and EC. However, motility of boar spermatozoa is not fully independent from mitochondrial activity as indicated by positive correlations with total and progressive motility. Similar rapid, transitory increases of mitochondrial activity with concomitant increase in ATP levels have been described after inducing the acrosome reaction in vitro with progesterone (Ramio-Lluch et al. 2011). Such patterns of short extra activation of mitochondria with later steady state maintenance, may provide tempero-spatial restricted extra energy supply in spermatozoa playing a pivotal role in various stages of sperm's journey in the female genital. Spermatozoa with insufficient increase in available ATP from mitochondria may fail to switch their motility pattern to a more activated motility pattern and thus may fail to actively pass the viscomucous barriers of the uterotubal junction or release from the oviductal sperm reservoir before ascending to the site of fertilization. In fact, spermatozoa bound to the oviduct epithelial cells in the sperm reservoir show a (hyper-)activated motility pattern upon release (reviewed by Suarez 2008). Such changes in motility may likewise be facilitated by spikes of mitochondrial activity to generate extra ATP.

ATP levels in this study have been assessed at the level of each individual sample. With respect to selection mechanisms in vivo, it would be interesting to know how heterogeneous single spermatozoa are with respect to their ATP content or energy charge. Recent comparisons between tail length adjusted ATP content in spermatozoa of mice breeds with different mating behaviours suggest that spermatozoa from mice breeds in which females mate with different males, i.e. sperm competition for oocyte fertilization is high, can maintain higher ATP levels over time (Tourmente et al. 2015). An extrapolation of ATP levels from a whole sample to a single spermatozoon is delicate. This would require a sample with approx. 100 % viable sperm and no ATP in medium. These conditions are most closely fulfilled at day 0 after 15 minute incubation at 38°C with 92.2 % motile and 92.4 % viable sperm with high mitochondrial transmembrane potential, respectively. At that time, each spermatozoon would have an average ATP content of 4.21 fMol.

The adenylate energy charge is regarded as a more destinct indicator of the metabolic energy status of living cells than ATP concentrations alone (Du Toit et al. 1993). An energy charge of more than 0.8 is considered as indicative for highly active proliferating somatic cells. Values of about 0.6 have been considered to be an indicator of somatic cells in the stationary phase of growth, while an energy charge below 0.5 is interpreted as being indicative for resting or dying cells (Ford and Leach 1998 and references therein). Semen samples in our study had an average energy charge between 0.7 and 0.8 at 17°C and an energy charge between 0.5 and 0.8 at 38°C, which is consistent with a majority of plasma membrane intact and motile cells. Similar values have been reported by others for diluted boar semen (Kamp et al. 2003; Jones and Milmlow 1997). Our experimental set up resembled semen processing and storage conditions similar to these used in routine laboratories of AI centers. Thus, data from our study provide a first reference range for judging energy charge in stored semen samples for purposes of quality assessment.

In conclusion, although storage does not affect the resting level of ATP and energy charge in liquid stored boar spermatozoa, the cells spend an increasing degree of energy for reactivation of motility with increasing storage length. A spike in mitochondrial activity is associated with motility activation. The increased expense of energy for reactivating motility is associated with deficits in keeping the energy metabolisms stable after prolonged storage with subsequent incubation at body temperature. This may be part of the explanation, why energy dependent sperm functions, e.g. capacitation, are hampered in long-term stored boar spermatozoa.

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Figures and Tables

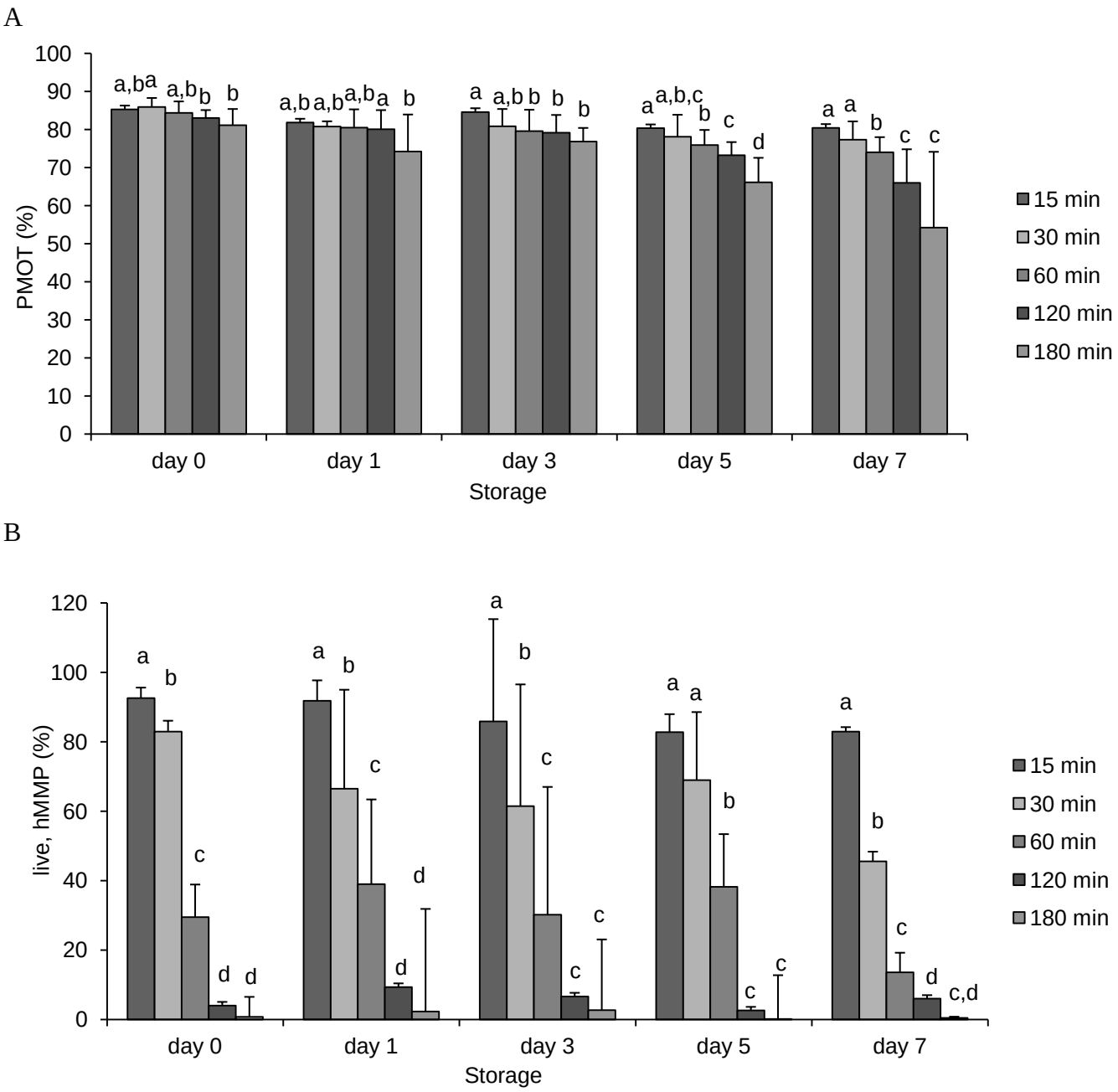
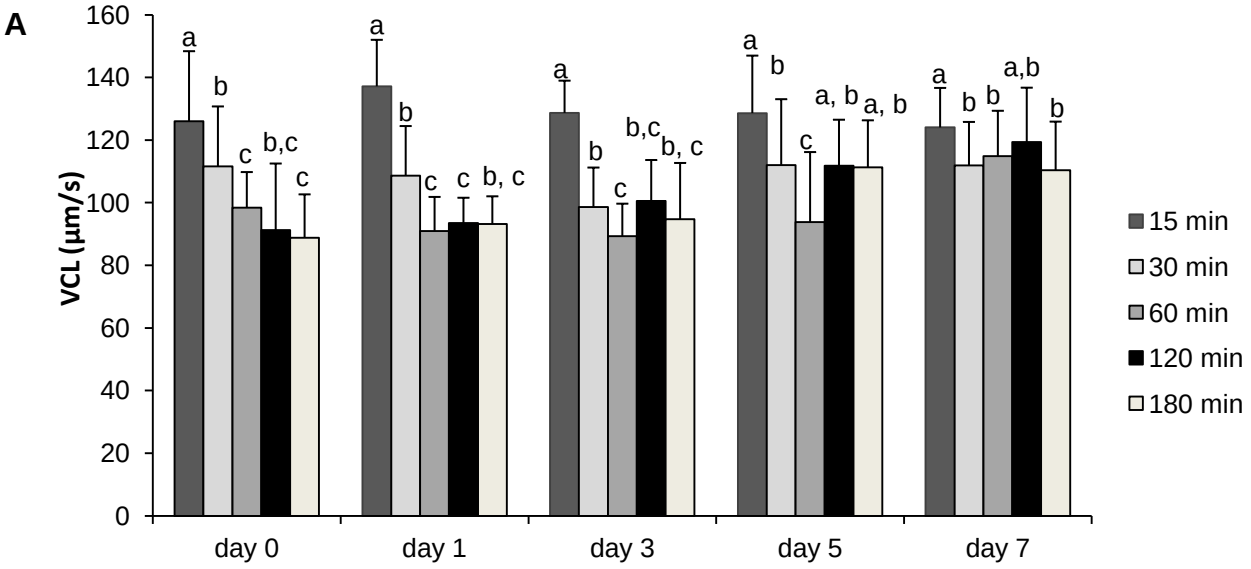


Figure 1. (A) Progressive motility, PMOT, and (B) amount of live spermatozoa with a high mitochondrial transmembrane potential, hMMP, of boar semen samples following incubation at 38°C at different time points. Values are expressed as means and standard deviations (n=7). Different letters (a-d) at a given storage day indicate significant differences between incubation times ($P < 0.05$).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VCL (µm/s)
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140
120
100
80
60
40
20
0
day 0
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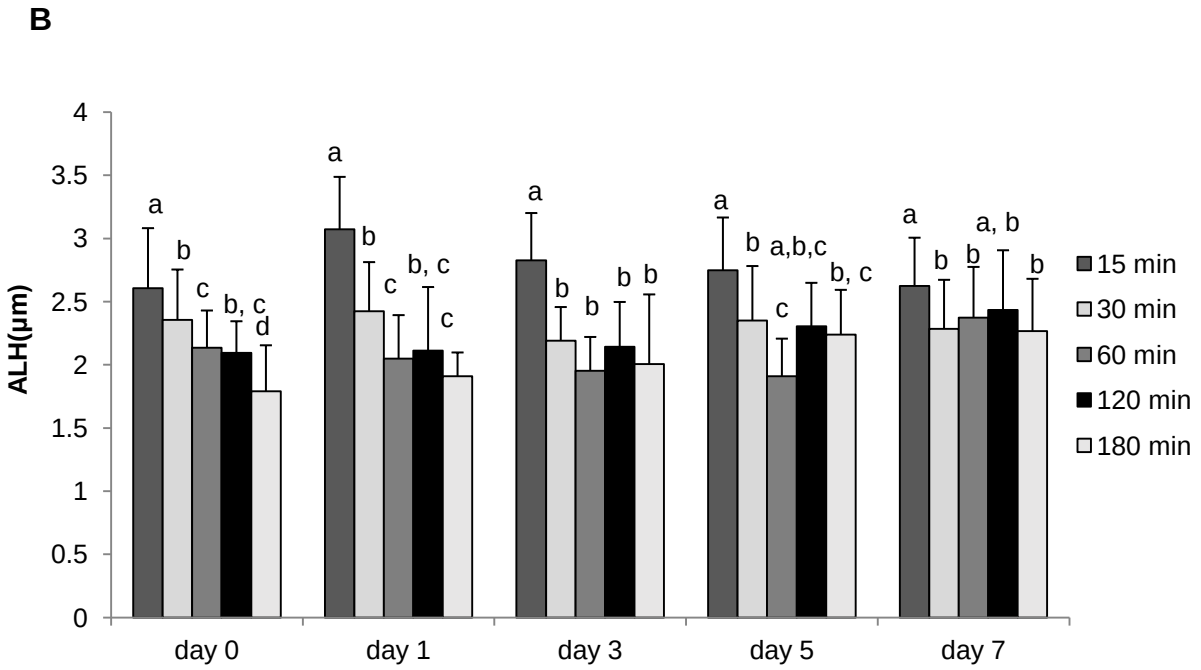
B
ALH(µm)
4
3.5
3
2.5
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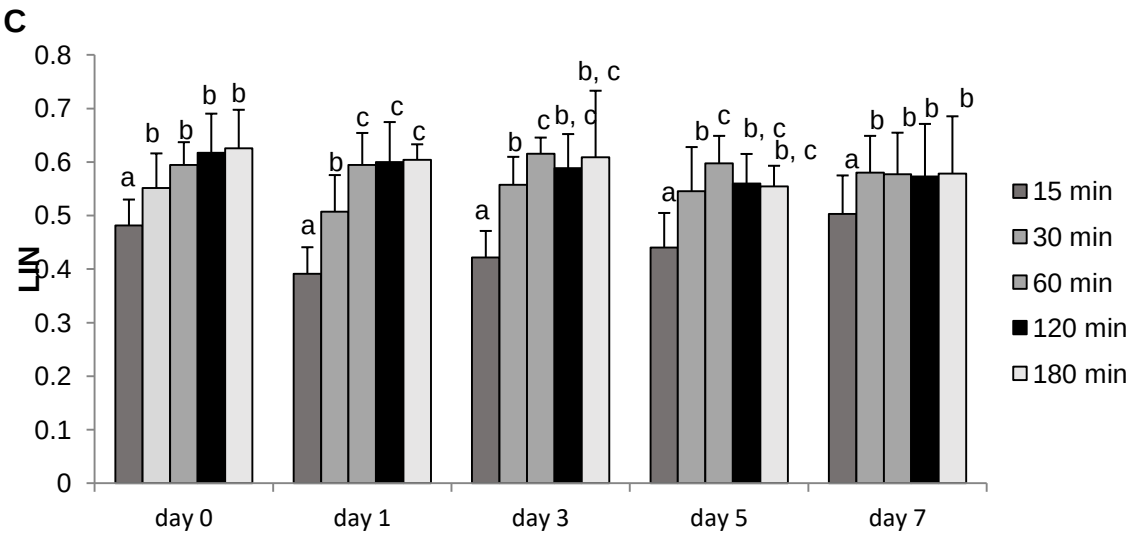


Figure 2. A) Curvilinear velocity (VCL), B) linearity (LIN), and C) amplitude of lateral head-displacement (ALH) of progressive motility spermatozoa following semen incubation at 38°C. Samples were incubated at the day of dilution (day 0) or after up to seven days of storage at 17°C. Values are expressed as means and standard deviations (n=7 ejaculates per boar and time). Different letters (a-c) on columns at a given day of storage indicate significant differences between incubation times ($P < 0.05$).

