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Freezing chicken semen: Influence of base medium osmolality, cryoprotectants, cryoprotectant concentration, and cooling rate on post-thaw sperm survival

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ABSTRACT

A classical chicken semen diluent (Lake's 7.1 diluent) was modified to have lowered osmolalities (ranging from 290 to 410 mOsm/kg). The modified medium with physiological osmolality of 325 mOsm/kg allowed cold storage of fresh semen for several days with very little loss of membrane integrity and motility, while high osmolalities inhibited motility. This modified medium was then used as base for freezing medium to test effects of the type and concentration of cryoprotective agent (CPA), and the cooling rate (CR). A number of CPAs (methylformamide, methylacetamide, dimethylformamide (DMF), dimethylacetamide (DMA), diethylformamide, and propylene glycol) were first compared by freezing semen with 0.6 mol/l of the respective CPA at a cooling rate of 250 °C/min. Post-thaw motility and membrane integrity were highest with DMA and DMF. Finally, in more detailed factorial experiments, semen from individual cocks or pooled semen was frozen using CRs of 4, 50, 250, and 440 °C/min and DMA concentrations ([DMA]) of 0.4, 0.6, 1.0, and 1.5 mol/l. Straws from each semen sample x treatment combination were divided for semen assessment at three different research groups for sperm motility, membrane integrity, kinked tails, and DNA fragmentation, using microscopy, computer assisted motility analysis, and flow cytometry. There were clear effects of both CR and [DMA] and their interaction. CRs 50 and 250 °C/min gave best post-thaw sperm performance. Higher DMA concentrations gave better post-thaw membrane integrity, but concentrations above 1.0 mol/l can decrease sperm velocity or even inhibit sperm motility. Therefore [DMA] may best be 0.6–1.0 mol/l at a CR of 50–250 °C/min.

1. Introduction

In bird species such as chicken or turkey, artificial insemination is largely restricted to 'fresh' semen. For longer-term preservation of semen, cryopreservation can be an effective method, provided that the frozen-thawed semen has sufficient fertilizing capacity. Frozen chicken semen is used in genetic diversity conservation programs [5,7,17,53], and can be used in the chicken breeding industry for import/export of genetics, conservation of specific breeding lines, and for maintaining semen of layer breeder cocks during the period of genetic testing of these males.

Viability and fertility of fresh (extended) chicken semen is known to decline during 24–48h cold storage [2,37,54]. Frozen semen can be stored for a very long duration, but the number of spermatozoa that gives maximal fertilization levels is much higher than for fresh semen [49]. Moreover, fertility results can be low, especially in the case of low fertility lines and many threatened native breeds [7,35,43]. A good semen diluent is important for cold storage and use of fresh extended semen, but also for cryopreservation. A 'base' medium for freezing semen should provide appropriate chemical and physico-chemical conditions that minimize cellular stress to provide the best possible starting point for cells to cope with the (additional) stresses of freezing and thawing [50]. In addition to the base medium, the type and concentration of the used cryoprotective agent (CPA), and the cooling rate

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and other freezing method characteristics are important for successful cryopreservation.

Glycerol is widely used as a suitable CPA, but it prevents fertilization if present together with the semen in the hen [32,40]. Consequently, the semen must be washed free of glycerol after thawing, e.g. by dialysis [10,32], stepwise dilution followed by centrifugation [21,43], or discontinuous density gradient centrifugation [24]. Alternatively, other CPAs have been tried instead of glycerol, such as dimethyl sulfoxide (DMSO) [3,47], and ethylene glycol (EG), [36]. The amides N, N-dimethylacetamide (DMA) and N,N-dimethylformamide (DMF) have been used successfully for a number of fowl and other bird species, including chicken [11,20,29,33,36,42,45,46], turkey [16,25,30], goose [26,41,48], duck [45], emu [39] and a number of falconiform birds [6]. Tselutin [45,46], and Chalah [11] reached excellent fertility with chicken semen frozen with DMA by a very rapid cooling technique that involves plunging 50 μl semen droplets straight into liquid nitrogen. However, the same semen, but frozen in parallel in straws in a programmable freezer at a low cooling rate (7 °C/min), had much lower fertility (26.7%) than the semen frozen with the rapid pellet freezing (84.7%) [46]. However, from this study it can't be seen if the difference was due to the type of packaging and freezing (straw vs. pellets) *per se*, or to the cooling rate.

