



Assessment of chilling injury in hypothermic stored boar spermatozoa by multicolor flow cytometry

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Abstract

Hypothermic storage of boar semen may allow antibiotic-free semen preservation but is limited due to chilling sensitivity of boar spermatozoa. Progress in this area requires sensitive tools to detect chilling injury. Therefore, multiparameter flow cytometry panels were evaluated to ascertain whether they are useful tools for identifying sublethal damage of sperm function at a single cell level, thus considering the high intrinsic sperm heterogeneity in a sample. The first fluorochrome panel consisted of Hoechst 33342 to identify DNA-containing events, Yo-Pro 1 to detect viability, merocyanine 540 to describe membrane fluidity, and PNA-Alexa Fluor™ 647 to identify acrosomic integrity. The second fluorochrome panel consisted of SiR700-DNA to identify DNA-containing events, JC-1 to characterize the mitochondrial transmembrane potential (MMP), and Calbryte 630 to assess the intracellular calcium level. Extended boar semen was stored either at 17°C (control) or 5°C (chilled). It is shown that chilling increased membrane fluidity in the viable (Yo-Pro 1 negative) sperm population at 24 h ($p < 0.05$). At 144 h, the viable, acrosomic intact sperm population with low membrane fluidity was similar for both storage temperatures. Moreover, chilling reduced the main sperm population with high MMP, medium fluorescence for JC-1 monomer and low intracellular calcium level ($p < 0.05$). However, after in vitro sperm capacitation, this population did not differ between the two storage temperatures. Exemplary computational data visualization in t-distributed stochastic neighbor embedding (t-SNE) maps and moving radar plots revealed similar subpopulations as identified by three-dimensional stacked bar charts. In conclusion, sperm surviving an initial chilling injury withstand long-term storage and respond in a similar manner to capacitation conditions as sperm stored conventionally at 17°C. Multicolor flow

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cytometry is a valuable tool for detecting chilling-induced alterations of cell function in sperm subpopulations.

KEYWORDS

chilling, hypothermic semen storage, multicolor flow cytometry, spermatozoa, boar semen, sublethal injury

1 | INTRODUCTION

Artificial insemination in pigs is the most used biotechnology in animal reproduction. Boar semen is commonly stored in the liquid stage at relatively high temperature (16–18°C). Consequently, antibiotics in semen extenders are mandatory to avoid bacterial growth of bacteria during storage [1, 2]. The intense search for sustainable alternative antimicrobial concepts has resulted in achieving bacteriostasis by hypothermic semen storage at 5°C. A limiting factor is the high chilling sensitivity of boar spermatozoa [3], which can be attenuated by protective extender media and controlled cooling [4, 5]. Progress in developing cooling concepts depends on sensitive measures for chilling injury.

Cold shock is lethal for a minor sperm population but more importantly, leads to sublethal functional changes in different amounts of spermatozoa. Cell compartments sensitively reacting to cold shock are the sperm's plasma membrane and the mitochondria. Cooling-induced phase transitions of lipids cause changes in membrane structure and fluidity, a disturbed ion transport and eventually a disruption of cell function [6–8]. Sperm mitochondria are involved in calcium homeostasis as they can accumulate calcium in the matrix space [9]. In addition, a peak in mitochondrial activity [10] and tyrosine phosphorylation of mitochondrial proteins [11] during *in vitro* capacitation indicate that mitochondrial activity is important for sperm function (reviewed in Durairajanayagam, Singh) [12]. The response to capacitating stimuli can be measured by a time-dependent increase in intracellular calcium [13]. So far, the chilling-induced changes of mitochondria activity and free intracellular calcium levels have been examined as a two-step approach in independent assays. Analyzing multiple traits at single cell level will be advantageous to gain more in depth knowledge on chilling stress affecting the interplay between membrane phase transitions, calcium homeostasis, and capacitation. Recently, multiparametric flow cytometry has been introduced in spermatology [14, 15]. It allows better prediction of fertility potential as shown lately for frozen bull sperm; the nonreturn rates were correlated ($r_s = 0.64$, $p < 0.01$) to a population of viable sperm with high mitochondrial transmembrane potential (MMP) and low intracellular calcium content [16].

Thus, the aim of the present study was to characterize chilling-induced alterations of cell functions at single cell level in viable boar sperm by means of multicolor flow cytometry. Special emphasis was laid on the dynamics of mitochondrial function and regulation of cytosolic calcium during *in vitro* capacitation of cold-stored sperm. Chilling effects were provoked by storage in a short-term extender at 5°C, a

temperature which is suitable to inhibit bacterial growth in an antibiotic-free environment [5, 17] but is commonly regarded as harmful for spermatozoa. Overall, the aim is to provide a sensitive flow cytometry panel for the assessment of sublethal cell injury which would allow to promote research in cold-storage of boar semen as a sustainable tool in pig reproduction.

2 | MATERIAL AND METHODS

2.1 | Semen preparation and analysis

Semen from nine fertile boars (Pietrain and Large White) housed at the Unit for Reproductive Medicine, University of Veterinary Medicine Hannover, Germany was manually collected using the “gloved hand” method. Since the animals were kept for semen collection and handled in accordance with the European Commission Directive for Pig Welfare, no ethical permission was required. Normospermic ejaculates ($\geq 20 \times 10^9$ spermatozoa, $\geq 70\%$ total motility, $> 75\%$ morphologically normal spermatozoa) were extended in Beltsville Thawing Solution (BTS;) [18] to 20×10^6 sperm/ml in semen tubes with a final volume of 90 ml. Semen tubes were kept at 21°C for 2 h and then stored at 17°C or chilled to 5°C as recommended by Paschoal et al. [4] Tubes were stored for 144 h in the dark.