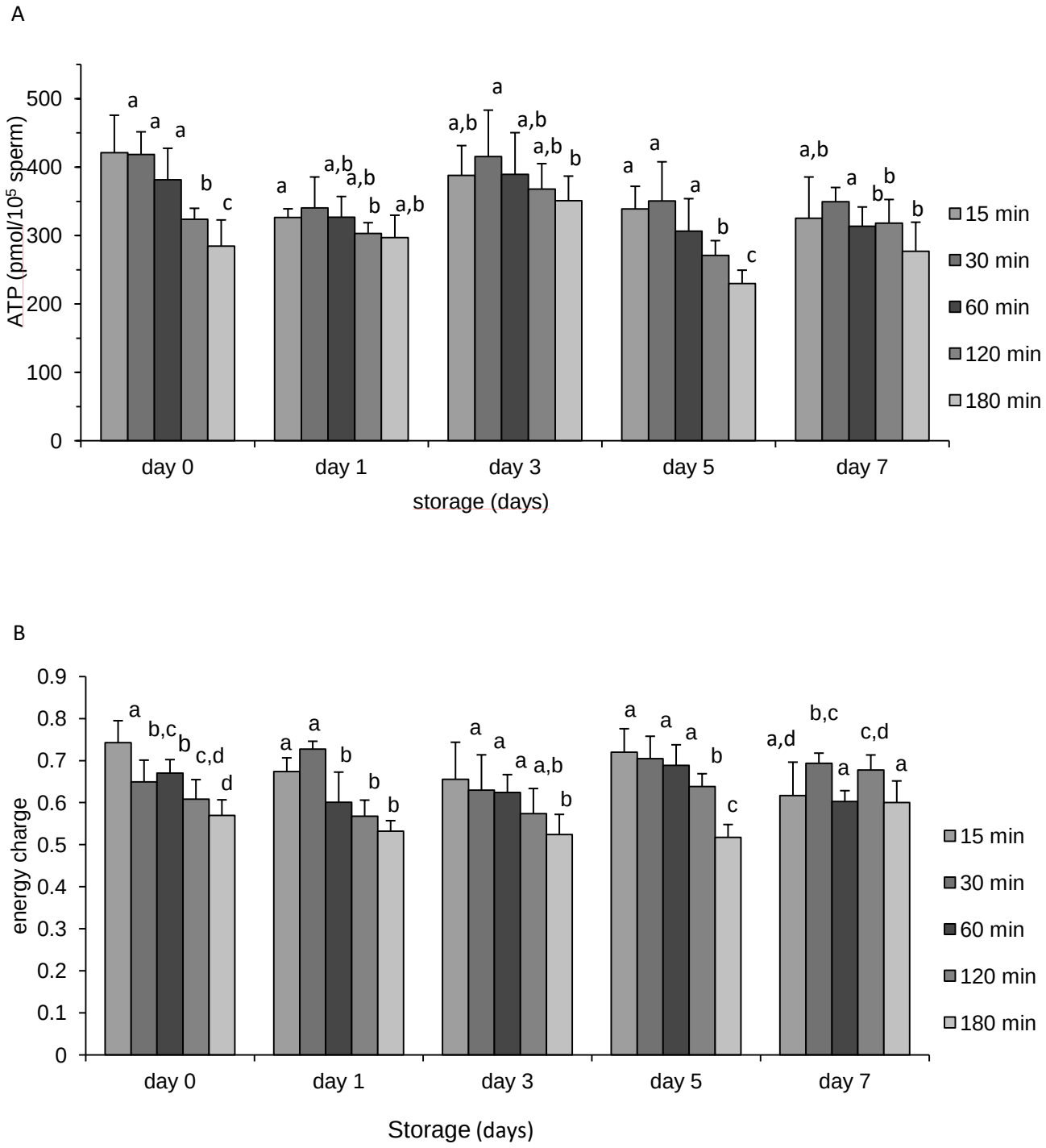


Fig 3. ATP (A) and energy charge (B) of boar spermatozoa following liquid semen incubation at 38°C at different time point. Values are expressed as means and standard deviations (n=7 ejaculates per boar and time). In the same incubation time, the value with different letter (a-e) differs significantly ($p < 0.05$).

Table 1. Parameters of semen samples stored at 17°C (n = 7). Different lowercase letters (a – c) within a column indicate significant differences (P < 0.05) between different days of storage. All values are means and standard deviations.

storage	PI & PNA-FITC negative (%)	ATP (pMol/10^5 sperm)	EC
Day 0	93.7 ± 0.49[a] *	438.5 ± 21.4 [a,b]	0.78 ± 0.05[a]
Day 1	92.1 ± 2.12[a]	384.5 ± 14.0[c]	0.70 ± 0.07[b]
Day 3	90.6 ± 2.69[a]	484.4 ± 44.0[a]	0.73 ± 0.12[a,b]
Day 5	90.7 ± 2.35[b]	451.8 ± 56.8[a]	0.77 ± 0.08[a,b]
Day 7	89.0 ± 3.11[c]	397.9 ± 67.1[b,c]	0.71 ± 0.07[b]

* n = 3

Table 2. Motility parameters of computer-assisted semen analysis (CASA) after 15, 30, 60, 120 and 180 minutes incubation in a water bath at 38°C (n = 7). All values are mean and standard deviation. Different lowercase alphabets (a-e) in each column show significant differences (P < 0.05) between storage days.

		Incubation time				
	Storage	15 min	30 min	60 min	120 min	180 min
TM (%)	Day 0	92.2 ± 1.6^{a}	91.9 ± 1.2^{a}	91.6 ± 2.1^{a}	90.1 ± 1.0^{a}	89.2 ± 1.8^{a}
	Day 1	89.6 ± 2.8^{b}	89.9 ± 1.9^{b}	88.3 ± 3.1^{b,c}	88.6 ± 2.4^{a,b}	84.9 ± 3.6^{b}
	Day 3	92.0 ± 1.9^{a,b}	90.1 ± 2.0^{a,b}	88.7 ± 3.0^{b}	88.0 ± 0.3^{b}	83.2 ± 3.0^{b}
	Day 5	89.1 ± 2.4^{c}	87.3 ± 3.4^{c}	87.5 ± 1.8^{b,c}	82.6 ± 4.0^{c}	76.9 ± 3.3^{c}
	Day 7	90.5 ± 1.2^{b,c}	87.0 ± 3.1^{c}	84.7 ± 3.6^{c}	80.3 ± 5.1^{c}	70.7 ± 1.7^{c}
PM (%)	Day 0	85.3 ± 2.8^{a,}	85.9 ± 2.4^{a}	84.4 ± 3.0^{a}	83.0 ± 2.1^{a}	81.1 ± 4.29^{a}
	Day 1	81.8 ± 4.1^{a,b}	80.8 ± 1.4^{b,c}	80.5 ± 4.8^{b}	80.1 ± 5.0^{a}	74.2 ± 9.7^{b}
	Day 3	84.6 ± 4.1^{a,b}	80.8 ± 4.6^{b}	79.6 ± 5.6^{a,b}	79.2 ± 4.7^{a}	76.9 ± 3.6^{b}
	Day 5	80.4 ± 4.7^{b}	78.1 ± 5.8^{b,c}	75.9 ± 4.9^{b}	73.3 ± 3.4^{b}	66.1 ± 6.5^{c}
	Day 7	80.4 ± 3.6^{b}	77.4 ± 4.8^{c}	74.0 ± 4.0^{c}	66.0 ± 8.8^{c}	54.2 ± 20.0^{c}

		Incubation time				
	Storage	15 min	30 min	60 min	120 min	180 min
VAP (μm/s)	Day 0	79.5 ± 4.8^{a,b}	74.9 ± 8.0^{a}	69.4 ± 7.2^{a}	65.5 ± 11.7^{a}	65.0 ± 8.6^{a}
	Day 1	79.3 ± 4.8^{a,b}	69.8 ± 5.8^{b}	63.7 ± 7.4^{a}	66.6 ± 3.1^{a}	66.2 ± 4.5^{a}
	Day 3	76.9 ± 3.0^{a}	67.1 ± 7.3^{a,b}	65.0 ± 8.4^{a}	71.4 ± 7.1^{a,b}	68.0 ± 8.8^{a,b}
	Day 5	77.1 ± 7.2^{a,b}	73.7 ± 8.1^{c}	65.5 ± 12.6^{a}	74.8 ± 7.4^{b}	74.8 ± 5.9^{b}
	Day 7	81.4 ± 4.6^{b}	74.7 ± 6.1^{c}	79.2 ± 5.5^{b}	81.4 ± 4.1^{b}	76.1 ± 9.8^{b}
VCL (μm/s)	Day 0	126.0 ± 22.4^{a,b}	111.6 ± 19.2^{a,b}	98.4 ± 11.4^{a}	91.27 ± 21.2^{a,b}	88.8 ± 13.8^{a,b}
	Day 1	137.2 ± 14.8^{a}	108.7 ± 15.8^{a,b}	91.0 ± 10.9^{a}	93.5 ± 8.09^{a}	93.2 ± 8.8^{a}
	Day 3	128.7 ± 10.3^{a,b}	98.6 ± 12.6^{a}	89.3 ± 10.4^{a}	100.6 ± 13.1^{a,b}	94.7 ± 18.0^{a,b}
	Day 5	128.6 ± 18.4^{a,b}	112.0 ± 21.0^{b}	93.8 ± 24.2^{a,b}	111.8 ± 14.7^{b}	111.3 ± 15.0^{b}
	Day 7	124.0 ± 12.6^{b}	112.0 ± 13.8^{b}	114.9 ± 14.5^{b}	119.4 ± 17.3^{c}	110.3 ± 15.6^{c}
VSL (μm/s)	Day 0	53.3 ± 4.4^{a}	61.2 ± 4.6^{a,c}	58.9 ± 5.7^{a}	55.6 ± 8.2^{a,b}	55.6 ± 6.8^{a,b}
	Day 1	53.9 ± 5.1^{a,b}	55.2 ± 5.2^{b}	54.4 ± 7.2^{a}	56.4 ± 3.5^{a}	56.8 ± 3.3^{a}
	Day 3	54.5 ± 3.6^{b}	55.0 ± 5.4^{a,b}	55.3 ± 6.5^{a}	59.1 ± 4. 9^{a,b}	56.8 ± 7.8^{a,b}
	Day 5	56.4 ± 6.5^{b,c}	60.4 ± 6.2^{c}	55.8 ± 11.1^{a}	61.9 ± 6.4^{b}	62.0 ± 5.5^{b}
	Day 7	62.5 ± 3.7^{c}	62.6 ± 4.6^{c}	66.2 ± 5.1^{b}	67.7 ± 3.1^{c}	63.8 ± 10.4^{b}

		Incubation time				
	Storage	15 min	30 min	60 min	120 min	180 min
STR	Day 0	0.74 ± 0.07^{a}	0.82 ± 0.05^{a}	0.85 ± 0.03^{a,b}	0.85 ± 0.04^{a}	0.85 ± 0.04^{a}
	Day 1	0.67 ± 0.05^{b}	0.79 ± 0.04^{b}	0.85 ± 0.03^{a}	0.84 ± 0.04^{a}	0.85 ± 0.02^{a}
	Day 3	0.70 ± 0.03^{a,b}	0.81 ± 0.02^{a,b}	0.85 ± 0.02^{a,b}	0.83 ± 0.04^{b}	0.83 ± 0.06^{a,b}
	Day 5	0.73 ± 0.05^{a}	0.81 ± 0.04^{a}	0.85 ± 0.02^{a,b}	0.82 ± 0.04^{b}	0.82 ± 0.02^{b}
	Day 7	0.76 ± 0.06^{c}	0.83 ± 0.03^{c}	0.83 ± 0.04^{b}	0.83 ± 0.05^{a,b}	0.83 ± 0.05^{a,b}
LIN	Day 0	0.48 ± 0.05^{a}	0.55 ± 0.06^{a}	0.59 ± 0.04^{a}	0.62 ± 0.07^{a}	0.63 ± 0.07^{a}
	Day 1	0.39 ± 0.05^{b}	0.51 ± 0.07^{b}	0.59 ± 0.06^{a}	0.60 ± 0.07^{a}	0.60 ± 0.03^{a}
	Day 3	0.42 ± 0.05^{b}	0.56 ± 0.05^{a,b}	0.62 ± 0.03^{a}	0.59 ± 0.06^{a}	0.61 ± 0.12^{a}
	Day 5	0.44 ± 0.06^{c}	0.55 ± 0.08^{a,b}	0.60 ± 0.05^{a}	0.56 ± 0.06^{a}	0.55 ± 0.04^{a}
	Day 7	0.50 ± 0.07^{a,d}	0.56 ± 0.07^{a}	0.58 ± 0.08^{a}	0.57 ± 0.01^{a}	0.58 ± 0.11^{a}
WOB	Day 0	0.62 ± 0.05^{a}	0.67 ± 0.05^{a}	0.70 ± 0.03^{a}	0.70 ± 0.01^{a}	0.73 ± 0.05^{a}
	Day 1	0.58 ± 0.04^{b}	0.64 ± 0.06^{b}	0.70 ± 0.05^{a,b}	0.71 ± 0.05^{a}	0.71 ± 0.03^{a,b}
	Day 3	0.59 ± 0.05^{b}	0.68 ± 0.05^{a,b}	0.72 ± 0.03^{b}	0.71 ± 0.04^{a}	0.72 ± 0.09^{a,b}
	Day 5	0.60 ± 0.06^{c}	0.66 ± 0.08^{a,b}	0.71 ± 0.06^{a,b}	0.68 ± 0.04^{a}	0.67 ± 0.05^{b}
	Day 7	0.66 ± 0.05^{d}	0.67 ± 0.06^{a}	0.69 ± 0.06^{a,b}	0.69 ± 0.08^{a}	0.69 ± 0.08^{a,b}

		Incubation time				
	Storage	15 min	30 min	60 min	120 min	180 min
ALH (µm)	Day 0	2.6 ± 0.5[a]	2.4 ± 0.4[a]	2.1 ± 0.3[a]	2.1 ± 0.2[a,b]	1.8 ± 0.4[a]
	Day 1	3.1 ± 0.4[b]	2.4 ± 0.4[a]	2.1 ± 0.3[a]	2.1 ± 0.5[a,b]	1.9 ± 0.2[a]
	Day 3	2.8 ± 0.4[a]	2.2 ± 0.3[a]	2.0 ± 0.3[a]	2.1 ± 0.4[a]	2.0 ± 0.6[a,b]
	Day 5	2.8 ± 0.4[a]	2.4 ± 0.4[a]	1.9 ± 0.3[a,b]	2.3 ± 0.3[a,b]	2.2 ± 0.4[a,b]
	Day 7	2.6 ± 0.4[a]	2.3 ± 0.4[a]	2.4 ± 0.4[b]	2.4 ± 0.5[b]	2.3 ± 0.4[b]
BCF (Hz)	Day 0	39.8 ± 1.0[a]	40.5 ± 3.0[a,b]	39.0 ± 2.7[a,b]	38.4 ± 2.3[a]	35.8 ± 3.1[a,b]
	Day 1	40.0 ± 2.1[a]	39.5 ± 2.5[a,b]	37.5 ± 2.5[a,b]	36.8 ± 1.5[a]	37.1 ± 1.8[a]
	Day 3	39.8 ± 1.3[a]	38.4 ± 2.6[a]	37.4 ± 1.8[a]	37.3 ± 1.3[a]	35.1 ± 2.0[b]
	Day 5	40.5 ± 2.5[a]	40.5 ± 3.4[a,b]	37.4 ± 5.7[a,b]	38.6 ± 2.7[a]	38.8 ± 2.3[a]
	Day 7	41.3 ± 1.8[a]	41.5 ± 2.2[b]	40.3 ± 2.3[b]	38.9 ± 2.9[a]	37.5 ± 3.5[a]

Table 3. Plasma membrane integrity of boar spermatozoa following semen incubation at 38°C (n=7). Different lowercase alphabets (a-d) in each column show significant differences (P < 0.05) among days of storage. All values are mean and standard deviation.