In 2006 we reported that very good fertility can be equally obtained using DMA as CPA with either pellet and straw freezing, using 240 million total sperm per insemination, provided that also the straws are frozen at a very high cooling rate (200 °C/min) [53]. Also, we obtained better post-thaw fertility with 'ASG' medium as base medium than with Lake's freezing medium [18,22] when both media were used with 0.6 mol/l DMA as CPA. 'ASG' medium is in fact modified Lake and Ravie medium 7.1 (L&R7.1, [19]) with osmolality reduced to 325 from 411 mOsm/kg water (see also [4,25,53]). This fertility trial had been done with sufficient number of replicates and hens per treatment, but the effects of osmolality of the base medium, the freezing rate, and the DMA concentration on post-thaw sperm characteristics had only been investigated tentatively in a pilot study. Therefore, in the current study we have readdressed these variables in a study with sufficient replicates and numbers of cocks per replicate. First, the effect of osmolality on sperm quality was investigated during cold storage of semen. In addition, we tested effects of BSA, as an earlier study showed that the presence of BSA can increase the apparent post-thaw sperm motility [53]. The current study confirmed that osmolalities above the sperm physiological range had a strong negative effect on sperm motility. Subsequently, we have investigated the suitability of cryoprotectants similar to DMA, comparing post-thaw survival of semen frozen with either DMA, DMF, N-methylacetamide (MA), or N-methylformamide (MF). Best post-thaw results were obtained with DMA. Therefore, in more detailed factorial experiments, post thaw sperm quality was investigated after freezing of semen from individual cocks and pooled semen with 0.4, 0.6, 1.0, or 1.5 mol/l (or 3.7, 5.6, 9.3, 14.0 vol%) DMA at cooling rates of 4, 50, 250, or 440 °C/min.

2. Materials and methods

2.1. Media and reagents

All chemicals were research grade chemicals purchased from Merck Sigma-Aldrich, unless specified otherwise.

The standard poultry extender (PE) was medium '7.1' from Lake and Ravie [19], but modified to have 325 mOsm/kg (instead of 411 mOsm/kg) by lowering the concentrations of all chemical ingredients equally (i.e. add more water). PE contained (gram per 100 ml, mmol/l between brackets): 1.21 g (64.7) sodium-L-glutamate.H2O, 0.102 g (3.14) tri-potassium-citrate.H2O, 0.064 g (2.97) magnesium acetate.4H2O, 0.53 g (26.5) D-(+)-glucose.H2O, 2.43 g (114) BES (N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid), 0.185 g (46.2) NaOH, and Milli-Q water to a final volume of 100 ml, resulting in pH 7.1 and osmolality of approximately 325 mosm/kg of water. For experiment 1, extenders were prepared with essentially the same recipe, but containing less or more water to achieve different osmolalities. Where indicated, bovine serum albumin fraction V (BSA) was added to PE or to the freezing media to a concentration of 10 mg/ml.

CPAs used were DMA, DMF, MA, MF, N,N-diethylformamide (DEF), or propane-1,2-diol (propylene glycol, PG). Freezing media were essentially PE with CPA. However, the CPAs are not just added to the ready PE, but rather added to replace an equal volume of water. To make, for example, 100 ml of CPA medium, a solution was made that contained the exact same amounts of all ingredients of 100 ml of PE base medium, plus the CPA, and then water was added to a final volume of 100 ml. Thus, the molar concentrations of all not-permeating solutes were the same in the freezing media as in PE base medium. This approach is different from just adding CPA to ready PE, which would lead to lower molar concentrations of the not permeating solutes (the PE is 'diluted' by the CPA), and could result in reswelling to higher than the original (isotonic) cell volumes. To reach the desired final CPA concentrations in the semen, a volume of cooled, prediluted semen (for predilution, see paragraph below) was mixed with half a volume of cooled PE-CPA with three times the final concentration of the CPA. For instance, in experiments 5 and 6 we used PE-DMA solutions of 1.2, 1.8, 3.0 and 4.5 mol/l DMA for final concentrations of 0.4, 0.6, 1.0 and 1.5 mol/l (or 3.7, 5.6, 9.3, 14.0 vol%) DMA, respectively. The freezing medium of 4.5 mol/l DMA contained no BSA, as BSA did not remain soluble at 4.5 mol/l DMA. Consequently, the final BSA concentration in straws with 1.5 mol/l DMA had been a bit lower (6.7 instead of 10 mg/ml in experiment 5 and approximately 5.3 instead of 8 mg/ml in experiment 6).