Sperm motility was assessed at 24, 72, and 144 h, respectively with the computer-assisted semen analysis (CASA)-System AndroVision® Version 1.1.6. (Minitüb GmbH, Tiefenbach, Germany) as previously described [4]. A minimum of 600 spermatozoa were analyzed with a rate of 60 frames per second. Spermatozoa were classified as motile when their curved-line velocity was greater than 24 $\mu\text{m/s}$, and their amplitude of lateral head displacement was greater than 1 μm .

2.2 | Multiparameter flow cytometry

A CytoFlex flow cytometer (Beckman Coulter GmbH) with CytExpert software version 2.3 (Beckman Coulter GmbH) was used. The flow cytometer was equipped with lasers of 405 nm (80 mW), 488 nm (50 mW), and 638 nm (50 mW) wavelength, respectively.

Two 4-color panels were applied with non-stained and single stained probes as controls (Figures S1 and S2). Spectral overlap was compensated during acquisition. Artificial mixtures of viable and nonviable sperm served to set thresholds between sperm with intact/defective

plasma membranes, intact/defective acrosomes and low/high MMP (Figure S3). Backgating was used to verify gating (Figures S4 and S5) and new fluorochrome combinations were compared with traditional fluorochromes (Tables S1 to S3).

2.2.1 | Acrosomic integrity and membrane fluidity

The diluted semen sample (480 μ l) was incubated for 15 min at 38°C and subsequently stained with Hoechst 33342 (H33342; final concentration: 1.69 μ M), Yo-Pro 1 (0.02 μ M), Merocyanine 540 (M540; 0.54 μ M) and PNA-Alexa Fluor™ 647 (1 μ g/ml), and incubated for a further 15 min in the dark at 38°C. Fifty microliters of the stained sample were transferred to 950 μ l HBS (ingredients see Henning, Petrunkina) [19]. A total of 10,000 events were acquired. Signals were detected with the following filters: 450/45 BP (H33342), 525/40 BP (Yo-Pro 1), 585/42 BP (M540) and 780/60 BP (PNA-Alexa Fluor™ 647). Spermatozoa were identified with H33342 positive staining. Agglutinations were excluded by Forward Scatter (FSC) gating. Viable spermatozoa (Yo-Pro 1 negative) were classified as being acrosomic intact (PNA-negative) or defective (PNA-positive) and as having either a low plasma membrane fluidity (M540 negative) or a high plasma membrane fluidity (M540 positive, Figures 1 and S1).

2.2.2 | Calcium influx and mitochondrial membrane potential

One milliliter of semen sample was loaded with Calbryte 630 (0.91 μ M) for a 60-min incubation period at 38°C. After 45 min, SiR700-DNA

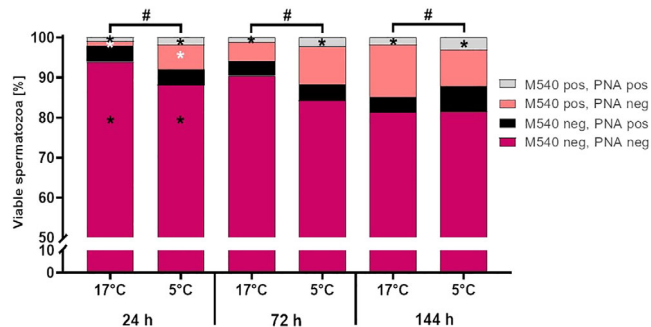


FIGURE 1 Analysis of membrane fluidity and acrosomic integrity in viable sperm by multiparameter flow cytometry to assess sublethal chilling injury. Extended semen samples ($n = 9$) were stored at 17°C (control) or at 5°C (chilled) and examined after 24, 72, and for 144 h. The distribution (%) of sperm with low or high membrane fluidity (M540 negative or positive) and intact or defective acrosome (PNA negative or positive) within the population of viable (H33342 positive, Yo-Pro-1 negative) spermatozoa is shown. *Values for the same sperm class differ between storage temperatures at a given time point ($p < 0.05$). #Relative abundances of the four class-levels from sperm loaded with M540 and PNA-Alexa differ between storage temperatures at a given time point ($p < 0.05$) [Color figure can be viewed at wileyonlinelibrary.com]

(0.01 μ M), Hoechst 33258 (H33258; 0.51 μ M) and JC-1 (0.56 μ M) were added and co-incubated for 15 min. Fifty microliters of stained sample were added to 950 μ l pre-warmed Tyrode's capacitating medium (CM; ingredients see Reference) [19] or a bicarbonate-free non-capacitating control medium (non-CM), and incubated at 38°C under 5% CO₂ and 100% humidity (CM) or in air (non-CM), respectively. Cytosolic calcium concentrations and changes in MMP were assessed after a 3- and 60-min incubation period. Signals were detected with the following filters: 712/25 BP (SiR700-DNA), 450/45BP (H33258), 660/20 BP (Calbryte 630), 525/40 BP (JC-1 monomer) and 585/42 BP (JC-1 aggregate). The SiR700-DNA positive stained particles identified spermatozoa. Agglutinations were excluded by FSC gating (Figure S2). For viable spermatozoa (H33258 negative), gates delineating cells with differing intracellular calcium content (low or high fluorescence intensity [FI] for Calbryte 630) and MMP (low or high FI for JC-1 aggregates) were defined. The FI of JC-1 monomer was considered because discrimination between JC-1 monomers and aggregates improves cytometric analysis of mitochondrial-related changes [20].