	Incubation (min)				
storage	15	30	60	120	180
Day 0	89.3 ± 3.59^{a,b,c}	89.2 ± 2.04^{a}	87.8 ± 2.44^{a}	85.1 ± 2.81^{a}	80.9 ± 6.14^{a,c}
Day 1	89.8± 1.69^{a}	88.5 ± 3.58^{a,b}	88.1 ± 2.81^{b}	74.5 ± 16.92^{a,b}	83.0 ± 5.84^{a,b}
Day 3	87.3 ± 1.96^{b,c}	86.5 ± 2.61^{b}	83.6 ± 2.07^{a,c}	80.1 ± 7.83^{b}	74.0 ± 12.14^{b,d}
Day 5	85.6 ± 2.96^{d}	86.3 ± 2.49^{b}	85.1 ± 3.69^{c}	81.2 ± 6.03^{a,b}	75.9 ± 9.94^{c,d}
Day 7	87.1 ± 2.59^{c}	84.4 ± 3.07^{b}	83.9 ± 3.25^{c}	80.4 ± 4.75^{b}	71.8 ± 8.78^{d}

Table 4 Pearson correlation coefficients between selected parameters for samples incubated at 38°C. Data from all incubation times and days of storage were combined (n = 175)

	PI & PNA-FITC neg. [%]	Total motility [%]	Progressive motility [%]	VAP [µm/s]	VCL [µm/s]	VSL [µm/s]	STR	LIN	WOB	ALH [µm]	BCF [Hz]	hMMP [%]
ATP pmol/10^5 sperm	0.29 ***	0.48 ***	0.45 ***	0.08	0.10	-0.03	-0.15	-0.13	-0.11	0.16 *	0.16 *	0.38 ***
EC	0.39 ***	0.26 ***	0.23 **	0.25 **	0.31 ***	0.09	-0.23 **	-0.26 ***	-0.28 ***	0.22 **	0.37 ***	0.47 ***
hMMP (%)	0.49 ***	0.43 ***	0.40 ***	0.17 *	0.41 ***	-0.25 ***	-0.60 ***	-0.59 ***	-0.55 ***	0.46 ***	0.34 ***	-

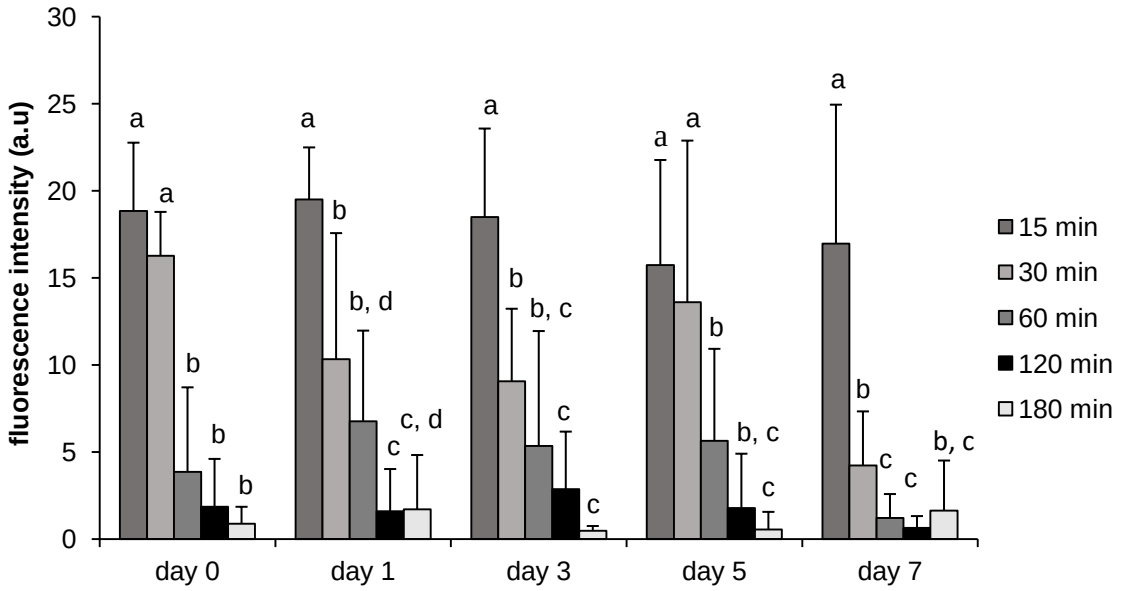
* P < 0.05 ** P < 0.01 *** P < 0.001

VAP = average path velocity VCL = curvilinear velocity VSL = straight-line velocity

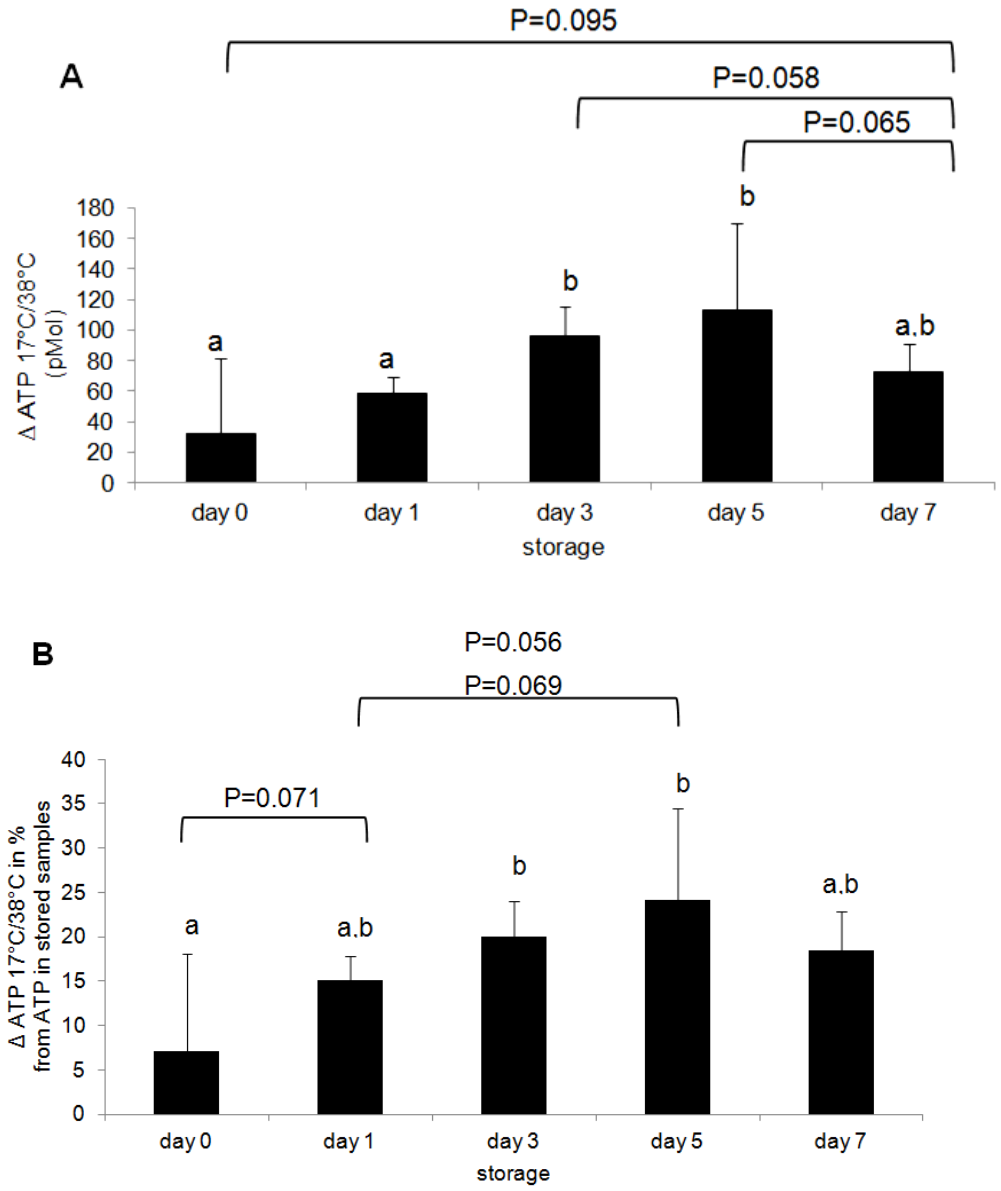
STR = straightness LIN = linearity WOB = wobble

ALH = amplitude of lateral head-displacement BCF = beat cross frequency EC = energy charge

hMMP = sperm with high mitochondrial transmembrane potential ATP = adenosine triphosphate



Supplemental Figure 1. Average fluorescence intensity for JC-1 aggregates (a.u. = arbitrary units) in live (PI negative) boar spermatozoa following liquid semen incubation at 38°C. Values are expressed as means and standard deviations (n=7 ejaculates per boar and time). Columns with different letters (a-e) at a given storage day indicate significant differences between incubation times ($P < 0.05$).



Supplemental Figure 2

(A) Difference (∆) in ATP concentration for semen samples at storage temperature (17°C) and after 15 min incubation at 38°C (n = 7). (B) Differences in ATP concentration expressed as percentage of ATP concentration in samples stored at 17°C (starting values). Different lowercase letters (a – b) indicate significant differences (P < 0.05) between different days of storage. All values are means and standard deviations.

Supplemental Table 1. Percentage of sperm with high mitochondrial transmembrane potential (hMMP), ATP content, and energy charge (EC) of semen samples after 15, 30, 60, 120 and 180 minutes incubation in a water bath at 38°C (n = 7). All values are mean and standard deviation. Different lowercase alphabets (a-d) in each column show significant differences ($P < 0.05$) between storage days.

		Incubation time				
	Storage	15 min	30 min	60 min	120 min	180 min
hMMP (%)	Day 0	92.5 ± 3.02^{a}	82.9 ± 5.87^{a}	32.95 ± 29.4^{a}	4.0 ± 5.14^{a}	0.7 ± 1.29^{a,b}
	Day 1	91.8 ± 3.08^{a}	66.5 ± 28.5^{a,b}	38.9 ± 35.0^{a,b}	9.3 ±19.56^{a}	2.3 ± 2.74^{a,b}
	Day 3	85.8 ± 9.38^{a,b}	61.5 ± 24.4^{b}	30.1 ± 36.81^{a,b}	6.6 ± 15.15^{a}	2.7 ± 5.64^{a,b}
	Day 5	82.8 ± 11.08^{a,b}	69.0 ± 33.49^{a,b}	38.2 ± 33.66^{a,b}	2.6 ± 4.64^{a}	0.1 ± 0.11^{a}
	Day 7	82.9 ± 5.75^{b}	45.6 ± 29.53^{b}	13.5 ± 20.32^{b}	6.0 ± 12.58^{a}	0.4 ± 0.3^{b}
ATP (pmol/10^5 sperm)	Day 0	421. 3 ± 54.6^{a}	418.5 ± 33.1^{a}	381.3 ± 46.2^{a}	323.8 ± 16.0^{a}	284.7 ± 38.1^{a,d}
	Day 1	326.5 ± 12.6^{b}	340.3 ± 45.3^{b}	326.8 ± 30.3^{b}	303.2 ± 15.6^{b}	296.9 ± 32.9^{a}
	Day 3	387.9 ± 43.6^{a,c}	415.5 ± 67.8^{a,c}	389.5 ± 60.9^{a}	367.9 ± 37.2^{c}	350.8 ± 36.0^{b}
	Day 5	338.9 ± 33.1^{b,c}	356.5 ± 57.3^{b,c}	306.5 ± 47.4^{b}	270.94 ± 21.6^{d}	222.95 ± 19.4^{c}
	Day 7	325.1 ± 60.5^{b}	349.3 ± 21.0^{b}	313.5 ± 28.3^{b}	318.1 ± 34.7^{a,b,c}	277.0 ± 42.4^{d}
EC	Day 0	0.74 ± 0.05^{a}	0.65 ± 0.05^{a,c}	0.67 ± 0.03^{a,b}	0.61 ± 0.05^{a,b}	0.57 ± 0.04^{a,c}
	Day 1	0.67 ± 0.03^{b}	0.72 ± 0.02^{b}	0.60 ± 0.07^{a,c}	0.57 ± 0.04^{a}	0.53 ± 0.02^{b}
	Day 3	0.66 ± 0.09^{a,b,c}	0.63 ± 0.08^{a}	0.62 ± 0.04^{a,c}	0.57 ± 0.06^{a}	0.52 ± 0.05^{b}
	Day 5	0.72 ± 0.06^{a,b}	0.70 ± 0.05^{b,c}	0.69 ± 0.05^{b}	0.64 ± 0.03^{b}	0.52 ± 0.03^{b}
	Day 7	0.62 ± 0.08^{c}	0.69 ± 0.02^{a,b,c}	0.60 ± 0.03^{c}	0.68 ± 0.04^{c}	0.60 ± 0.05^{c}

Chapter 3

Revisiting storage temperature for liquid preserved boar semen from the perspective of energy metabolism

Abstract

The optimum storage temperature for liquid preservation of boar semen has been empirically determined to be between 16°C and 18°C. The aim of the present study was to revisit the current critical storage temperature recommendations from the perspective of sperm energy metabolism. The relationship between energy measures and sperm function at different storage conditions was examined. Moreover, extender influences were considered. Split ejaculates from seven boars were diluted with a standard extender (Beltsville Thawing Solution, BTS) and cold-shock protective extender (Androstar® Plus; Minitüb GmbH, Tiefenbach), cooled down to 25, 17, 10 and 5°C and stored up to 120 h. After 24 h, 72 h and 120 h. ATP levels and energy charge were assessed at storage temperature and after re-warming of spermatozoa to body temperature. Semen samples were assessed for sperm motility (CASA), integrity of plasma and acrosomal membranes (PI and FITC-PNA staining), and mitochondria activity (JC-1/PI assay). Motility and membrane status were significantly influenced by storage length, storage temperature and extender ($P< 0.05$). Average total motility and viability were higher than 75 % in both extenders for samples stored up to 5 days at 25°C, 17°C or 10°C. Neither ATP levels, nor energy charge or motility differed for samples stored at 17°C or 25°C. Hypothermic storage at 10°C and 5°C, respectively, lead to a storage-length related decrease of both intracellular ATP levels and AEC and to a concomitant appearance of adenine nucleotides in the extender medium. The energy charge found in Androstar Plus samples at 5°C and 10°C storage was significantly higher than that in samples diluted in BTS, but no did not differ after re-warming of samples, although Androstar Plus samples contained more motile and viable spermatozoa ($P<0.05$). The percentage of live sperm with high mitochondrial transmembrane potential decreased with lower storage temperatures ($P<0.05$). In conclusion, contrary to common expectation, an elevated cell activity during long-term storage at 25°C does not lead to a metabolic exhaustion or loss of boar sperm quality. Long-term cooling

stress at 10 and 5°C affects sperm energy metabolism which may contribute to irreversible failure of cell function. The extent of this can be influenced by cold-shock protecting extenders.

Keywords: energy metabolism, cold shock, chilling, long-term storage

3.1. Introduction

Liquid preservation of boar spermatozoa at 16-18°C for up to three days is common practice in porcine artificial insemination (Johnson et al. 2000; Riesenbeck 2011). Although spermatozoa from other species are commonly stored at temperatures as low as 5°C for artificial insemination (horse: Brogan et al. 2015, bull: Vishwanath and Shannon 2000), this has not become common practice for boar spermatozoa owing to their high cold-shock sensitivity (Drobnis et al. 1993). A lower cholesterol to phospholipid ratio in spermatozoa from pigs compared to less cold-shock sensitive spermatozoa from other species is assumed to be the underlying cause (De Leeuw et al. 1990).

The upper and lower temperature limits for storage of liquid-preserved boar semen have been empirically determined. The common view is that storage at room temperature (20°C to 25°C) has the disadvantage that sperm metabolism is not fully inhibited and thus leads to a depletion of sperm energy reserves (Paulenz et al. 2000, Althouse et al. 1998). In agreement with this theory, the upper temperature limit for storage of liquid boar semen has been suggested to be 20°C (Paulenz et al. 2000). A higher percentage of spermatozoa with low mitochondrial membrane potential and a lower average oxidoreductive capability of spermatozoa at 25°C compared to 17°C point in the same direction (Gaczarewicz et al. 2015).

Lowering the storage temperature below 16-18°C would, in theory, further slow-down sperm metabolism. At the same time the probability for a sub-lethal chilling injury increases. The lower temperature limit for boar semen storage has been suggested to be at 12°C to 10°C which is in agreement with the lower boundary for lipid phase transition processes in whole spermatozoa (Althouse et al. 1998; Schmid et al. 2013).

Chilling of boar spermatozoa below 10°C is associated with leakage of ions, ATP and enzymes into the surrounding medium due to membrane disruption (reviewed in White 1993). Recent data indicate that spermatozoa require an increasing amount of ATP to re-activate a fully motile state when stored at 17°C (Henning H, Nguyen QT, Wallner U, Beyerbach M, Waberski D, unpublished). Low motility in cold-shocked samples is commonly associated with low ATP levels and with a loss in specific

regulation of ATP-dependent processes, such as calcium influx during capacitation (Dziekońska et al. 2009, Schmid et al. 2013). A non-compensable loss of ATP from the intracellular ATP pool or an unbalanced energy charge may be causative factors in a gradual loss of sperm function in cold-shocked spermatozoa. The sensitivity of boar spermatozoa to cold-shock can be modulated by the composition of the extender (Dziekońska et al. 2009, Schmid et al. 2014, Schulze et al. 2013). Higher percentage of motile and viable spermatozoa at storage temperatures of 10°C and 5°C have for Androstar Plus extender have been recently reported (Schmid et al. 2014). Whether such protective effects are mediated by a stabilization of the sperm energy metabolism during cooling and storage has not yet been investigated.