2.2. Semen collection and pre-processing

Semen was kindly provided by Cobb from a local research facility in The Netherlands. The semen was obtained from broiler-breeder cocks at the age of 33–39 weeks. Semen was collected by the abdominal massage technique [9] and was diluted 1:1 directly (in the barn) after collection with PE at room temperature. The prediluted semen was then, still on location, further diluted with an equal volume of PE or PE-BSA as indicated per experiment. The samples were placed in 15-ml Falcon tubes in a 5 °C cool box. In experiments in which smaller semen samples (e.g. 200–500 μl) were used, the samples were placed in the cool box in a 2.0-ml Eppendorf tube inside a 15-ml Falcon tube. The samples were transported to the institute in approximately 30 min time. A small subsample of the 1:1 prediluted semen was kept apart for measurement of the sperm concentration. The semen samples were further processed in a refrigerated work bench set at 5 °C (4–7 °C).

2.3. Specific experiments

Experiment 1. Three replicates were done with semen from 5 cocks per replicate (different cocks were used each replicate, i.e. 15 different cocks in total). Of each cock, 100 μl prediluted semen (prediluted in the barn 1:1 with PE-325) was then, still on location, mixed with 100 μl of modified PE with osmolalities of 283, 325, 373, or 427 mOsm/kg, or with PE-325 with BSA (10 mg/ml). After transport and cooling to 5 °C, the semen samples were further diluted with 400 μl of the same respective extender at 5 °C, resulting in final osmolalities of 290, 325, 365, 410 mOsm/kg. These samples were then further diluted to 200 x 10^6 sperm/ml with PE with osmolalities of 290, 325, 365, or 410 mOsm/kg, respectively, of 5 °C. The semen samples were stored in 2-ml Eppendorf tubes in a refrigerated incubator (Sanyo MIR-253) at 5 °C for 4 days. Motility, tail normalcy, and membrane integrity was assessed on days 0, 1, 2, and 4. At day 4, a subsample of 50 μl of the semen samples with osmolality 410 mOsm/kg was mixed with 100 μl of PE of 283 mOsm/kg (at 5 °C) to restore osmolality to 325 mOsm/kg. The

sperm were allowed to recover for 20 min at 5 °C and the sperm motility was assessed.

Experiment 2. Two replicates were done with semen from 5 cocks per replicate (different cocks were used each replicate, i.e. 10 different cocks in total). Each ejaculate was diluted 1:1 with PE at room temperature directly after collection. The prediluted semen samples were then each split into two subsamples, and one subsample was further diluted with an equal volume of PE and the other with PE-BSA. After cooling to 5 °C, the semen was further diluted to a sperm concentration of 300 x 10^6 sperm/ml with PE or PE-BSA, respectively, mixed with PE-CPA or PE-BSA-CPA to final concentrations of 0.6 mol/l CPA and 200 x 10^6 sperm/ml, packed in 0.25-ml straws and frozen directly (within 7 min after adding CPA) at 250 °C/min, as detailed below. Motility, tail normalcy, and membrane integrity in pre-freeze and post-thaw samples were assessed as detailed below.

Experiment 3. One replicate was done with semen from 10 cocks. Each ejaculate was diluted 1:1 with PE (i.e. no BSA) directly after collection, but media in all further steps contained BSA. Other methods were the same as in experiment 2, except now only DMA was used as CPA, and straws were frozen at 250 °C/min at 0h (within 7 min), 4h, or 20h after adding DMA.