An overlay of all data sets from one semen storage temperature group was created to categorize viable spermatozoa into classes according to their FI for JC-1 aggregates indicative for high MMP, JC-1 monomers indicative for low MMP and Calbryte 630 as a calcium probe in two-dimensional density plots (Figure 2A–D). Data for JC-1 aggregates or JC-1 monomers were plotted against Calbryte 630. Three classes for each fluorochrome were delineated: Class 1 (low FI), Class 2 (medium FI), Class 3 (high FI), with Classes 2 and 3 representing subclassifications of the conventionally defined JC-1 or Calbryte 630 positive population. The density plots of samples stored at 17°C were used to define the three classes for JC-1 aggregates (Figure 2A) and JC-1 monomer (Figure 2B). The density plots of samples stored at 5°C depicting JC-1 aggregates against Calbryte 630 were used to subdivide Calbryte 630 signals into three classes (Figure 2C). Subsequently, thresholds for all defined classes were applied to the individual density plots. Thus, gates or classes were kept constant for all samples (Table S4).

The three classes for each of the three different fluorochromes were then used to define 27 different subpopulations of viable spermatozoa (Figure 3). To each of the subpopulations, a triplet code was assigned according to the classes with numbers from 1 to 3 for low to high FI, for example, 2-3-3 encoded for medium FI for JC-1 aggregate, high FI for JC-1 monomer and high FI for Calbryte 630 (Table S5).

Computational flow cytometry was applied using Cytobank Premium 7.3.0 (<https://cytobank.org>, Cytobank, Inc.) to visualize subpopulations of viable sperm in t-SNE overlays [21]. Data are exemplarily shown for one animal in Figure 4. Spermatozoa were defined and gates were manually applied for low and high FI. The procedure “viSNE” was run to reduce the high-parameter data down to two dimensions [22] (https://support.cytobank.org/hc/en-us/articles/204369428-Introduction-to-viSNE-in-Cytobank_). Moreover, continuous, non-gated FI for the stained sperm were visualized for each fluorochrome and are exemplarily shown for semen samples of one animal as heat maps in Figure 5.

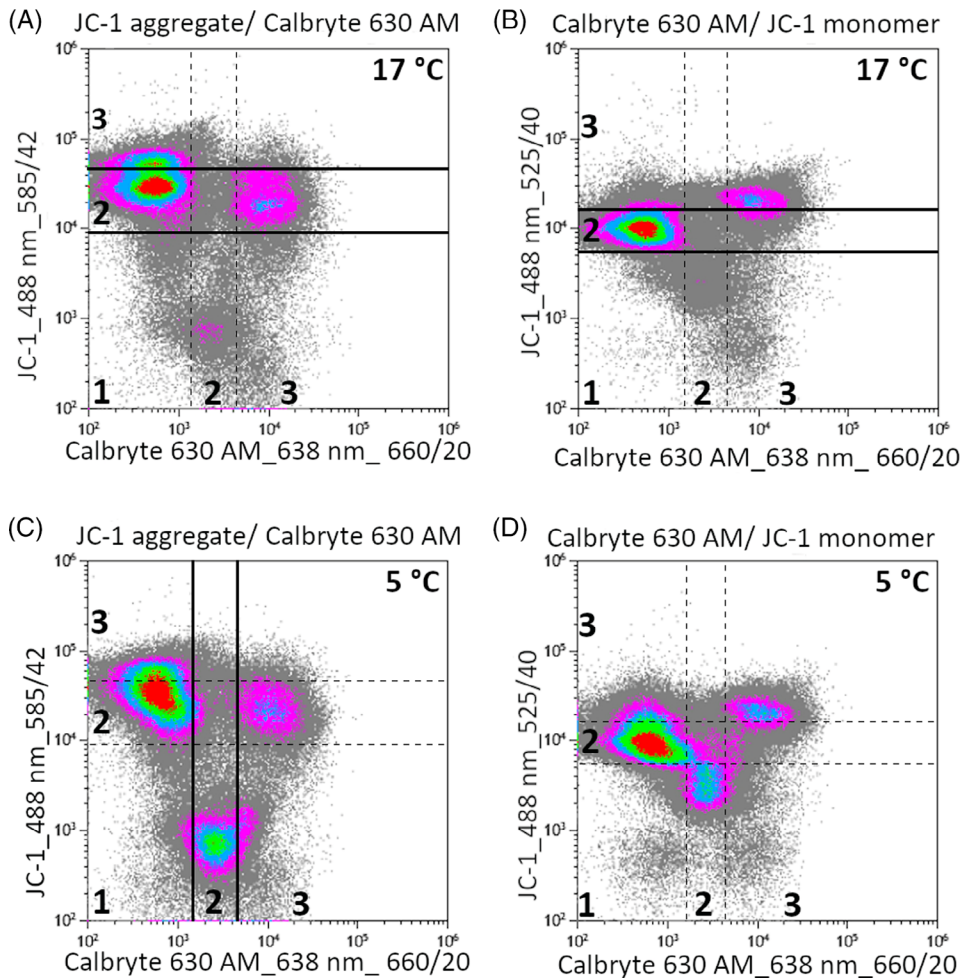


FIGURE 2 Categorization of viable (SiR700 DNA positive, H33258 negative) sperm into subpopulations to analyze mitochondrial membrane potential and calcium influx in the multiparameter assay. Overlays of all events in semen samples ($n = 9$) stored at 17°C (A, B) and at 5°C (C, D) were manually gated in three classes for each fluorochrome according to their fluorescence intensity (FI). The density plots of 17°C-samples were used to define classes for JC-1 aggregate (A) and JC-1 monomer (B). The density plots of 5°C-samples depicting FI of JC-1 aggregates against Calbryte 630 were used to define classes for Calbryte 630 signals (C). (D) All defined classes are plotted [Color figure can be viewed at wileyonlinelibrary.com]

The software Kaluza analysis 2.1 (Beckman Coulter GmbH) was used to visualize viable sperm in a moving three-dimensional radar plot with axes for the JC-1 aggregate, the JC-1 monomer and Calbryte 630. An example is presented in Figure 6A,B. Acquired data were gated for low and high FI (Calbryte 630, JC-1 monomer) as stated above or for low, medium, and high FI (JC-1 aggregates).