The aim of the present study was to revisit the current critical storage temperature recommendations from the perspective of sperm energy metabolism. Therefore, the influence of different storage temperatures, i.e. 25°C, 17°C, 10°C and 5°C, and storage times on energy metabolism of boar spermatozoa was investigated. The relation between sperm energy metabolism and sperm functional parameters was then assessed. In addition, it was tested whether stabilization of membrane integrity and motility at low storage temperatures by using a cold-shock protective extender is associated with a stabilization of the energy metabolism.

3. 2. Material and Methods

3. 2. 1. Experimental design

Boar semen (n=7) was split in two parts and diluted in Beltsville Thawing Solution (BTS) or Androstar®Plus (ASP) to a sperm concentration of $20x10^6$ sperm/ml. Split samples were stored at 25°C, 17°C, 10°C and 5°C, respectively, and analyzed after 24 h, 72 h, and 120 h storage, respectively. ATP concentration, adenylate energy charge (EC), as well as viability and acrosome integrity were assessed in stored samples and after 15 min incubation of the samples at 38°C. The percentage of live sperm with high mitochondrial transmembrane potential, and motility parameters as assessed by computer assisted semen analysis (CASA) were determined only in samples incubated for 15 min at 38°C.

3. 2. 2. Chemicals

All chemicals were of analytical grade and, unless otherwise stated, obtained from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), and Roth (Karlsruhe, Germany), respectively. Propidium iodide (PI) was obtained from Sigma Aldrich (Steinheim, Germany), while Hoechst 33342 (H342) was purchased from life technologies (Darmstadt, Germany). Peanut agglutinin conjugated to fluorescein-isothiocyanate (PNA-FITC) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) were both from Enzo Life Sciences (Lörrach, Germany).

3. 2. 3. Semen processing

Semen was collected from seven healthy, mature boars housed at the facilities of the Unit for Reproductive Medicine, University of Veterinary Medicine Hannover. From each boar, one ejaculate was collected by the gloved hand method into disposable collection bags with integrated filter to remove the gel fraction. Collection bags were mounted in pre-warmed thermos cups (38°C) to prevent any cold-shock for the spermatozoa. After collection, the semen was immediately transferred to the laboratory in Styrofoam boxes. Sperm motility was evaluated subjectively for native ejaculates within 15 min after collection. Sperm concentration was determined using a “Thoma neu” counting chamber with phase-contrast microscope (Zeiss, Jena, Germany) at 200x magnification (ocular 10x, objective 20x, phase 1). Morphology of fixed spermatozoa was evaluated as described in Nguyen et al. (unpublished). Ejaculates with more than 70% motile and less than 25% morphologically abnormal sperm were diluted isothermically (33°C) on a split-sample basis with Beltsville Thawing Solution (BTS) or Androstar® Plus (both from Minitube, Tiefenbach, Germany) to a concentration of 20×10^6 cells/ml. Four samples of 100 mL semen for each extender were prepared in screw cap bottles for semen storage (Minitube), i.e. one bottle for each storage temperature. The samples were stepwise cooled to 25°C, 17°C, 10°C, and 5°C, respectively. Bottles were kept at room temperature for 1 hour. Thereafter, one bottle from each extender was transferred to an incubator and stored at 25°C. After a total of 1.5 h at room temperature, all remaining samples were transferred to a 17°C storage unit. A subset of samples was kept for 60 min at 17°C and then transferred to a storage unit set at 10°C. After a further 60 min at 10°C, one

bottle each of BTS or Androstar®Plus diluted semen was finally transferred to a 5°C storage unit. Semen samples were analysed after 24 h, 72 h, and 120 h storage, respectively. During storage semen samples were gently mixed once daily.

3. 2. 4. Computer-assisted semen analysis (CASA)

Semen samples were analysed as described in Nguyen et al. (unpublished). Briefly, 2 ml semen were incubated at 38°C for 15 min before motility was analysed on a computer-assisted sperm analysis system (Sperm Vision®; Minitube, Tiefenbach, Germany). Total motility (TM; %) and progressive motility (PM; %) were recorded as well as average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL; µm/s), straightness (STR = VSL/VAP), linearity (LIN = VSL/VCL), wobble (WOB = VAP/VCL), amplitude of lateral head-displacement (ALH; µm), and beat cross frequency (BCF; Hz) for each single spermatozoon. Average values of all sperm kinematic parameters were calculated for progressive motile spermatozoa. A spermatozoon was defined by a head area between 23 μm^2 and 120 μm^2. It 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 (VSL) exceeded 4.5 µm

3. 2. 5. Assessment of viability and acrosome integrity

The integrity of the plasma and acrosomal membrane was assessed using a triple stain procedure with propidium iodide (PI), fluorescein-isothiocyanate-conjugated peanut agglutinin (PNA-FITC) and Hoechst 33342. Spermatozoa with intact plasma membrane and damaged plasma membrane are differentially stained with propidium iodide (PI). PNA-FITC was used to distinguish between spermatozoa with intact and defective outer acrosomal membrane. Hoechst 33342 stains the chromatin of all DNA-containing particles in the sample, thereby allowing to distinguish between cells and debris.

Aliquots of 2 ml diluted semen were incubated for 10 minutes in a water bath at 38°C. An aliquot of incubated semen was mixed with PI (final concentration: 5 µg/mL), PNA-FITC (final concentration: 3 µg/mL) and Hoechst 33342 (final concentration: 0.75 µg/mL). Samples were incubated for another 5 minutes at 38°C

and then transferred to a HEPES-buffered saline solution (HBS; 137 mM NaCl, 20 mM HEPES, 10 mM glucose, 2.5 mM KOH (300 ± 5 mOsmol/kg) for assessment. Analysis of stained samples was performed on a DAKO Galaxy flow cytometer (Dako Cytomation GmbH, Hamburg, Germany), equipped with a 488-nm blue argon laser (15 mW), an HBO lamp (excitation main peak at 365 nm) and filters for green (537.5/22.5 nm, FL-1), orange (590/25 nm, FL-2), red (630 nm LP, FL-3) and blue emitted light (465 nm BP, FL-4), respectively. In total, 10,000 events were recorded per sample. The sperm population was defined as DNA-containing events (H342 positive) in the size range of single spermatozoa as assessed by characteristic forward scatter distribution patterns. The percentage of viable (PI negative) spermatozoa with an intact acrosome (PNA-FITC negative) was evaluated using FloMax®" software (version 2.8, Partec, Münster, Germany). Overlap of emission spectra between FITC and PI was compensated post acquisition.

3. 2. 6. Assessment of mitochondrial transmembrane potential in live spermatozoa

The mitochondrial transmembrane potential (MMP) in spermatozoa was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) in combination with propidium iodide (PI) and Hoechst 33342 (H342) as described in Nguyen et al. (2015, unpublished). Hoechst 33342 (H342) allowed to distinguish between DNA-containing events (predominantly spermatozoa) and debris. The use of PI allowed to restrict data evaluation to live (PI negative) spermatozoa. A semen sample (970 µl) was mixed with 1 µl JC-1 stock solution (1.53 mM), 10 µl Hoechst 33342 stock solution (150 µg/ml), and 20 µl PI stock solution (1 mg/ml). Subsequently, samples were incubated for 15 min at 38°C in a water bath. A 5 µl subsample was transferred into preheated HBS solution (38°C) and 10,000 events were analysed on the DAKO Galaxy flow cytometer described above. Data evaluation was restricted to live, single spermatozoa, i.e. H342 positive, but PI negative events with a forward scatter signal in the range of single spermatozoa. Live spermatozoa with a high green, but low orange fluorescence intensity were considered as having a low mitochondrial transmembrane potential (lMMP) while spermatozoa with low green, but high orange fluorescence intensity were considered as having a high mitochondrial transmembrane potential (hMMP).

3. 2. 7. ATP and energy charge assay

The ATP concentration and adenylate energy charge (EC) were determined using a luciferase reaction kit according as described in Henning et al. (unpublished; FL-AA kit, Sigma-Aldrich, Steinheim, Germany). Samples of diluted boar semen (100 µl) were directly taken at the storage temperatures of 25, 17, 10 and 5°C and after having incubated the samples for 15 min at 38°C. They were mixed with 1 µl phosphatase inhibitor cocktail (P5726, Sigma-Aldrich, Steinheim, Germany) and kept on ice for 30 min. After inhibitor treatment samples were stored at -20°C for later ATP and EC assessment. For extraction of nucleotides, a heated boiling buffer (50 mM Tricine, 10 mM $MgSO_4$, 2 mM EDTA, pH =7,8) was added to aliquots of frozen semen samples and the mixture was kept for 10 min at 95°C to 96°C. Thereafter, the tubes were chilled on ice for 10 min and subsequently centrifuging at 5000 x g for 30 min at 4°C (Universal 30 RF, Hettich, Germany). The supernatant was used for determination of ATP and EC. All procedures were carried out as detailed in Henning et al (unpublished). ATP concentration was calculated as pmol ATP per 10^6 spermatozoa. The energy charge was calculated as described by Ball and Atkinson (1975) by the formula:

$$\text{Adenylate energy charge (EC)} = \frac{[\text{ATP}] + 0.5\,[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

3. 2. 8. Statistical analysis

Data were analysed with Excel (Microsoft Office 2010, Microsoft Corporation, Washington, USA) and Statistical Analysis Software (SAS, version 9.3, Cary, NC, USA). Data from all parameters were tested for normal distribution with a Shapiro Wilk test. The majority of parameters was not normal distributed even after logarithmic or quadratic transformation. Pair-wise comparisons of data from different storage temperatures and extenders were done with Wilcoxon's signed rank test for paired samples. Spearman rank correlation coefficients were calculated for selected parameters. The significance level was set at $P<0.05$.

3. 3. Results

3. 3. 1. ATP content, energy charge, and membrane integrity of boar sperm at different storage temperatures

Samples stored at 17°C were considered as reference point for all comparisons, because this temperature has been accepted as optimal storage temperature for liquid preserved pig spermatozoa. The ATP content of semen samples at 5°C in BTS extender was on all days of storage lower ($P<0.05$) when compared to samples at 17°C (Figure 1A). The ATP content did not differ between semen stored at 25°C, 17°C and 10°C at each day of storage for a given extender. On day 1 and day 5, samples stored at 5°C in Androstar® Plus had even similar ATP levels when compared to samples at higher storage temperatures. A significant difference between both extenders was noted on day 1 for samples stored at 5°C. Samples stored in Androstar® Plus had a higher ATP content than samples stored in BTS ($P<0.05$).

The energy charge did not differ between samples stored at 17°C or 25°C for both extenders (Figure 1B). The average value was on all days higher than 0.65 for each combination of extender and storage temperature (Figure 1B). At all days, Samples stored at 10°C or 5°C had always a lower energy charge when compared to samples at 17°C and 25°C ($P<0.05$). The energy charge in samples stored at 5°C was lower than the energy charge for samples at 10°C when BTS extender was used ($P<0.05$). When samples were stored at 5°C and 10°C in Androstar®Plus, the energy charge did not differ ($P>0.05$). A drop in energy charge to values of less or equal than 0.50 was present on day 3 for samples stored at 5°C, and on day 5 for samples stored at 5°C and 10°C, respectively. A difference in energy charge for samples stored either in BTS or Androstar® Plus was only noted on day 1 at 10°C and 5°C.

The sum of nucleotides, i.e. AMP, ADP, and ATP, that could be detected in the extender was the higher, the lower the storage temperature was (Supplemental Figure 1). Almost no nucleotides could be detected in samples stored at 25°C and 17°C. The nucleotide concentration was between 50 and 100 pMol per 25 µl extender at 10°C and higher than 200 pmol per 25 µl extender for samples stored at 5°C. Fluctuations over time at a given storage temperature were not detected.

The percentage of viable sperm with intact acrosomes (PI & PNA-FITC negative) was on average higher than 65% for all combinations of storage temperature and storage time in both extenders (Figure 1C). For samples stored at 25°C, 17°C and 10°C, respectively, the average values were higher than 80% throughout the whole experiment irrespectively of the extender. On day1 and day 3, samples stored at 10°C or 5°C in BTS contained less viable, acrosome-intact sperm than samples stored at 17°C or 25°C in BTS ($P<0.05$). On day 5, only samples at 5°C (67.2 ± 12.10 %) differed from the samples at 17°C (89.2 ± 5.39 %) and 25°C (87.3 ± 6.81 %; both $P<0.05$). A differences between samples stored at 10°C and 5°C was present on all days of storage. In BTS extender, no difference in the percentage of viable, acrosome intact spermatozoa was detected between samples stored at 25°C or 17°C. When Androstar®Plus was used, samples stored at 25°C contained less viable, acrosome intact spermatozoa than samples stored at 17°C. At day 1 and 3, samples stored at 10°C in Androstar®Plus had less viable, acrosome intact sperm than samples at 17°C (Δ day 1: 2.2%; Δ day 3: 1.5 %; both $P<0.05$). On day 5 samples in Androstar®Plus did not differ at 17°C (90.3 ± 3.76 %) and 10°C (87.8 ± 5.78 %; $P>0.05$). Samples at 5°C had always lower values when compared to samples at 17°C or 10°C, but were still on a high level (day 5: 81.6 ± 6.03 %) in Androstar®Plus. Differences between both extenders were most prominent for samples stored at 5°C, with values of 85.0 ± 4.16 % for Androstar®Plus and 70.2 ± 8.09 % for BTS already at day 1. Smaller differences between both extenders for samples stored at 10°C were detectable on day 1 and day 3 with a maximum average difference of 7 % on day 3.

3. 3. 2. ATP content, energy charge, mitochondrial transmembrane potential and membrane integrity after incubation at 38°C

The ATP content of the semen samples after incubation at 38°C on day 1 was similar irrespectively of the storage temperature (Figure 2A; $P>0.05$). On day 3 and day 5, samples stored at 5°C in BTS had lower ATP content than samples stored at 17°C. On day 3, this was only a trend for samples stored in Androstar®Plus ($P=0.0781$), but became significant on day 5 ($P<0.05$). The ATP levels for samples stored at 10°C where lower compared to samples stored at 17°C only on day 5 of

storage in BTS. Samples at 25°C and 17°C did not differ at the different days of storage for both extenders.

In general, the energy charge tended to be lower after incubation at 38°C when compared to values at storage temperature. This was especially evident for samples stored at 25°C and 17°C. On day 1, values ranged between 0.71 ± 0.09 and 0.45 ± 0.12 for samples stored in BTS at 25°C and 5°C, respectively. After incubation at 38°C, the values for the same samples were 0.60 ± 0.09 and 0.46 ± 0.13, respectively. However, at day 1, no differences were detected between samples stored at different temperatures in BTS after incubation at 38°C (Figure 2). On day 3 and 5, samples stored at 5°C had a lower energy charge compared to samples at 17°C ($P<0.05$). On day 5, also samples stored at 10°C (0.50 ± 0.09) had a distinct lower energy charge compared to samples at 17°C (0.68 ± 0.1; $P<0.05$). A similar pattern was observed for spermatozoa preserved in Androstar®Plus . Only exception was that already on day 1, samples stored at 5°C (0.49 ± 0.10) had a lower energy charge after incubation than samples stored at 17°C (0.67 ± 0.10 $P<0.05$).