Experiment 4. One replicate was done with semen from 5 cocks. Methods were essentially the same as in experiment 3, except that four different CPAs were used (DMA, DMF, DEF, or PG), and straws were frozen at 0h (within 7 min) or 1h after adding CPA.

Experiment 5. Two replicates were done with semen from 5 cocks per replicate (different cocks were used each replicate, i.e. 10 different cocks in total). The initial steps of semen processing were essentially as in experiment 3, except that each ejaculate was diluted more strongly with PE-BSA, to accommodate the large number of [DMA] x freezing method combinations, resulting in final sperm concentrations ranging from 50 to 95 x 10^6 sperm/ml. Four final DMA concentrations (0.4, 0.6, 1.0 and 1.5 mol/l) times four different freezing methods with different cooling rates were used. In addition, a fifth freezing method (pellet freezing) was used with 0.6 mol/l DMA, giving a total of 17 combinations per cock. Freezing methods are detailed below. Addition of DMA, packaging in 0.25-ml straws and freezing was done separately and sequentially for each freezing method, such that freezing occurred approximately 1h after addition of DMA. Sperm assessment was carried out in parallel in three different institutes as detailed below.

Experiment 6. Effect of [DMA] and CR on post-thaw sperm quality of pooled semen at high sperm concentration. Four replicates were done with pooled semen from 20 to 30 cocks per replicate. Semen processing, freezing, and pre-freeze and post-thaw sperm assessment were essentially the same as in experiment 5, except that pooled semen was used that was diluted to a lesser extent with PE-BSA, to give a high final sperm concentration (1200 x 10^6 sperm/ml).

2.4. Freezing methods

Pellet freezing [45]: Semen droplets of 50 µl were dropped into a dewar with LN_2. Freezing was complete (nitrogen stopped boiling) in about 20s, i.e. the overall CR (over the complete freezing trajectory) was approximately 600 °C/min.

CR440: A Cryoson freezing cabinet, controlled by the control unit of a Planer Kryo 10 controlled-rate freezer (Planer Ltd. Sunbury-On-Thames, UK) was used, programmed at a constant temperature of −160 °C. Straws were placed on steel straw racks (IMV, L'Aigle, France) and held in place by a thin rubber band stretching along the length of the rack (which is necessary at this extreme high CR, with high ventilation rate and strong jets of injected LN_2). The racks were swiftly placed inside the freezing cabinet. The CR inside the straws reached approximately 440 °C/min, measured as the slope of temperature over time (ΔT/Δt) between −10 °C (after the dissipation of heat of fusion) and −60 °C. After at least 2 min, the straws were removed from the cabinet and plunged into LN_2.

CR250: Straws were placed on a steel straw rack and placed in a custom built ventilated nitrogen vapor freezer, set at a constant vapor temperature of −140 °C [50], resulting in a CR inside the straws of approximately 250 °C/min, measured as ΔT/Δt between −10 °C (after the dissipation of heat of fusion) and −60 °C. After at least 2 min, the straws were plunged into LN_2.

CR50: Straws were placed in a 'contact freezer', a self-designed, custom built tabletop freezing device. The device consists of a thermally isolated base made of EPDM (ethylene propylene diene monomer) closed-cell foam that can accommodate the straws, and a stainless steel top part filled with liquid nitrogen that serves as the heat sink. The straws are covered with a thin insulating sheet and the heat sink is put on top of that. In the current experiment, we used a stack of 22 sheets of printer paper as insulator between the straws and the heat sink, which resulted in a CR inside the straws of approximately 50 °C/min, measured as ΔT/Δt between −10 °C (after the dissipation of heat of fusion) and −60 °C. After 5–10 min, the straws were removed and plunged into LN_2.