2.3 | Statistical analysis

For data analysis and graphical description CytExpert 2.3, Excel, Kaluza Analysis 2.1 and SAS-Software 9.4m5 with the Enterprise Guide Client 7.15 (SAS Institute Inc.) were used. The Kolmogorov–Smirnov test was used to assess model residuals for normal and lognormal distribution. Additionally, Q-Q-plots were assessed visually. Data were analyzed with Wilcoxon's Signed Rank test for paired observations (PROC UNIVARIATE). Data were compared for storage temperatures at each time point and afterwards for storage time points or for incubation time points within storage temperatures. For analyzing the multiparametric assay of membrane fluidity and acrosomic integrity, additionally, a χ^2 test of homogeneity of overall sperm distribution in all four subpopulations between storage temperatures was performed. In the multiparametric assay of calcium influx and MMP, triplet-encoded subpopulations were

only considered for statistical analysis if a subpopulation contained on average $\geq 2\%$ of the viable spermatozoa for any time \times medium \times storage temperature combination. p -values were considered as statistically significant when $p < 0.05$. Data are shown as means \pm SEM.

3 | RESULTS

3.1 | Sperm motility

The total motility for diluted samples stored at 17°C ranged from $87.2 \pm 1.7\%$ at 24 h to $85.4 \pm 2.2\%$ at 144 h. For 5°C-stored semen samples, total motility declined from $73.9 \pm 3.1\%$ at 24 h to $60.0 \pm 3.3\%$ at 144 h. Values for 5°C-stored semen were significantly lower at all time points compared with 17°C-stored semen (Table S6).

3.2 | Multiparametric assays

3.2.1 | Acrosomic integrity and membrane fluidity

All samples maintained a viable sperm population of $\geq 70\%$ in 5°C stored samples and $\geq 84\%$ in 17°C-stored samples. The amount of