The percentage of live spermatozoa with high mitochondrial transmembrane potential (hMMP) after incubation at 38°C was on day 1 and 3 always highest in samples stored at 25°C and 17°C (Figure 2C). On day 1, the percentage of live sperm with hMMP was the lower, the colder the spermatozoa had been stored in BTS (17°C: 85.3 ± 5.9 %, 10°C: 77.4 ± 8.8 %, 5°C 69.2 ± 12.1 %; all $P<0.05$). The gradual differences between storage temperatures became the more evident the loner semen was stored. On day 5, gradual differences between samples stored at 25°C (85.5 ± 10.2 %,), 17°C (76.5 ± 18.6 %) and 10°C (67.2 ± 22.5 %) were observed, samples stored at 5°C (60.4 ± 22.5 %, contained less live sperm with hMMP than samples stored at 25°C and 17°C ($P<0.05$), but were not different from samples stored at 10°C ($P>0.05$). In samples diluted with Androstar®Plus , storage at 10°C resulted at all days in lower percentages of live sperm with hMMP (day 1: 77.4 ± 8.8 %, day 3: 62.8 ± 23.6 %, day 5: 67.3 ± 22.5 %) compared to samples stored at 17°C (day 1: 85.4 ± 5.9 %, day 3: 71.1 ± 14.7 %, day 5: 76.5 ± 18.6 %; all $P<0.05$; Figure 2C). For samples stored at 5°C this was only the case on day 3 and day 5 of storage. Differences between extenders were not detected for any combination of storage temperature and storage time.

The percentage of viable, acrosome intact spermatozoa after incubation at 38°C was on average higher than 80 % for all combinations of extender, storage temperature and storage length, except for samples stored in BTS at 5°C (day 1: 70.2 ± 8.1 %, day 3: 67.6 ± 15.9 %, day 5: 67.2 ± 12.1 %; Supplemental Figure 2). Samples stored at 25°C did not differ from samples stored at 17°C. Samples stored BTS at 17°C had higher values for live, acrosome intact spermatozoa than samples stored at 10°C. Samples stored at 5°C contained always the lowest percentage of live, acrosome intact sperm. A similar relation was present on day 1 and day 3 for samples stored in Androstar®Plus. Samples stored in BTS had lower values for live, acrosome intact spermatozoa at 10°C and 5°C compared to samples stored in Androstar®Plus on all days of storage ($P<0.05$).

3. 3. 3. Motility of samples stored at different temperatures

The average total motility was higher than 75 % in both extenders for samples stored up to 5 days at 25°C, 17°C or 10°C (Supplemental Table 1 and 2). Samples stored at 5°C had a total motility of less than 60% in BTS, but of more than 70 % in samples stored in Androstar®Plus (c.f. Supplemental Table 1 and 2). Progressive motility was chosen for a detailed comparisons between storage temperatures, because average parameters for motility descriptors are given for progressive motile sperm.

Similar to total motility, progressive motility was higher than 65% for all samples stored up to five days at 25°C, 17°C or 10°C (Figure 3A). No difference between samples stored at 25°C or 17°C were noted at any day of storage. Samples stored at 5°C had a progressive motility of less than 40% in BTS, but of more than 55 % in samples stored in Androstar®Plus (Figure 3A). On all days of storage, progressive motility was lower for samples stored in BTS at 10°C when compared to 17°C. Likewise, motility after storage at 5°C was lower when compared to motility after storage at 10°C. The same relations were observed for samples stored in Androstar®Plus on day 3 and 5 of storage. On day 1, only samples stored at 5°C had a lower progressive motility when compared to samples from higher storage temperatures. Samples in BTS had a lower progressive motility on day 1 and day 3 after storage at 5°C or 10°C compared to samples stored in Androstar®Plus ($P<0.05$). On day 5, this was only the case for samples stored at 5°C.

The curvilinear velocity of progressive motile spermatozoa is on day 1 and day 5 lower for samples stored at 25°C (day 1: 85.2 ± 7.9 µm/s, day 5: 82.0 ± 13.2 µm/s) and 5°C (day 1: 82.9 ± 9.4 µm/s, day 5: 80.1 ± 13.3 µm/s) when compared to samples stored at 17°C (day 1: 91.2 ± 12.3 µm/s, day 5: 95.0 ± 15.5 µm/s) in BTS (Figure 3B, all $P<0.05$). There is at no day a difference between samples stored at 17°C or 10°C in BTS ($P>0.05$). On the contrary, progressive motile sperm in Androstar® Plus are significantly faster after storage at 10°C and 5°C for 3 or 5 days compared to samples held at 17°C. Their VCL increases during storage at 10°C or 5°C (Supplemental Table 1). At the same time the linearity, straightness and wobble for spermatozoa stored in Androstar®Plus at 10°C and 5°C decreased while their amplitude of lateral head-displacement increased (c.f. Supplemental Table 1). For sperm stored in BTS only an increase in VCL between day 1 and day 3 of storage was noted (Supplemental Table 2). Progressive motile spermatozoa displayed a higher VCL in Androstar® Plus at 10°C (day 1) and 5°C (day 3 and day 5) when compared to samples stored in BTS ($P<0.05$).

The storage temperature had no influence on linearity of the sperm track in BTS-diluted samples in day 1 (Figure 3C). On day 3 and day 5, LIN was lower for samples stored at 10°C when compared to 17°C ($P<0.05$). In Androstar®Plus, samples stored at 5°C or 10°C had always lower LIN when compared to samples at 17°C or 25°C ($P<0.05$).

The amplitude of lateral head-displacement showed no difference for BTS-stored semen from all temperatures (Figure 3D). On day 3, the ALH was higher for spermatozoa stored at 10°C (2.35 ± 0.35 µm) compard to cells stored at 25°C (1.98 ± 0.3 µm), 17°C (2.09 ± 0.38 µm) or 5°C (2.05 ± 0.33 µm; all $P<0.05$). On day 5, ALH for spermatozoa stored at 10°C differed only from those stored at 25°C and 5°C. In Androstar®Plus, values for ALH were already significantly increased after 5°C storage (2.11 ± 0.18 µm) when compared to samples stored at 17°C (1.91 ± 0.25 µm; $P<0.05$). After 3 days storage, ALH was increased in both, samples at 10°C (2.47 ± 0.41 µm) and 5°C (2.49 ± 0.35 µm), compared to 17°C (2.15 ± 0.28 µm) or 25°C (2.17 ± 0.34 µm).The most distinct differences were observed after five days storage. Samples stored at 25°C (2.12 ± 0.23 µm) and 17°C (2.11 ± 0.32 µm) had still similar values, while ALH in samples from 10°C had increased further (2.47 ±

0.34 µm). Samples stored at 5°C had the highest ALH (2.72 ± 0.44 µm) on day 5 (Figure 3D).

3. 3. 4. Correlation of energy metabolism with sperm function and integrity

The data from both extenders and all storage temperatures were combined in one data set to calculate correlations for ATP levels and energy charge with parameters of sperm function and integrity (Table 1). The energy charge of samples at storage temperature was significantly correlated with total motility ($r=0.70$; $p<0.001$), progressive motility ($r=0.71$; $p<0.001$), the percentage of live spermatozoa with hMMP ($r=0.49$; $p<0.001$), and the percentage of live acrosome intact cells at 38°C ($r=0.62$; $p<0.001$). The ATP content of samples at storage temperature was also positive correlated to the afore-mentioned parameters, but all correlations coefficients were lower (0.23 to 0.42; c.f. Table 1). The energy charge and ATP levels measured at storage temperature were also weak to moderate correlated to descriptors of the sperm motility trajectory. In addition to Table 1, correlations were also calculated separately for samples at each different storage temperature (Supplemental Table 3). Correlations between ATP levels or energy charge at storage temperature with sperm parameters similar to the full data set were only present for samples at 5°C. Correlations of ATP levels at 5°C with total motility ($r=0.53$; $p<0.001$), progressive motility ($r=0.54$; $p<0.001$), the percentage of live spermatozoa with hMMP ($r=0.33$; $p<0.001$), and the percentage of live acrosome intact cells at 38°C ($r=0.55$; $p<0.001$) were higher when compared to the full data set. Also correlations of ATP levels with sperm velocity and ALH and BCF were higher when compared to the full data set. Notably, energy charge at 5°C, 10°C or 25°C, but not 17°C, was consistently positive correlated with progressive motility ($r=0.33$ to 0.45; $p<0.01$) and the percentage of live spermatozoa with hMMP ($r=0.34$, $p<0.001$ to $r=0.56$; $p<0.001$). Similarly, ATP levels at 5°C ($r=0.49$; $p<0.001$), 17°C ($r=0.46$; $p<0.001$), and 25°C ($r=0.57$; $p<0.001$) were positively correlated with ALH.

Surprisingly, the correlations between energy charge after incubation at 38°C and total motility ($r=0.58$; $p<0.001$), progressive motility ($r=0.59$; $p<0.001$), the percentage of live spermatozoa with hMMP ($r=0.39$; $p<0.001$), and the percentage of live acrosome intact cells at 38°C ($r=0.20$; $p<0.01$) were weaker than for energy charge at storage temperature. The correlations between ATP levels after incubation

at 38°C and total motility (r=0.42; p<0.001), progressive motility (r=0.48; p<0.001), the percentage of live spermatozoa with hMMP (r=0.20; p<0.01), and the percentage of live acrosome intact cells at 38°C (r=0.38; p<0.001) were similar to those for ATP levels measured at storage temperature.

3. 4. Discussion

In the present study, diluted boar semen samples were subjected to different storage temperatures between 25°C and 5°C to revisit the storage temperature limits for liquid preserved boar spermatozoa from the perspective of cell energy metabolism. The optimum temperature for storage of boar spermatozoa has been determined empirically to be 16-18°C (Johnson et al. 2000). This temperature range is a compromise between slowing down sperm activity (as visually judged by motility) and disruption of sperm integrity due to cold shock. Storage temperatures above 20°C have been thought to insufficiently slow down sperm metabolism leading to an exhaustion of sperm (Paulenz et al. 2000). Indeed, in individual samples a flagella beating of sperm can be observed when stored at 25°C. However, the current data clearly show that the potential risk of higher sperm activity and exhaustion due to a lack in energy cannot be confirmed. Neither ATP levels, nor energy charge, nor motility differed for samples stored at 17°C or 25°C in BTS extender. Mitochondrial function in terms of a stable mitochondrial transmembrane potential in live cells is even slightly better preserved at the higher storage temperature.

The critical lower storage temperature for boar spermatozoa has been suggested to be 12°C when using Androhep extender (Althouse et al. 1998) or 10°C when using Androstar®Plus (Schmid et al. 2014). At both temperatures, fertility data obtained under field conditions did not differ compared to samples stored at 17°C. Noteworthy, 9°C to 10°C is also the lower end point for major phase transition processes in boar spermatozoa (Schmid et al. 2014; Drobnis et al. 1993). The classical view on chilling injury is that due to lipid phase transition and phase separation processes the sperm membranes become leaky (White 1993). In agreement with these observations, a decrease in viable and motile spermatozoa at 10°C and 5°C was accompanied with a leakage of adenine nucleotides into the extender. The most abundant nucleotide in the extender was AMP. It is most likely that also ATP and ADP leaked into the extracellular environment, but were readily

degraded to AMP. The presence of AMP in the medium might explain that ATP levels in stored spermatozoa did not differ for samples at 10°C and 17°C, but energy charge was already lower in samples stored at 10°C. In this respect, samples preserved in an extender with a cold-shock protective substance (Androstar®Plus), had a higher energy charge at 10°C and 5°C which might be due to the stabilization of the plasma membrane.

An important aspect of the study is that ATP levels and energy charge were not only assessed at a given storage temperature, but also after re-warming of samples to body temperature. Surprisingly, lower ATP levels and EC for samples stored at 5°C for 24 h could not be confirmed once samples had been incubated at 38°C after. At the same time, the percentage of motile cells in BTS extender was considerably decreased while only a moderate decrease in live sperm with high MMP was found. Earlier research found a high activity of mitochondria shortly after re-warming which may produce a spatio-temporal overshoot in available ATP (Henning H, Nguyen QT, Wallner U, Beyerbach M, Waberski D,unpublished). However, ATP produced by oxidative phosphorylation in the mitochondria has been found dispensable for maintenance of general flagellar beating (Miki et al. 2004). The lack of a phosphagen system in porcine spermatozoa which acts as a transport shuttle for transporting ATP from the mitochondria to the distal dynein-ATPases would preclude the usage of mitochondria-derived ATP (Kamp et al. 1996). The relative high ATP levels after re-warming of samples stored for 24 h at 5°C cannot solely be attributed to mitochondria activity. Glycolysis is supposed to be the main source of ATP in boar spermatozoa in the presence of glucose (Marin et al. 2003). Assuming that glycolysis is still active in sperm after chilling and re-warming, the discrepancy between ATP levels and motility points to a disruption of motility regulating cellular processes by irreversible failure in utilization of ATP. With progressing storage time, also energy balance and production of ATP become highly disturbed.

The differences between extenders for progressive motile and viable, acrosome intact sperm were quite prominent for samples stored at 5°C. These differences were not reflected in energy charge or ATP content. Only after the most extreme combination of temperature and time (120 h, 5°C), small differences were also found in energy charge. This may point to a stabilizing mechanism of Androstar®Plus on

the level of the plasma membrane which has, if any, only indirect influence on the intracellular energy balance. Values for energy charge below 0.5 after re-warming of samples stored at 5°C to 38°C suggest a seriously impaired energy metabolism. The data indicate that motility at 38°C can be maintained over a wide range of ATP levels and energy charge. Using an extender with a cold-shock protective substance resulted in the preservation of a high percentage of (progressive) motile and viable sperm in the present study. A protective effect against cold shock has also been reported in other studies (Schmid et al. 2014, Schulze et al. 2013). The discrepancy in observations from measures of sperm energy metabolism and functional parameters might be due the fact that ATP and EC are not related to a single spermatozoon. Both parameters are assessed for the whole sample. An extrapolation of values to single spermatozoa is hardly possible, because of an increasing number of non-viable, leaky spermatozoa and the presence of nucleotides in the extender. Probably, very heterogeneous subpopulations of spermatozoa are present in a given sample with respect to energy metabolism

Chilling and rewarming of spermatozoa below 15°C has been associated with capacitation-like changes (Green and Watson 2001). Samples in Androstar®Plus show signs of hyperactivation-like motility patterns that were not detected in BTS stored spermatozoa. The fact that this observation was restricted to one extender is most likely to due to the fact that in Androstar®Plus a much higher percentage of spermatozoa were still able to exhibit progressive motility at later storage times. Intracellular changes may occur due to the temperature decrease that cannot be compensated for.

Spermatozoa require ATP for reactivation of motility (Henning H,Nguyen QT, Wallner U, Beyerbach M, Waberski D, unpublished).The increasing demand of samples for ATP with storage time at 17°C to reactivate motility could not be confirmed in this study (data not shown). However, the relatively strong correlations between EC on storage temperature level and motility after subsequent re-warming at 38°C suggest that EC at storage temperature might to some extent be determinative for success of motility activation. The relevance of ATP levels for sperm velocity and movement characteristics remains unclear. Correlation coefficients indicate that the relation is rather weak. Indeed, speed of sperm motility and the flagella wave pattern are not

only dependent on ATP but are highly regulated amongst others by intracellular pH, calcium concentration and protein phosphorylation (reviewed by Harayama 2013; Suarez 2008).

In conclusion, storing semen samples at temperatures between 25°C and 10°C results only in minor effects on sperm quality. Semen samples exposed to long-term cooling stress at 10 and 5°C exhibit a gradual loss of ATP and energy charge which may contribute to irreversible failure of cell function. Extending the possible range of storage temperatures to 5°C is facilitated by cold-chock protective extenders. However, the choice of extender had only a minor impact on energy metabolism and ATP levels after rewarming of spermatozoa. Contrary to common expectation, an elevated cell activity during long-term storage at 25°C does not lead to a lack of energy and metabolic exhaustion of sperm.