CR4: The Cryoson freezing cabinet, controlled by the Planer Kryo 10 control unit, was used at a CR of 4 °C/min. The cooling program was basically a linear cooling at a CR of 4 °C/min. However, the program featured a brief burst of nitrogen vapor when the chamber temperature reached −9 °C (T inside the straws 5–6 °C) to seed the straws, and the cooling was then paused for 1 min, and continued with linear cooling at 4 °C/min until −105 °C. The pause allowed the dissipation of heat of fusion, and thus prevented a very large temperature difference between the contents of the straws and the freezing chamber, as this would lead to substantially higher CRs (after the plateau of heat of fusion) than the intended 4 °C/min. At T −105 °C, the straws were removed from the cabinet and plunged into LN_2.

The time course of temperature change inside the 0.25-ml straws during freezing was registered in a number of 'dummy' straws with PFA-insulated copper-constantan thermocouples (TCs) with a diameter of 76 µm ((Omega, type 5SC-TT-TI-40-1 M), using an Omega TC-08 thermocouple data logger and Logging Software for Windows (Omega, Norwalk, CT 06854, USA). The freezing chamber temperature sensor (Pt100) and all TCs were calibrated using melting ice as 0 °C reference. The thermocouple probes were inserted through a small opening, made with a fine needle, in the middle of the straws and fixed with a small plug of glue. Then, the straws were filled with semen in PE-DMA (0.6 mol/l DMA) and the straws were sealed with a plug of polyvinyl alcohol. The straws with TCs were placed on the straw rack surrounded by other straws, and the respective freezing method was started. Typical cooling curves for the four straw methods are shown in Fig. 1.

2.5. Thawing

Straws were taken from LN_2 and briefly (2s) exposed to air, to allow LN_2 on the outside of the straw to evaporate, plunged into a 4-5-°C water bath, and moved through the water vigorously for 30 s. Straws were wiped dry, cut open, and contents were collected in a collection tube. Pellets were thawed by placing 2–3 pellets on a portable heated microscope stage at 50 °C, allowing the semen to drip off the stage into a collection tube in a 5 °C water bath as soon as it became liquid. All post-thaw semen samples were kept at 4–5 °C unless specified otherwise.

2.6. Semen assessment

Sperm concentration was determined using an Accucell (IMV, L' Aigle, France), using the settings supplied for Turkey semen (a = 0, b = 66, c = 5136 and d = −179). Calibration of the Accucell with chicken semen, using a Bürker Türk counting chamber, revealed that the concentration obtained with these Accucell settings needed to be adjusted by multiplication by 1.12.