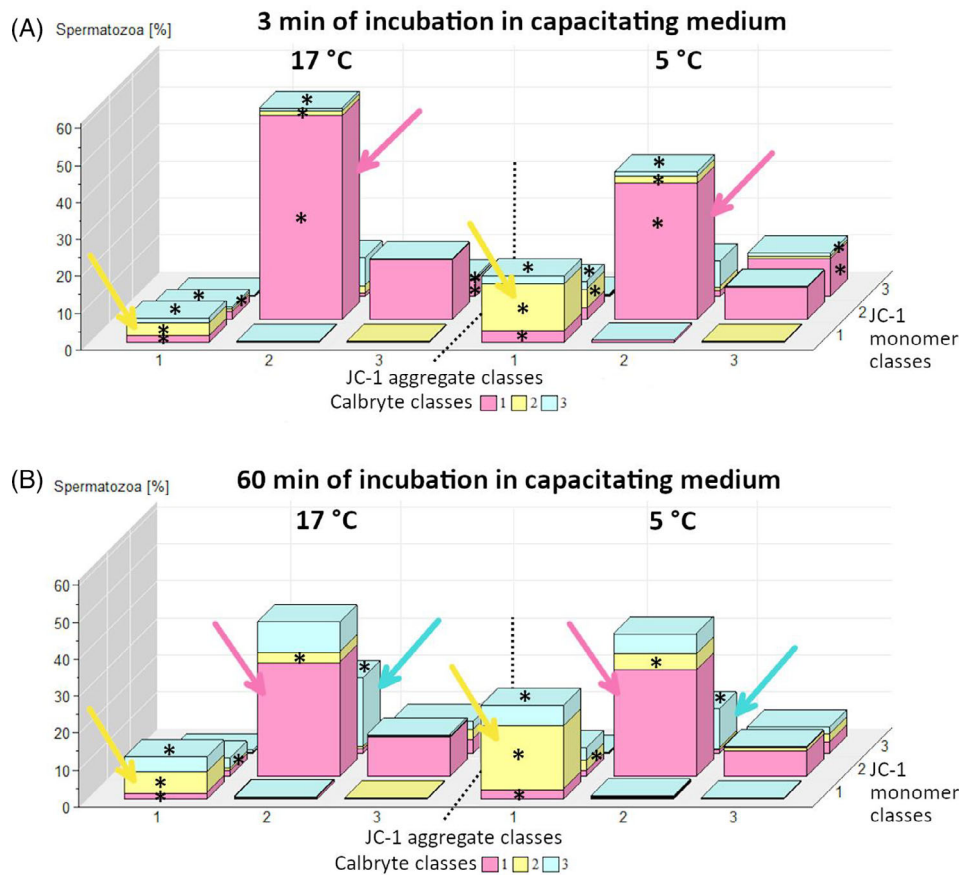


FIGURE 3 Three-dimensional stacked bar charts showing mitochondrial membrane potential (MMP; JC-1 aggregate FI, JC-1 monomer FI) and calcium content (Calbryte 630 FI) in subpopulations of viable (Sir700 DNA positive, H333258 negative) sperm to assess sublethal chilling effects. Semen was stored in Beltsville thawing solution for 72 h at 17°C (control) or 5°C (chilled) and then incubated in a capacitating medium. Subpopulations are encoded in triplet codes (classes 1 to 3) according to their FI: 1 = low, 2 = medium, 3 = high. The main population (triplet 2-2-1 →) consisted of viable sperm with medium MMP, medium FI for JC-1 monomers and low calcium. 5°C-storage resulted in a greater population of viable sperm with low MMP and medium calcium (triplet 1-1-2 →) after 3 min (A) and after 60 min of incubation (B) compared with samples stored at 17°C. A greater percentage of sperm stored at 17°C responded to capacitating conditions with an increase in calcium (triplet 2-3-3 →) compared with sperm stored at 5°C (B). *Values of same sperm triplet differ between storage temperatures ($p < 0.05$); $n = 9$ samples. FI: Fluorescence intensity. Results after incubation in a non-capacitating medium (control) are presented in Figure S6 [Color figure can be viewed at wileyonlinelibrary.com]

viable spermatozoa was significantly higher in samples stored at 17°C compared with 5°C at all time points. (Table S6).

Temperature- and storage time related changes in subpopulations of viable spermatozoa with respect to acrosomic integrity and membrane fluidity are depicted in Figure 1. The distribution of spermatozoa within the subpopulations differed significantly between the storage temperatures at all time points. For all samples, the main population consisted of sperm with a low membrane fluidity and intact acrosomes (M540 neg, PNA neg). After 24 h, the amount of spermatozoa in this subpopulation was significantly higher for samples stored at 17°C ($93.9 \pm 1.1\%$) compared with 5°C ($88.2 \pm 3.6\%$). Furthermore, the amount of spermatozoa with high membrane fluidity and intact acrosomes (M540 pos, PNA neg) was significantly lower in samples stored at 17°C ($1.2 \pm 0.4\%$) compared with 5°C ($6.2 \pm 3.6\%$). After 144 h storage, the sperm population with low membrane fluidity and intact acrosomes (M540 neg, PNA pos) was at a similar level (81%) for both storage temperatures.

3.2.2 | Calcium influx and mitochondrial membrane potential

At the onset of incubation in CM (time point 3 min), the main population of spermatozoa for both storage temperatures was categorized into triplet 2-2-1 (Figure 3A,B, red arrow), which encodes for a medium MMP (medium FI of JC-1 aggregate), medium JC-1 monomer level and low cytosolic calcium levels (low FI of Calbryte 630). In 5°C-stored samples, this population was significantly smaller ($36.8 \pm 6.8\%$) compared with 17°C-stored samples ($55.1 \pm 9.0\%$). After a 60-min incubation period, the main population decreased to lower values (17°C: $30.9 \pm 5.6\%$; 5°C: $28.8 \pm 4.9\%$) and did not differ between the temperature groups ($p > 0.05$). Incubation under non-capacitating control conditions did not affect the size of the main population (Figure S6, Table S5).

Viable sperm subpopulations with high intracellular calcium levels (triplet code X-X-3) increased during incubation in the CM. In samples

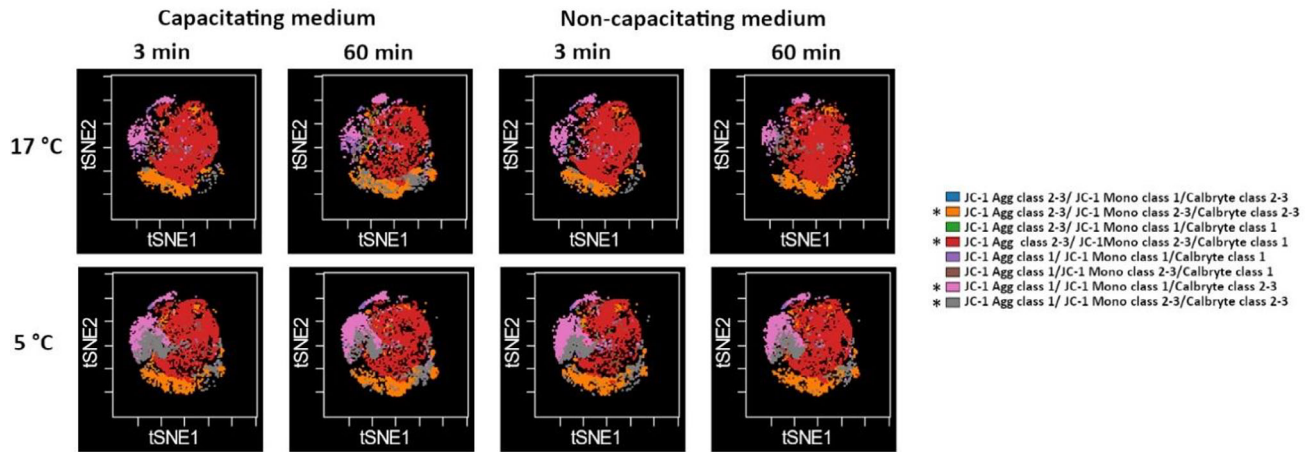


FIGURE 4 Allocation of viable (SiR700 DNA positive, H33258 negative) sperm in a t-SNE overlay map to visualize sublethal chilling effects. Data are exemplarily depicted for one boar. Semen samples were stored in Beltsville thawing solution for 72 h at 17°C (control) or 5°C (chilled) and then incubated in a capacitating or in a non-capacitating medium. Subpopulations are shown in different colors based on their mitochondrial membrane potential (JC-1 aggregates and monomers) and intracellular calcium content with low (1), medium (2) or high (3) fluorescence intensity. *Subpopulations correspond to the main subpopulations identified in Figure 3 [Color figure can be viewed at wileyonlinelibrary.com]