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Figures and Tables

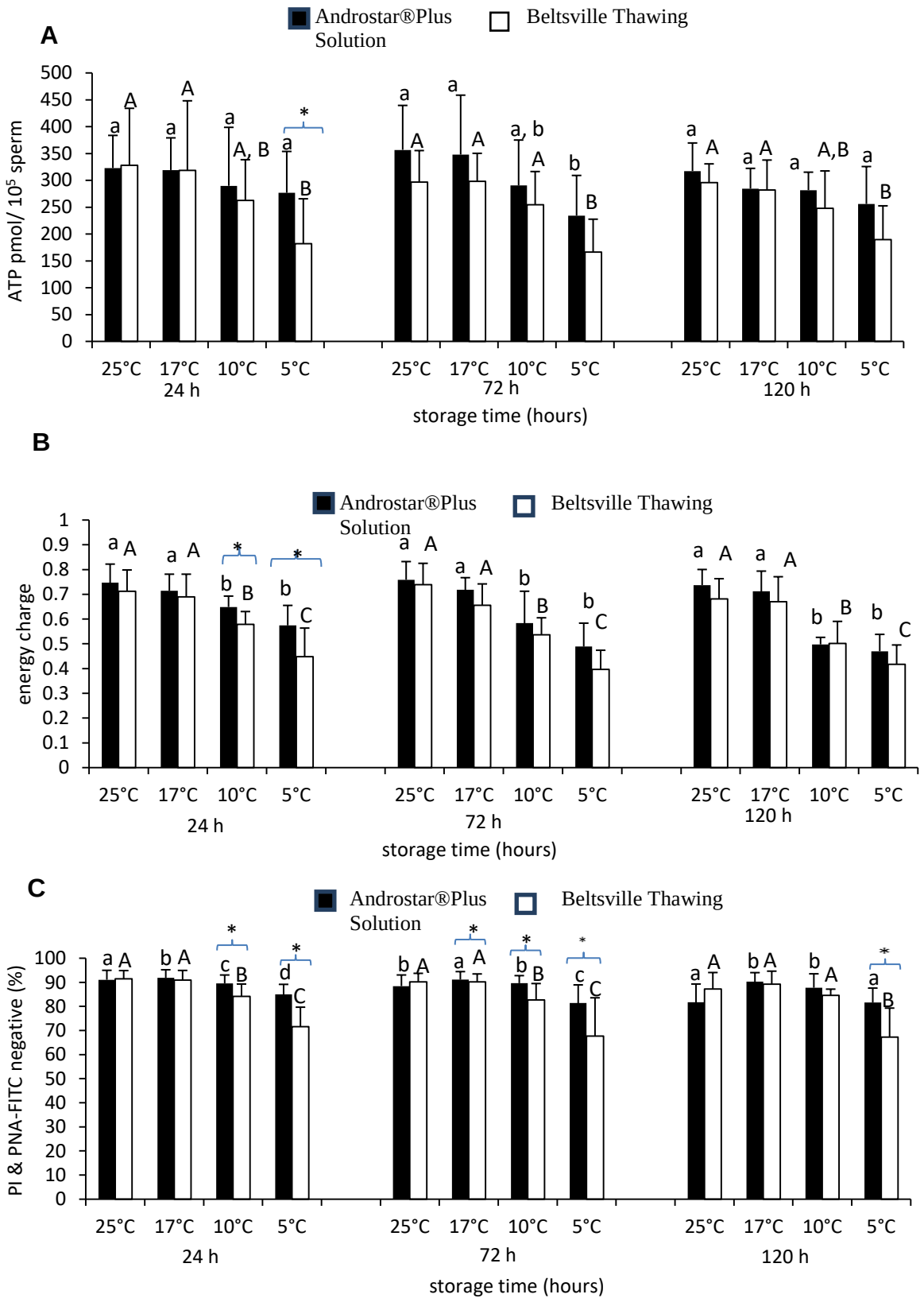


Figure 1. ATP content (A), energy charge (B) and percentage live and acrosome intact sperm (PI & PNA-FITC negative; C) of boar semen samples stored at various temperatures in either Beltsville Thawing Solution (BTS) or Androstar®Plus (ASP). Samples were analysed directly after storage. Data are presented as means ± standard deviation (n=7). Different letters indicate significant differences between storage temperatures within extenders and days ($P< 0.05$): $^{a-c}$ for samples diluted in Androstar Plus and $^{A-C}$ for samples diluted in BTS ($P< 0.05$). An asterisk indicates significant differences between extenders ($P< 0.05$).

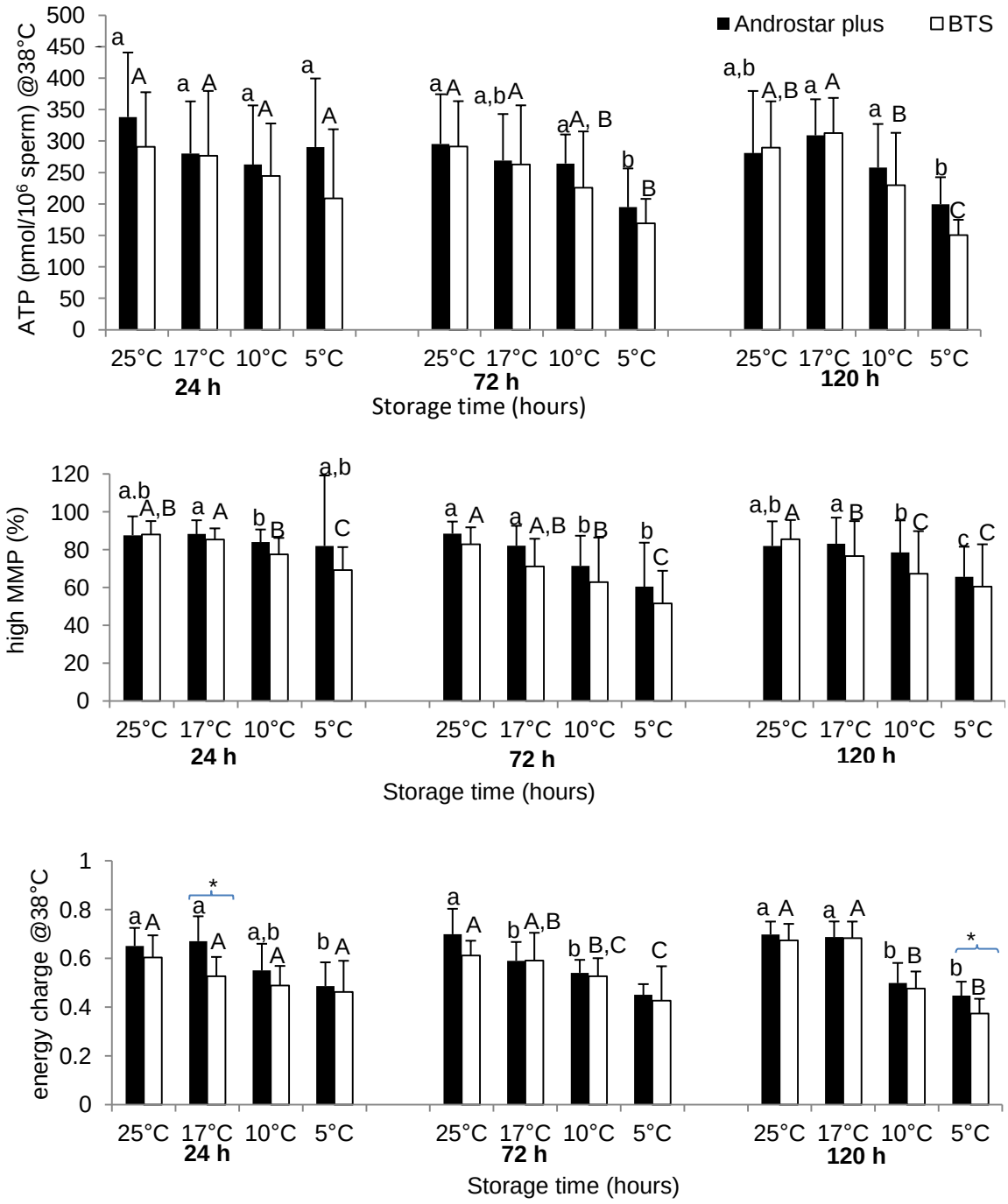


Figure. 2. ATP content (A), energy charge (B) and percentage of sperm with high mitochondrial transmembrane potential (C) after 15 min incubation at 38°C for boar semen samples stored at various temperatures in either Beltsville Thawing Solution (BTS) or Androstar® Plus (ASP). Data are presented as means ± standard deviation (n=7). Different letters indicate significant differences between storage temperatures within extenders and days (P< 0.05): $^{a\text{-}c}$ for samples diluted in Androstar Plus and $^{A\text{-}C}$ for samples diluted in BTS (P< 0.05). An asterisk indicates significant differences between extenders (P< 0.05).

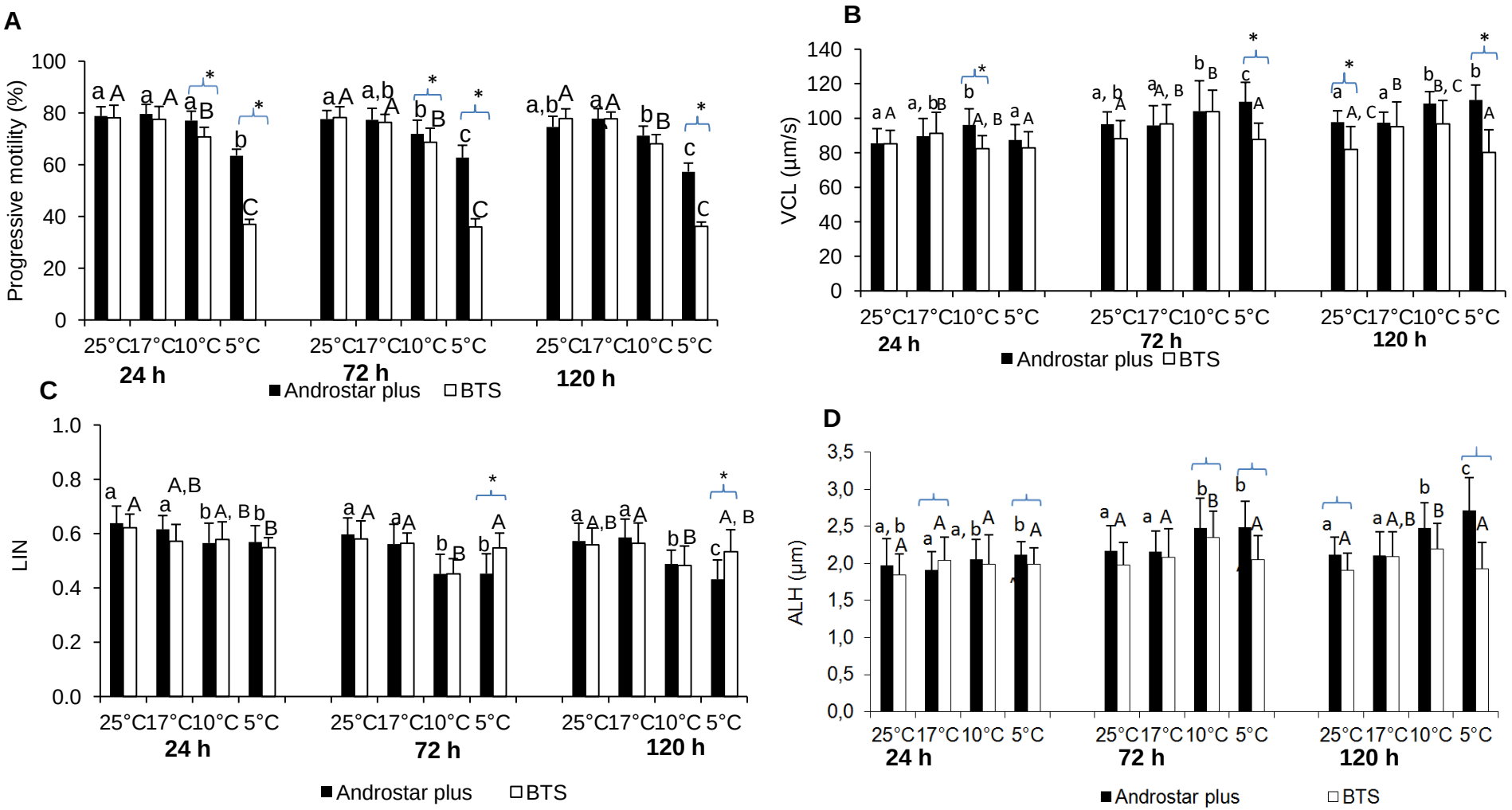
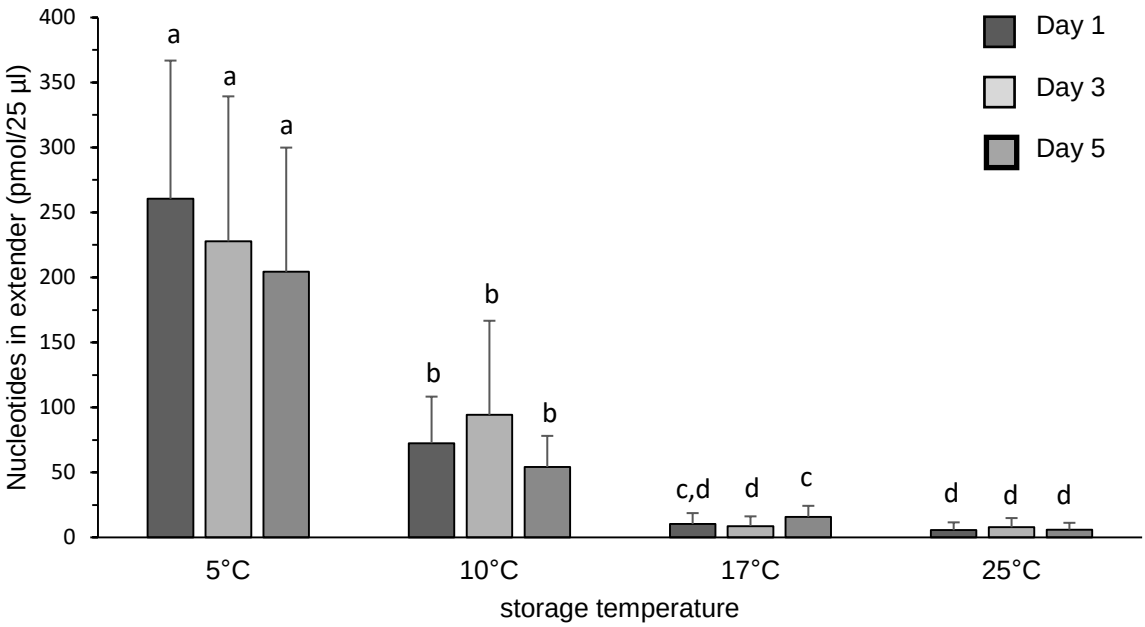
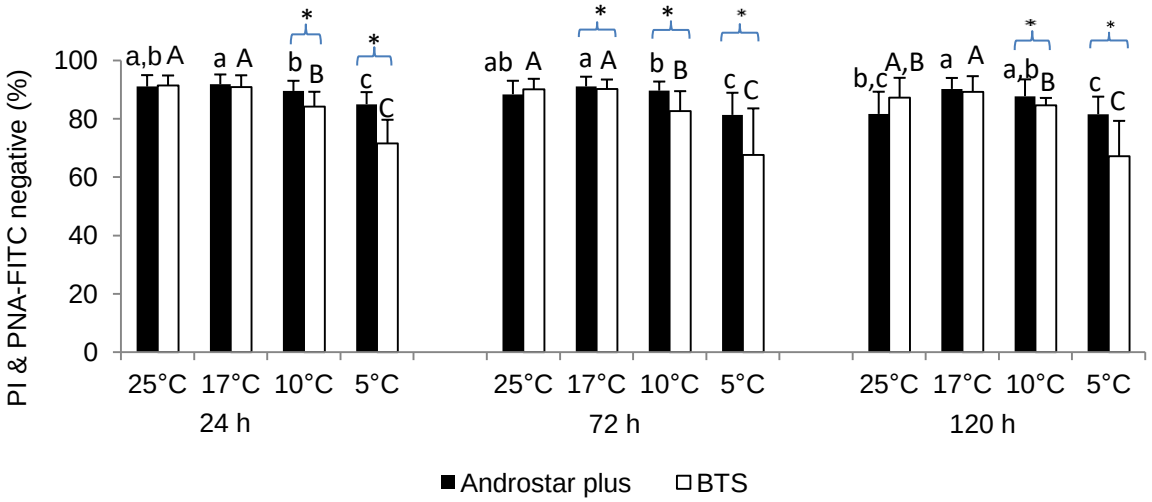