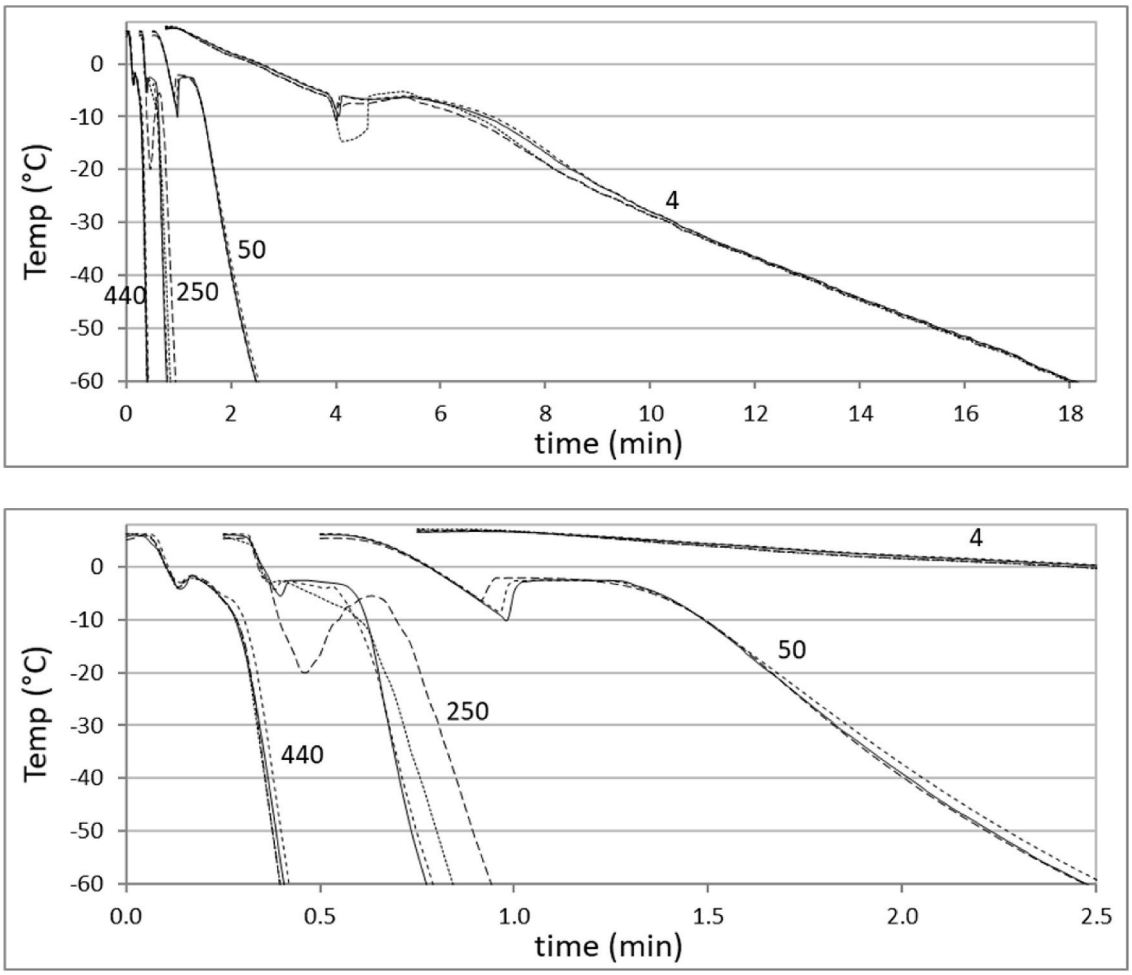

Fig. 1. Typical freezing curves for the four straw freezing methods used (four straws for each method; curves shown until −60 °C). Per method/CR, the freezing curves are shifted horizontally relative to each other in order to visualize them separately, and are shown over 18 min and 2.5 min time scales (upper and lower panels, respectively) because of the large range of steepness (CR).

Semen straws had been labeled with a random number only, so that the cock and treatment were not known to the researchers doing the assessment.

In experiments 2-6, per semen/treatment combination, two straws (except experiment 4, 1h holding, for which it was 1 straw), or two times 2–3 thawed pellets, were assessed by Wageningen University and Research for microscopic assessment of motility, tail normalcy, and membrane integrity (DAPI exclusion). In addition, in experiments 5 and 6, 2–4 straws per semen/treatment combination were shipped to INRAe (France) for flow-cytometric assessment of membrane integrity, and 2–4 straws per semen/treatment combination were shipped to INIA (Spain) for sperm motility assessment by computer assisted sperm analysis (CASA) and analysis of DNA fragmentation by deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay by flow-cytometry.

2.7. Microscopic assessment of motility, tail normalcy, and membrane integrity (DAPI exclusion)

In experiment 5, frozen/thawed semen with 1.5 mol/l DMA was diluted 1:1 with PE-BSA prior to motility assessment, as motility was almost completely blocked at the highest [DMA], but was restored when [DMA] was lowered. Pre-freeze and post-thaw semen of experiment 6 was stepwise diluted 10 times with PE-BSA prior to motility assessment, as this semen was very concentrated. Four µl of a semen sample was placed on a slide under an 18 × 18 mm coverslip, and the proportion (percentage) of motile sperm cells was determined by phase contrast microscopy at room temperature in five locations of the cover slip (center and four corners). The mean of the values of the five locations was taken. In the same slides (same five locations per slide), 200 cells were evaluated for having a normal tail or a 'kinked' tail (hairpin bent tail or 180° bent tail). Another aliquot of 20 µl of semen was mixed with 20 µl of 10 µmol/l DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) in PE, held for 5 min at room temperature in the dark, and 10 µl of glutaraldehyde (0.5 vol%) was added to immobilize the sperm. Two-hundred cells per sample were evaluated for DAPI staining by epifluorescence microscopy, to determine the percentage sperm cells that excluded DAPI, i.e. cells with membrane integrity (%MI-DAPI).