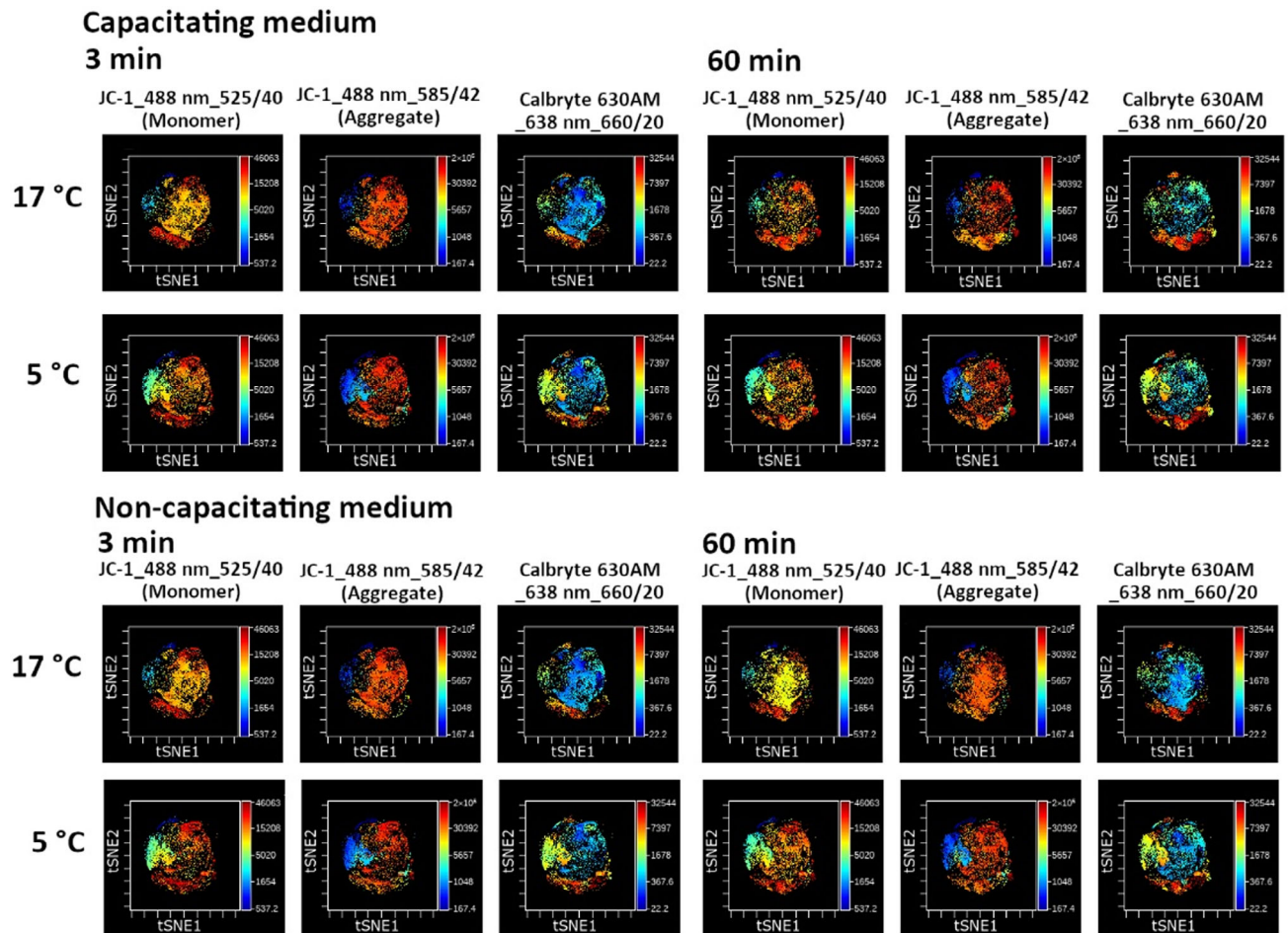


FIGURE 5 Allocation of viable (SiR700 DNA positive, H33258 negative) sperm in a t-SNE heat map exemplary for one boar. Continuous fluorescence intensities (FI) are visualized by channel for JC-1 aggregates and JC-1 monomers to assess the mitochondrial membrane potential, and Calbryte 630 to assess the intracellular calcium content. Semen samples were stored in Beltsville thawing solution for 72 h at 17°C (control) or 5°C (chilled) and then incubated in a capacitating or in a non-capacitating medium. In 5°C-stored semen, a greater proportion of sperm with low FI for JC-1 aggregates (blue) and monomers (turquoise) with high calcium (yellow-green) can be distinguished. After 60 min incubation in capacitating medium, in the sperm population that maintained a high MMP (red population), an increase in calcium was visualized by a shift from blue (3 min) to a greenish color (60 min) in both storage temperature groups [Color figure can be viewed at wileyonlinelibrary.com]