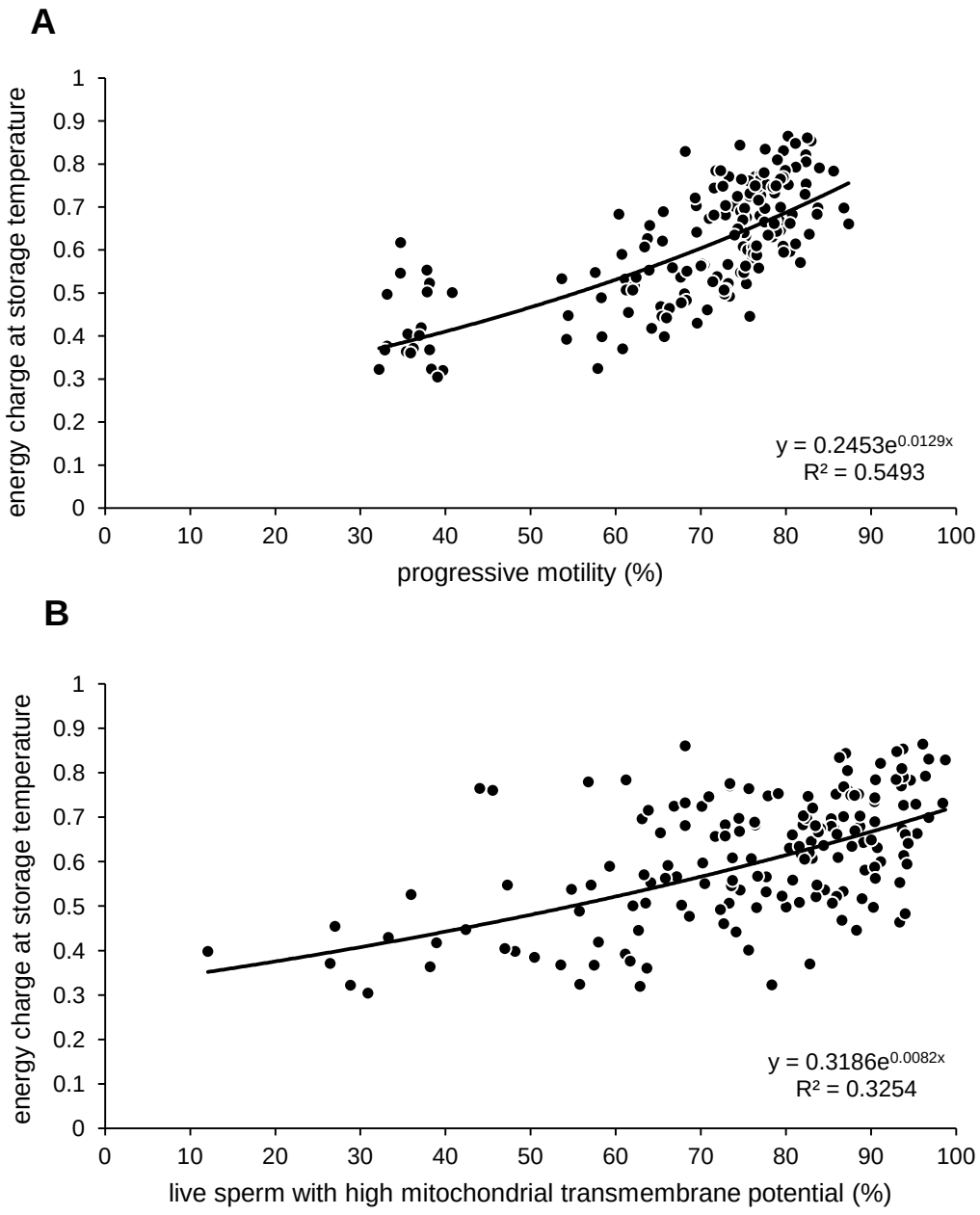
Figure 3. Progressive motility (A), curvilinear velocity (B) and linearity (B) of boar spermatozoa stored at various temperatures in either Beltsville Thawing Solution (BTS) or Androstar®Plus (ASP). Data are presented as means ± standard deviation (n=7). Different letters indicate significant differences between storage temperatures within extenders and days (P< 0.05): $^{a-c}$ for samples diluted in Androstar Plus and $^{A-C}$ for samples diluted in BTS (P< 0.05). An asterisk indicates significant differences between extenders (P< 0.05).



Supplemental Figure 1 Total amount of free nucleotides (AMP, ADP and ATP) in extender of semen samples after storage at different temperatures. Results from both extenders have been combined (n=14). All values are means and standard deviations. Different letters (a-d) indicate significant differences (P<0.05).



Supplement Figure 2.Membrane integrity of spermatozoa after 15 min incubation at 38°C for boar semen samples stored at various temperatures in either Beltsville Thawing Solution (BTS) or Androstar®Plus. Data are presented as means ± standard deviation (n=7). Different letters indicate significant differences between storage temperatures within extenders and days (P< 0.05): $^{a\text{-}c}$ for samples diluted in Androstar®Plus and $^{A\text{-}C}$ for samples diluted in BTS (P< 0.05). An asterisk indicates significant differences between extenders (P< 0.05).



Supplemental Figure 03 Correlation between energy charge assessed at storage temperature with progressive motility (r=0.71; P<0.001) and the percentage of live spermatozoa with high mitochondrial transmembrane potential (r=0.49; P<0.001) after incubation at 38°C. Data from all storage temperatures and days of storage were combined (n = 168).

Table 1 Spearman correlation coefficients for ATP content and energy charge, assessed at storage temperature and after 15 minute incubation at 38°C, with parameters after incubation at 38°C. Data from all storage temperatures and days of storage were combined (n = 168).

		At 38°C											
		Total motility [%]	Progressive motility [%]	VAP [µm/s]	VCL [µm/s]	VSL [µm/s]	STR	LIN	WOB	ALH [µm]	BCF [Hz]	Live, hMMP (%)	PI & PNA-FITC neg. [%]
At storage temperature	ATP (pmol/10^5 sperm)	**0.49 *****	**0.51 *****	**0.21 ***	0.16 *	**0.33 *****	**0.27 *****	0.07	0.09	0.20 **	0.18 *	**0.32 *****	**0.48 *****
	Energy charge	**0.71 *****	**0.71 *****	-0.00	-0.07	0.22 **	**0.39 *****	**0.30 *****	0.18*	-0.18 **	0.27***	**0.54*****	**0.57 *****
At 38°C	ATP (pmol/10^5 sperm)	**0.45 *****	**0.47 *****	0.18*	0.05	0.28***	0.23**	0.17*	0.15*	0.13	0.10	0.23**	**0.36 *****
	Energy charge	**0.59*****	**0.58 *****	0.08	-0.00	0.20**	0.24 **	0.20 **	0.09	-0.02	0.15 *	**0.39*****	**0.39*****

* P < 0.05 ** P < 0.01 *** P < 0.001

VAP = average path velocity VCL = curvilinear velocity VSL = straight-line velocity

STR = straightness LIN = linearity WOB = wobble

ALH = amplitude of lateral head-displacement BCF = beat cross frequency EC = energy charge

Live, hMMP = Pi negative sperm with high mitochondrial transmembrane potential ATP = adenosine triphosphate

Supplemental Table 1. Motility parameters from computer-assisted semen analysis (CASA) for samples stored at different temperatures in Androstar®Plus (n = 7). All values are mean and standard deviation. Different lowercase alphabets (a-e) in each column show significant differences (P < 0.05) between storage days.

		Storage temperature			
	storage	25°C	17°C	10°C	5°C
TM (%)	Day 1	89.3 ± 2.9[a]	88.7 ± 3.3[a]	86.2 ± 3.2[a]	75.0 ± 3.1[a]
	Day 3	88.3 ± 3.8[a]	88.9 ± 3.9[a]	86.0 ± 5.0[a]	74.8 ± 4.4[a]
	Day 5	86.0 ± 3.9[a]	88.4 ± 3.4[a]	82.7 ± 3.9[a]	71.3 ± 3.9[a]
PM (%)	Day 1	78.8 ± 3.6[a]	79.6 ± 3.7[a]	77.0 ± 3.6[a]	63.5 ± 2.5[a]
	Day 3	77.6 ± 3.3[a]	77.3 ± 4.4[a]	71.9 ± 5.3[b]	62.7 ± 4.8[a]
	Day 5	74.6 ± 4.1[a]	77.8 ± 3.9[a]	71.2 ± 3.7[b]	57.3 ± 3.3[b]
VAP (μm/s)	Day 1	64.3 ± 4.9[a]	65.3 ± 6.3[a]	65.0 ± 6.3[a]	64.1 ± 8.2[a]
	Day 3	67.1 ± 7.8[a]	66.9 ± 5.5[a]	68.8 ± 6.4[a]	66.9 ± 8.9[a]
	Day 5	65.4 ± 3.6[a]	69.4 ± 2.8[a]	69.7 ± 3.0[a]	75.3 ± 5.1[a]
VCL (μm/s)	Day 1	85.6 ± 8.5[a]	89.6 ± 10.3[a]	96.2 ± 9.4[a]	87.4 ± 9.0[a]
	Day 3	96.6 ± 7.1[a]	95.8 ± 11.5[a]	104.1 ± 17.7[a,b]	109.5 ± 11.6[b]
	Day 5	97.7 ± 6.7[a]	97.5 ± 6.1[b]	108.5 ± 47.7[b]	110.6 ± 8.7[b]
VSL (μm/s)	Day 1	54.9 ± 4.4[a]	54.6 ± 6.4[a]	51.9 ± 5.1[a]	50.9 ± 6.7[a]
	Day 3	57.4 ± 8.0[a]	56.1 ± 5.9[a]	52.8 ± 5.7[a]	51.6 ± 8.6[a]
	Day 5	54.8 ± 5.5[a]	57.5 ± 4.5[a]	50.2 ± 7.2[a]	52.1 ± 4.5[a]

		Storage temperature			
	storage	25°C	17°C	10°C	5°C
STR	Day 1	0.9 ± 0.04[a]	0.83 ± 0.05[a]	0.80 ±0.06[b]	0.79 ± 0.06[b]
	Day 3	0.85 ± 0.04[a]	0.83 ± 0.04[a]	0.77 ± 0.08[a,b]	0.77 ± 0.07[a,b]
	Day 5	0.83 ± 0.06[a]	0.82 ± 0.04[a]	0.71 ± 0.08[a]	0.69 ± 0.06[a]
LIN	Day 1	0.64 ± 0.06[a]	0.62 ± 0.05[a]	0.57 ± 0.07[a]	0.59 ± 0.06[a]
	Day 3	0.60 ± 0.06[a]	0.56 ± 0.07[a]	0.45 ± 0.07[b]	0.45 ± 0.07[b]
	Day 5	0.57 ± 0.07[a]	0.59 ± 0.07[a]	0.49 ± 0.05[a,b]	0.43 ± 0.07[b]
WOB	Day 1	0.75 ± 0.04[a]	0.73 ± 0.04[a]	0.71 ± 0.06[a]	0.70 ± 0.05[a]
	Day 3	0.72 ± 0.05[a]	0.71 ± 0.06[a]	0.68 ± 0.07[a,b]	0.66 ± 0.07[a,b]
	Day 5	0.66 ± 0.06[a]	0.71 ± 0.06[a]	0.63 ± 0.07[b]	0.61 ± 0.07[b]
ALH (µm)	Day 1	1.97 ± 0.36[a]	1.91 ± 0.25[a]	2.06 ± 0.26[a]	2.11 ± 0.18[a]
	Day 3	2.17 ± 0.34[a]	2.15 ± 0.28[a]	2.47 ± 0.41[b]	2.49 ± 0.35[b]
	Day 5	2.12 ± 0.23[a]	2.11 ± 0.32[a]	2.47 ± 0.34[b]	2.72 ± 0.44[b]
BCF (Hz)	Day 1	35.3 ± 1.5[a]	37.2 ± 2.1[a]	37.2 ± 1.8[a]	35.6 ± 1.9[a]
	Day 3	37.7± 3.8[a]	37.1 ± 3.4[a]	36.3 ± 4.6[a]	37.0 ± 2.8[a]
	Day 5	37.4 ± 2.1[a]	38.3 ± 2.1[a]	37.8 ± 2.7[a]	37.8 ± 2.8[a]

Supplemental Table 2 Motility parameters from computer-assisted semen analysis (CASA) for samples stored at different temperatures in BTS (n = 7). All values are mean and standard deviation. Different lowercase alphabets (A- C) in each column show significant differences (P < 0.05) between storage days.

		Storage temperature			
	storage	25°C	17°C	10°C	5°C
TM (%)	Day 1	88.3 ± 2.5^{A}	87.9 ± 3.3^{A}	79.6 ± 3.7^{A}	55.3 ± 2.9^{A}
	Day 3	87.7 ± 2.6^{A}	88.1 ± 3.4^{A}	80.8 ± 3.4^{A}	51.5 ± 2.4^{B}
	Day 5	86.6 ± 3.3^{A}	87.1 ± 3.2^{A}	78.8 ± 4.4^{A}	47.3 ± 2.6^{C}
PM (%)	Day 1	78.1 ± 4.9^{A}	77.5 ± 5.0^{A}	70.7 ± 3.8^{A}	40.0 ± 1.9^{A}
	Day 3	78.2 ± 4.2^{A}	76.4 ± 3.1^{A}	68.7 ± 5.4^{A}	36.0 ± 3.1^{A}
	Day 5	77.8 ± 3.8^{A}	77.7 ± 2.6^{A}	68.1 ± 3.6^{A}	36.1 ± 1.7^{A}
VAP (μm/s)	Day 1	62.8 ± 4.4^{A}	63.0 ± 7.4^{A}	64.5 ± 11.5^{A}	62.2 ± 8.7^{A}
	Day 3	62.5 ± 5.6^{A}	66.9 ± 9.5^{A}	64.7 ± 13.2^{A}	59.3 ± 8.8^{A}
	Day 5	54.8 ±8.2^{A}	64.7 ± 7.6^{A}	60.8 ± 12.1^{A}	55.1 ± 14.7^{A}
VCL (μm/s)	Day 1	85.2 ± 7.8^{A}	91.2 ± 12.3^{A}	82.3 ± 7.7^{A}	82.9 ± 9.4^{A}
	Day 3	88.2 ± 10.5^{A}	96.7 ± 11.3^{A}	103.9 ± 12.5^{B}	87.7 ± 9.5^{B}
	Day 5	82.0 ± 13.2^{A}	95.1 ± 14.5^{A}	96.8 ± 13.6^{C}	80.1 ± 13.3^{A,B}
VSL (μm/s)	Day 1	54.1 ± 4.8^{A}	51.9 ± 7.7^{A}	51.8 ± 11.9^{A}	48.9 ± 7.6^{A}
	Day 3	53.4 ± 5.8^{A}	55.0 ± 9.5^{A}	49.8 ± 14.5^{A}	47.0 ± 10.4^{A}
	Day 5	46.0 ± 7.7^{A}	53.4 ± 7.0^{A}	45.1 ± 10.9^{A}	44.2 ± 13.3^{A}

		Storage temperature			
	storage	25°C	17°C	10°C	5°C
STR	Day 1	0.86 ± 0.03^{A}	0.82 ± 0.04^{A}	0.79 ± 0.07^{A}	0.78 ± 0.06^{A}
	Day 3	0.85 ± 0.04^{A}	0.82 ± 0.04^{A}	0.78 ± 0.11^{A}	0.78 ± 0.08^{A}
	Day 5	0.83 ± 0.03^{A}	0.82 ± 0.04^{A}	0.73 ± 0.08^{A}	0.79 ± 0.05^{A}
LIN	Day 1	0.62 ± 0.05^{A}	0.57 ± 0.06^{A}	0.58 ± 0.07^{A}	0.55 ± 0.04^{A}
	Day 3	0.58 ± 0.07^{A}	0.56 ± 0.04^{A}	0.45 ± 0.06^{B}	0.55 ± 0.06^{A}
	Day 5	0.56 ± 0.06^{A}	0.56 ± 0.08^{A}	0.48 ± 0.07^{A,B}	0.53 ± 0.08^{A}
WOB	Day 1	0.73 ± 0.03^{A}	0.69 ± 0.06^{A}	0.69 ± 0.07^{A}	0.68 ± 0.06^{A}
	Day 3	0.71 ± 0.05^{A}	0.69 ± 0.06^{A}	0.66 ± 0.1^{A}	0.67 ± 0.08^{A}
	Day 5	0.67 ± 0.06^{A}	0.68 ± 0.07^{A}	0.63 ± 0.08^{A}	0.67 ± 0.07^{A}
ALH (µm)	Day 1	1.9 ± 0.3^{A}	2.0 ± 0.3^{A}	2.0 ± 0.4^{A}	2.0 ± 0.2^{A}
	Day 3	2.0 ± 0.3^{A}	2.1 ± 0.4^{A}	2.4 ± 0.4^{A}	2.1 ± 0.3^{A}
	Day 5	1.9 ± 0.2^{A}	2.1 ± 0.3^{A}	2.2 ± 0.3^{A}	1.9 ± 0.4^{A}
BCF (Hz)	Day 1	35.9 ± 2.3^{A}	37.1 ± 2.1^{A}	36.9 ± 2.3^{A}	34.3 ± 2.0^{A}
	Day 3	37.8 ± 1.4^{A}	38.3 ± 3.8^{A}	36.6 ± 3.5^{A}	31.5 ± 4.5^{A}
	Day 5	34.3 ± 4.1^{A}	37.9 ± 2.7^{A}	37.8 ± 3.0^{A}	31.3 ± 4.0^{A}

Supplement table 3 Spearman correlation coefficients for ATP content and energy charge measured at storage temperature and after 15 minute incubation at 38°C.with parameters for samples incubated at 38°C. Data from both extenders and all days of storage were combined for a given storage temperature (n = 42).