2.8. Motility assessment by CASA

Thawed semen was stepwise diluted with PE-BSA to a sperm concentration of approximately 40 million sperm/ml. Sperm motility was assessed by CASA using the Sperm Class Analyzer® v.4.0 software (Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope (negative contrast) as previously described [34], using the settings for chicken semen provided by the CASA system. CASA motility analysis was done using standard glass slides, with 5 µl of semen under a coverslip of 20 × 20 mm. A minimum of three fields and 500 sperm tracks were evaluated at a magnification of 100x for each sample (image acquisition rate 25 frames/s).

2.9. Assessment of DNA integrity

DNA integrity was assessed using the APO-BrdU™ TUNEL Assay Kit (Thermo fisher Scientific). Briefly, an aliquot of 50 µl of semen was mixed with 450 µl formaldehyde (4 vol%), washed twice in PBS, resuspended in ice-cold ethanol (70% v/v) and stored at −20 °C until analysis. For analysis, the sperm concentration was adjusted to 1-2 x 10^6 sperm ml in PBS-BSA. As positive control, fixed and ethanol-permeabilized sperm were treated with RQ1 RNase-Free DNase (Promega) (24 U/ml) for 60 min at room temperature, and washed with PBS-BSA. Labelling and antibody staining of 200-µl sperm samples was done in a 96-well plate (V-shaped wells), essentially following manufacturer's instructions. The plates were analyzed with a FACSCanto II flow cytometer (BD Biosciences, USA) using 404 nm and 488 nm lasers for excitation, and 450/50 nm and 530/30 nm filters for emission. Events were gated in the FSC/SSC dot plot to exclude debris. Subsequently, sperm cells were gated on the basis of blue emission of the H33342 staining, and TUNEL staining (green emission) was assessed in 2–10 × 10^3 spermatozoa per sample. Acquired data were analyzed using the FlowJo software ((BD Biosciences).

2.10. Membrane integrity (PI exclusion, flow cytometry)

Post-thaw sperm concentration was adjusted to 25 x 10^6 cells/ml with PE. Sperm membrane integrity was assessed by flow cytometry as described by Thélie et al. [44]. Sperm cells were incubated in 96 wells plates with 50 µmol/l SYBR-14 for 10 min at 4 °C in darkness; 48 µmol/l propidium iodide was added and incubation was continued for an additional 5 min. Flow cytometry was performed using an EasyCyte Guava flow cytometer (Millipore, Molsheim, France). 5000 events were acquired per sample, using the blue laser (488 nm) and photomultiplier tubes PMT-Green (525/30 nm), -Yellow (583/26 nm), and -Red (695/50 nm). Events were gated in the FSC/SSC dot plot to exclude cellular debris. Subsequently, sperm cells were gated on the basis of the SYBR-14 staining, and the percentage sperm cells that excluded PI, i.e. cells with membrane integrity (%MI-PI), was determined.

2.11. Statistical analyses

Data expressed as percentages (e.g. %Mot for motility), were re-expressed as proportions and analyzed by a generalized linear mixed model (GLMM). Other data, like VAP for sperm velocity, were analyzed by a linear mixed model (LMM). The GLMM comprised a logit link function and a beta distribution at the 'residual level'. In addition there were fixed effects and random effects on the logit scale. For instance, in experiment 1 fixed effects were main effects for replicates, main effects for media, main effects for days, and interaction between media and days. Random effects in experiment 1 comprised random effects for cocks, to account for repeated measures of the same cock. When there were an equal number of repeats, e.g. two samples per medium, day and cock in experiment 1, the mean of the two samples was used for analysis. When the number of repeats was not the same, e.g. two straws at 0 h and one straw at 1 h in experiment 4, data were analyzed per straw and additional random interactions, including interaction between cocks within replicates, hours and CPA, were introduced on the logit scale. In experiment 6, where pooled semen was used, there were no random effects on the logit scale. The LMM comprised fixed and random effects, next to the usual residual error terms. Both in the GLMM and LMM random effects (e.g. random cock effects) were assumed to be independently normally distributed around 0 with constant component of variance. For analysis, R routine glmmTMB from library glmmTMB was used [8]. For the LMM this amounted to restricted maximum likelihood (REML). For the GLMM, approximate maximum likelihood was used, employing Laplacian integration. Joint test results, e.g. for interaction between media and days in experiment 1, were based on the Wald test (referring to a chi-square distribution). Pairwise comparisons were based on the z-test (referring to the standard normal distribution). In the latter case multiplication of P-values by the number of pairs considered (rounding values exceeding 1 to 1) yielded Bonferroni P-values (P_{Bonf}) that take multiple testing into account. For the Wald test, the R routine 'wald.test' from library 'aov' was used and for pairwise comparisons the R routine 'emmeans' from library 'emmeans' was used.