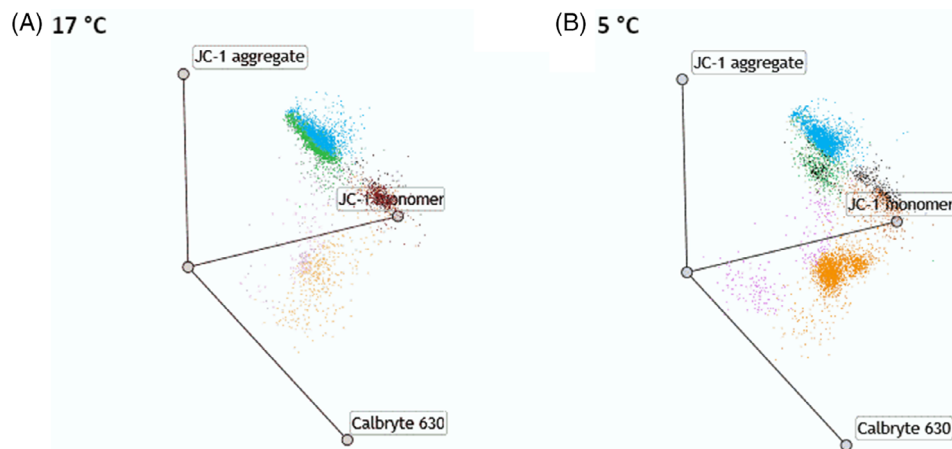


FIGURE 6 The moving radar plot depicts viable spermatozoa (SIR700 DNA positive, H33258 negative) stained with the fluorochromes JC-1 to assess the mitochondrial membrane potential (MMP) and Calbryte 630 to assess the intracellular calcium content. Semen was stored for 72 h in Beltsville thawing solution at 17°C (control; A) or 5°C (chilled; B). Data are exemplarily shown for one boar after 3 min of incubation in a capacitating medium. Fluorochromes were gated for low/high fluorescence intensity (FI; Calbryte 630) or low/medium/high FI (JC-1 aggregate). Sperm subpopulations are plotted in different colors: Purple: low calcium, high MMP, Orange: high calcium and low MMP, Green: low calcium and medium MMP, Blue: low calcium and high MMP, Red: high calcium and medium MMP, Black: high calcium and high MMP [Color figure can be viewed at wileyonlinelibrary.com]

stored at 17°C, the most obvious increase was noted for spermatozoa in triplet 2-3-3 (3 min: $7.6 \pm 0.6\%$, 60 min: $18.7 \pm 3.1\%$, $p < 0.05$), that is, calcium-rich sperm with a medium MMP and high level of JC-1 monomers (Figure 3B, blue arrow), followed by spermatozoa in triplet 2-2-3 (3 min: $0.9 \pm 0.2\%$; 60 min: $8.1 \pm 1.4\%$, $p < 0.05$), that is, with medium MMP and medium level of JC-1 monomer. In 5°C-stored samples, the population encoded by triplet 2-3-3 was smaller compared with 17°C-stored samples ($p < 0.05$). In controls (non-CM), the size of these populations remained low for both storage temperature groups (Figure S6, Table S5).

After a 3-min incubation period in CM, samples stored at 5°C had significantly more spermatozoa with low MMP, low presence of JC-1 monomers and medium (triplet 1-1-2) or high (triplet 1-1-3) intracellular calcium levels compared with samples stored at 17°C (Figure 3A,B, yellow arrow). After a 60-min incubation period in CM, the increase in these subpopulations was higher for samples stored at 5°C compared with 17°C (values at 60 min for triplets 1-1-2: $17.2 \pm 1.9\%$, vs. $5.8 \pm 0.7\%$ ($p < 0.05$) and for triplets 1-1-3: $5.8 \pm 0.6\%$ vs. $3.9 \pm 0.3\%$ ($p < 0.05$), Table S5).

Computational analysis as exemplarily shown in t-SNE map overlays for one boar (Figure 4) revealed similar subpopulations compared with the 3D stacked bar chart description. The capacitation-induced changes in four main populations in relation to the semen storage temperature correspond to those presented in the 3D stacked bar charts shown in Figure 3. Similarly, changes in the subpopulations during incubation in CM were visualized in heat maps with continuous FI for the applied fluorochromes (Figure 5). The visualized distances between the subpopulations were not influenced by the storage temperature or capacitation treatment.

Likewise, in the moving radar plot, main sperm populations and consistent distances between subpopulation were visualized for the two storage temperatures exemplary for one boar (Figure 6A,B). The type and size of the main subpopulations were similar to the 3D

stacked bar charts and t-SNE maps, thus reflecting a similar effect of storage temperature on the sperm heterogeneity in a semen sample.

4 | DISCUSSION

The present study gives new insight into chilling effects on stored boar spermatozoa using multiparameter flow cytometry. Chilling stress was provoked by long-term semen storage at 5°C in a simple short-term extender medium, and was exclusively studied in viable (plasma membrane intact) sperm. Determining multiple sperm attributes on a single cell basis allows to visualize the sperm subpopulations. Thus, the physiological heterogeneity of spermatozoa in a semen sample [23] is mirrored, and additionally, links between structural and functional sperm attributes can be established. Overall, our results indicate that sperm surviving cooling to 5°C maintain membrane characteristics and capacitation behavior with associated mitochondrial function similar to sperm stored at 17°C, which is the usual storage temperature for boar semen. This strengthens the notion that hypothermic storage is an option for boar semen preservation if sufficient numbers of viable sperm are present in the semen dose.