		At 38°C											
		Total motility [%]	Progressive motility [%]	VAP [µm/s]	VCL [µm/s]	VSL [µm/s]	STR	LIN	WOB	ALH [µm]	BCF [Hz]	Live, hMMP (%)	PI & PNA-FITC neg. [%]
At 5°C	ATP (pmol/10^5 sperm)	**0.53 *****	**0.54 *****	**0.42 ****	**0.43 ****	**0.34 ****	-0.11	**-0.33 ***	-0.20	**0.49 ****	**0.37 ***	**0.33 ***	**0.55 ****
	Energy charge	**0.53 *****	**0.45 ****	0.17	0.21	0.16	- 0.17	**- 0.34 ***	- 0.21	0.21	**0.41 ****	**0.56 *****	**0.54 ****
At 10°C	ATP (pmol/10^5 sperm)	0.15	0.20	0.02	-0.19	0.22	**0.42 ****	0.10	0.27	0.04	-0.22	0.12	0.10
	Energy charge	0.29	**0.42 ****	- 0.22	- 0.27	- 0.02	**0.32 ***	0.10	0.16	-0.19	0.08	**0.42 ****	0.29
At 17°C	ATP (pmol/10^5 sperm)	**-0.37 ****	-0.24	0.18	**0.33 ***	0.13	0.01	-0.29	-0.20	**0.46 ****	-0.03	-0.06	0.05
	Energy charge	-0.0004	0.06	-0.01	-0.05	0.05	0.10	0.01	0.10	0.002	-0.15	-0.03	0.05
At 25°C	ATP (pmol/10^5 sperm)	0.02	0.07	0.15	0.26	0.05	-0.10	-0.15	-0.01	**0.57 *****	0.02	- 0.24	-0.02
	Energy charge	**0.46 ****	**0.32 ****	- 0.01	0.05	-0.04	-0.17	-0.08	-0.10	0.05	0.04	**0.34 ***	0.29

* P < 0.05

** P < 0.01

*** P < 0.001

VAP = average path velocity VCL = curvilinear velocity VSL = straight-line velocity

STR = straightness LIN = linearity WOB = wobble

ALH = amplitude of lateral head-displacement BCF = beat cross frequency EC = energy charge

Live, hMMP = sperm with high mitochondrial transmembrane potential ATP = adenosine triphosphate

Chemicals

Substances	Source	Article number
Adenylate kinase	Sigma-Aldrich, Steinheim	M 3003
ATP assay mix	Sigma-Aldrich, Steinheim	FL-AAM
ATP assay mix dilution buffer	Sigma-Aldrich, Steinheim	FL-AAB
ATP standard	Sigma-Aldrich, Steinheim	FL-AAS
BSA (fraction V)	Sigma-Aldrich, Steinheim	A3803
$CaCl_2$	Serva, Heidelberg	15585
DMSO	Roth, Karlsruhe	6643
EGTA	Roth, Karlsruhe	3054
Formaldehyde 37%	Roth, Karlsruhe	4979
FITC-PNA	Axxora, Lörrach	VC-FL-1071
Fluo-4/AM	Invitrogen, Oregon	F14201
Gentamycinsulfat	Serva, Heidelberg	22185
D(+)-Glucose-monohydrate	Merck, Darmstadt	104074
HCl	Merck, Darmstadt	09057
HEPES	AppliChem, Darmstadt	A106
KCl	Merck, Darmstadt	104939
KH_2PO_4	Merck, Darmstadt	105108
Na-(DL)-lactate 60%(w/w)	Sigma-Aldrich, Steinheim	L1375
$MgSO_4*7H_2O$	Sigma-Aldrich, Steinheim	M5921
tri-Natriumcitrat-dihydrate	Merck, Darmstadt	106448
NaCl	AppliChem, Darmstadt	A4661
NaOH	Merck, Darmstadt	106462
Percoll®	GE Healthcare, München	17-0891
Pyruvate kinase	Sigma-Aldrich, Steinheim	P 1506

Equipment

Counting chamber Thoma "neu"	Fa. Jürgens, Hannover
Votex, Reax top	Fa. Heidolph, Schwabach
Flow cytometer "Galaxy"	Partec GmbH. Münster
pH-meter, Multiplex 3000/pMX	Fa. WTW, Weilheim
Freezing point osmometer (osmomat 030)	Fa. Gonotec, Berlin
Water bath	
Typ 1013	Fa. GFL, Burgwedel
WNB 745	Fa. Memmert, Schwabach
Slides	IDL GmbH&Co. KG, Nidderau
Cover slip	IDL GmbH&Co. KG, Nidderau
Leja chamber 4-chamber slide	Leja Products B.V., Nieuw-Vennep, The Netherlands
Phase contract microscope	Fa. Zeiss, Jena
Semen bottle with cap	Fa. Minitüb GmbH&Co KG, Tiefenbach
Semen collection bag, US-bag	Fa. Minitüb GmbH&Co KG, Tiefenbach
Semen collection cup	Fa. Minitüb GmbH&Co KG, Tiefenbach
Variable Single Chanel pipet	
0.5 - 10 µl	
2.0 - 20 µl	Eppendort AG, Hamburg
10 - 100 µl	
100 - 1000 µl	
Pipette tips (10 µl)	Biozym Scientific GmbH, Hessich Oldendorf
P Pipette tips (100 and 1000 µl)	Landgraf Laborsysteme GmbH, Langenhagen
Parafilm	Landgraf Laborsysteme GmbH,

	Langenhagen
Centrifugation, Megafuge 2.0 R	Fa. Hareaus, Hanau
Reactor tube (3.5 ml)	Fa. Sarstedt, Nürnbrecht
Laboratory weigh (PT12)	Sartorius AG, Göttingen
Laboratory weigh (ACL-80.4)	Sartorius AG, Göttingen
multi-channel pipette, Biohit eLine E 1000	Biohit GmbH, Rosbach
Thermomixer 5436	Eppendorf, Hamburg
96 well microtiter plate	Greiner Bio-One, Frickenhausen
Tecan GENios Pro plate reader	Tecan Group Ltd., Männedorf,

Summary

Nguyen Thu Quynh (2015)

Energy metabolism during long-term storage and subsequent thermal stress in liquid preserved boar spermatozoa

Boar spermatozoa are susceptible to cold shock and therefore are stored between 16 and 18°C before use in artificial insemination. Storage of extended semen alters essential sperm function which may affect fertility of sows. The aim of the present thesis was to examine the impact of hypothermic storage and subsequent thermal stress on sperm quality and energy metabolism in liquid-preserved boar semen. In addition, it was investigated whether deficiencies in the energy metabolism are causative for impaired sperm function of stored semen.

In the first study (Chapter 1), a revised protocol for efficient ATP extraction of diluted boar spermatozoa was established which allows a highly repeatable measurement of adenine nucleotides and adenylate energy charge (AEC) by the luciferin-luciferase reaction. Boar semen samples (n=6) diluted in Beltsville Thawing Solution (BTS) were incubated with phosphatase inhibitor cocktail at room temperature or on ice. Boiling for ATP extraction with or without boiling buffer, centrifugation, and subsequent ATP assessment from the supernatant was done either directly after the inhibition step in fresh semen samples or after frozen-storage of subsamples at -20°C. Both, inhibition on ice and the use of a boiling buffer, increased the amount of extracted ATP ($P<0.05$). Combination of both steps resulted in the highest efficiency of ATP extraction. In fresh samples, measured ATP concentration was approximately tenfold higher when both steps were combined ($P<0.05$). Freezing had no impact on ATP concentration compared to freshly analyzed samples when phosphatase inhibition was done on ice and a boiling buffer was used ($p>0.05$). ATP content of fresh and frozen samples correlated best for this treatment ($r=0.83$; $p<0.05$). AEC levels in fresh (0.9) and extended (0.7) spermatozoa were on high level indicating an active energy status in viable cells. The observed positive correlation between ATP ($r = 0.9$, $P<0.001$)/ AEC levels ($r = 0.7$, $P <0.001$) and the proportion of membrane intact spermatozoa as determined by flow cytometry using Propidium iodide (PI)/FITC-PNA staining emphasize that cell viability must be

considered for interpretation of energetic data. Increased membrane permeability induced by storage of extended boar semen at 5°C lead to higher ($P<0.05$) levels of AMP in the extracellular medium whereas no adenine nucleotides were measured in the seminal plasma of fresh semen samples. Overall, a revised protocol for efficient and highly repeatable ATP extraction was developed which allows storage of samples at -20°C prior to the luciferin-luciferase reaction without affecting ATP content and AEC. This protocol was then used for Studies 2 and 3 of the present thesis.

In the second study (Chapter 2), the impact of a long-term storage at +17°C and subsequent thermal stress on the cellular ATP level and AEC of extended boar spermatozoa was investigated. Metabolic energy data were related to mitochondrial membrane potential (MMP) of live sperm as assessed by flow cytometry using the JC-1/PI assay and to sperm kinematics evaluated by computer-assisted semen analysis (CASA). Ejaculates of seven boars were diluted in BTS, cooled to 17°C and stored for 24 h (day 1), 72 h (day 3), 120 h (day 5), and 168 h (day 7). At each time, samples were analyzed before and after 15, 30, 60, 120, and 180 min incubation at 38°C. During storage at 17°C, ATP content (day of collection (= day 0):454 pmol/10^5 sperm, day 5: 398 pmol/10^5 sperm) and AEC (day 0: 0.78 pmol/10^5 sperm, day 5: 0.71 pmol/10^5 sperm) remained stable ($P>0.05$). ATP levels and AEC of spermatozoa during incubation at 38°C were significantly affected by storage length and incubation time ($P<0.05$). ATP content and total motility of sperm declined moderately and earliest after 60 min incubation ($P<0.05$) while the percentage of live sperm with high MMP remained high (> 80 %) throughout semen storage, but decreased dramatically already after 30 min of incubation at 38°C ($P < 0.05$). Concomitantly with the decrease of MMP, motility patterns based on curvolinear velocity, linearity and lateral head displacement changed from an activated to a steady state pattern. ATP values of samples stored at 17°C decreased during incubation for 15 min at 38°C ($P<0.05$). With ongoing storage time the relative difference between ATP levels before and after incubation increased (day 0: 7.1, day 5: 24.2 pmol/10^5 sperm, $P<0.05$) indicating an increasing relative expense of ATP to regain a functional (motile) state during re-warming. Total motility correlated moderately with the ATP content ($r = 0.48$, $P<0.05$) and low to moderate to % live sperm with high MMP ($r = 0.38$, $P< 0.05$).

The results demonstrate that energy reserves in liquid preserved boar spermatozoa remain stable during prolonged storage at 17°C, though the contribution of oxidative phosphorylation in mitochondria seems to be rapid and only short-term compared to glycolysis. The transitory increase in ATP levels yield by mitochondrial activity may modulate wave patterns and trajectories of motile sperm. Moreover, the storage-associated increase of the ATP expense of energy for reactivation of motility indicates that ATP dependent cell functions become increasingly vulnerable during semen storage.

The aim of the third study (Chapter 3) was to investigate whether maintenance of ATP levels and energy charge are dependent on the storage temperature and storage time during liquid preservation of boar spermatozoa. The relationship to mitochondrial activity, motility and viability of spermatozoa after re-warming of spermatozoa was monitored. Moreover, it was tested whether stabilisation of membrane integrity and motility at low storage temperature by the use of a cold-shock protective extender is associated with a stable energy metabolism. Split ejaculates from seven boars were diluted with standard extender BTS and protective extender Androstar® Plus (Minitüb GmbH, Tiefenbach), cooled down to 25, 17, 10 and 5°C and stored up to 120 h. After 24 h, 72 h and 120 h preserved samples were checked for motility (CASA) and integrity of plasma and acrosomal membranes (PI and FITC-PNA/PI staining), MMP (JC1/ PI assay), ATP and energy charge (luciferin-luciferase assay). Motility and membrane status were significantly influenced by storage length, storage temperature and extender ($P< 0.05$). Neither ATP levels, nor energy charge or motility differed for samples stored at 17°C or 25°C. Hypothermic storage at 10°C and 5°C, respectively, lead to a storage-length related decrease of both intracellular ATP levels and AEC and to a concomitant appearance of adenine nucleotides in the extender medium. The percentage of live sperm with high MMP decreased with lower storage temperatures ($P<0.05$). Storage temperature and extender had a significant influence ($P< 0.05$) on ATP content and energy charge of preserved spermatozoa whereas storage time did not reveal an influence. The energy charge obtained in Androstar Plus samples at 5°C and 10°C storage was significantly higher than that in samples diluted in BTS, but dropped below 0.6 in both extenders indicating a deficient energy metabolic state. These results indicate a pronounced temperature sensitivity of sperm energy metabolism when sperm are

exposed to cooling stress. The extent of it can be modified by extender media. Cooling induced rearrangement of membrane lipids increase permeability and leads to a loss of adenine nucleotides alongside with other solutes. Presumably, low motility in chilled boar spermatozoa cannot simply be attributed to a lack of energy substrates but also to irreversible failure in utilization of ATP and disruption of motility regulating cellular processes. Contrary to common expectation, an elevated cell activity during long-term storage at 25°C does not lead to a lack of energy and metabolic exhaustion of sperm.

In conclusion, using a revised validated ATP assay protocol novel information on the influence of storage temperature and length on the energy metabolism in liquid preserved boar spermatozoa was obtained. The energy status of spermatozoa is maintained on a high level during long-term storage between 17 and 25°C and subsequent thermal stress, and consequently is not limiting cell function. Oxidative phosphorylation in mitochondria is not decisive for the maintenance of energy status and motility of thermically stressed boar spermatozoa after long-term storage in vitro. In spermatozoa exposed to long-term cooling stress at 10 and 5°C loss of ATP and energy charge may contribute to irreversible failure of cell function, ultimately leading to reduced fertility.

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