Combined detection of acrosomic integrity and membrane fluidity in semen stored for 24 h showed that chilling to 5°C reduces the proportion of viable, acrosome intact sperm with low membrane fluidity, thus confirming earlier observations with dual-labeled (viability and membrane fluidity) sperm [24, 25]. It is to note that with ongoing storage time, the majority of cells remained in this category reflecting the membrane-stable sperm population, and that the proportion of cells after 144h of storage no longer differed between the two storage temperatures. This suggests that membranes of sperm surviving the initial cooling stress withstand long-term storage as is the case for sperm experiencing conventional storage at 17°C.

The use of four fluorochromes, SiR700-DNA, H33258, JC-1 and Calbryte 630, allowed the simultaneous assessment of functional sublethal alterations related to calcium homeostasis and mitochondrial function. Taking into account the sperm heterogeneity in ejaculates, we deviated from the traditional dual classification for emitted fluorescence into negative (=low intensity) and positive (=high intensity) toward a classification into three groups: Low, medium and high fluorescence intensity, whereby the latter two correspond to the traditionally “positive” class. Viable sperm with high MMP and low intracellular calcium levels are considered as fertilizing competent [26, 27]. The integrative multiparametric analysis of viable sperm in the present study displayed a main sperm population of low calcium cells with medium MMP in both storage groups, albeit with a lower size in semen stored at 5°C compared with 17°C. This confirms the idea of chilling-induced destabilization reported in previous studies [5, 25, 28].

Previous flow cytometric assessment of cytosolic calcium levels in viable sperm demonstrated that chilling and storage reduce the proportion of sperm responding to capacitation stimuli *in vitro* [19, 25]. Capacitation is regarded as a transient stage of progressive membrane destabilization, which small sperm cohorts pass through in individual time-frames [13, 26]. In the present multiparametric study, the functional response to capacitating conditions was shown by the sperm population with enhanced (medium/high) calcium and concomitantly maintained MMP (medium/high FI for JC-1 agg). Remarkably, after exposure to capacitation conditions for 60 min, the sizes of this sperm population were on a similar level in both temperature groups. This indicates that the sperm population surviving the initial chilling stress maintains its ability to capacitate, including mitochondrial function, which is required for controlling oxido-reductive states during capacitation and acrosome reaction [10, 27]. Subtle chilling effects, however, were visualized by a significantly higher proportion of sperm with increased calcium, but a loss of MMP in the 5°C storage group compared with the 17°C controls. Interestingly, calcium-rich sperm stored at 17°C with maintained MMP accumulated more JC-monomers, indicating a capacitation-induced change in the plasma membrane or MMP leading to a higher accumulation of this cationic dye.

Automatic identification of cell populations is available (reviewed by Mair) [29]. Nevertheless, it was not used in the present study because it might have masked the connection among the main sperm populations of interest by disclosing further subpopulations. Instead, the populations of interest were manually classified in cumulative scatter plots from all samples, resulting in triplet descriptions for each sperm subpopulation (Figures 3 and S6; Table S5), which then were subjected to data analysis using conventional statistic software. In addition, computational flow cytometry was exemplarily applied to visualize the heterogenous nature of the semen samples in t-distributed stochastic neighbor embedding (t-SNE) maps. The technique was recently introduced in spermatology [14, 30]. However, to date, it is only scarcely used, probably due to the complexity of tools requiring advanced computational skills and the uncertainty of providing additional information [29]. In the t-SNE map, the 2D scatter plots visualize not only the size of the subpopulations but also the proximity of cells reflected by their distances in the high-dimensional space.

In the present example, phenotype overlays of viable sperm revealed similar sperm subpopulations and changes caused by sperm storage and capacitation treatment corresponding to the triplet code presented in the bar charts (Figure 4). It was reported that freeze-thawing of stallion semen induced a shift in population size and proximity of cells as assessed with viability, oxidative stress and apoptosis marker and visualized in t-SNE maps [14]. In contrast, in our example, the position of sperm population remained constant, indicating that chilling of boar spermatozoa does not affect the heterogenic pattern of sperm subpopulations exposed to storage and *in vitro* capacitation. Heat maps can be applied in a t-SNE map to demonstrate the relative population size, as shown in Figure 5. In addition, multi-dimensional data were exemplarily mapped in three dimensions as a moving radar plot (Figure 6). As axes are moved, the relationship between subpopulations becomes apparent. Again, the basic pattern was similar between the two storage groups. Nonetheless, in chilled sperm the green (low calcium, medium MMP) and blue (low calcium, high MMP) populations were slightly more apart from each other compared with the conventionally stored semen, indicating an advanced separation of these cell populations.

In conclusion, multiparametric assessment of sperm traits gives a profound insight into the impairment of cell function induced by hypothermic semen storage. The viable sperm population in chilled semen is partly destabilized as expressed by an increased number of spermatozoa with collapsed MMP and high cytosolic calcium levels. More importantly, a distinct population of viable sperm showed resistance to chilling stress by maintaining low intracellular calcium levels and high MMP. Extension of validated multiparametric panels considering oxidative stress and apoptosis markers is desirable to further characterize the population of functionally intact spermatozoa. Overall, our results suggest that the surviving sperm population keeps its functional integrity for capacitation and linked mitochondrial activity, thus encouraging the use of cool-stored boar semen in pig AI.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Helen Jäkel performed the research, Helen Jäkel and Karl Rohn analyzed and visualized the data. Anne-Marie Luther and Heiko Henning assisted with data interpretation. Dagmar Waberski and Anne-Marie Luther designed the study. Helen Jäkel wrote a draft of the manuscript, and Dagmar Waberski and Heiko Henning critically revised the drafted manuscript. All authors read, revised and approved the final manuscript.

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SUPPORTING INFORMATION

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

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