3. Results

3.1. *Experiment 1.* Base medium, effect of osmolality and the presence of BSA in fresh semen

Osmolalities 365 and 410 mOsm/kg had an immediate strong negative effect on motility (%Mot) of fresh semen (Fig. 2, Table 1). The interaction of media x days cold storage was significant for %Mot ($P < 0.001$), as the decline of %Mot over time was steeper at elevated than at physiological osmolalities. In a model without the interaction, the main effects for media and days were significant ($P < 0.001$) (Table 1A). Restoring physiological osmolality after four days cold storage at 410 mOsm, by stepwise lowering the osmolality of these samples to 325 mOsm/kg, increased the mean %Mot from 10.6% to 34.2%, but this was still strongly and significantly lower than that of samples held for four days at 325 mOsm/kg (51.9%). Media 325 and 325-BSA had no significant differences in %Mot on any of the days (Table 1B).

Membrane integrity (%MI-DAPI) was not strongly affected by days or media. The means (over cocks) of %MI-DAPI for all medium x day combinations were in the range of 80–90% (Table 1C). Media had no effect on %MI-DAPI on any of the days when analyzing the separate days (not shown). The analysis combining media and days showed no significant interaction of days x media (Table 1A). Main effects for media ($P = 0.008$) and days ($P = 0.002$) were significant, but effects were small, with somewhat (significantly) higher %MI-DAPI in medium 325-BSA than in media 325, 365 and 410 when analyzing means over all days (Table 1C). After Bonferroni correction, %MI-DAPI of medium 325-BSA was still significantly higher than that of media 325 and 410.

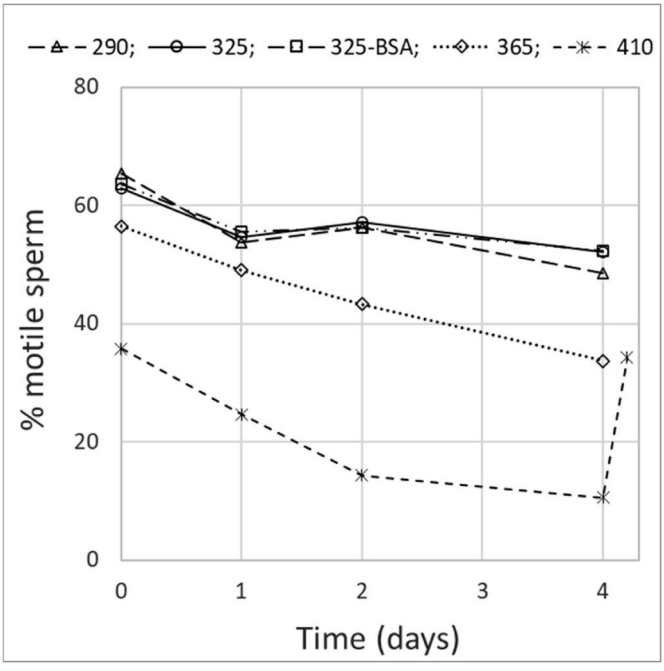

Fig. 2. Sperm motility (%Mot) after 0, 1, 2, and 4 days of 'fresh' storage at 5 °C in media of varying osmolalities. "325B" denotes medium 325 plus BSA. The time point at 4.2 days represents %Mot for medium 410, but brought back gradually to 325 mOsm/kg on day 4. P-values of interaction, main effects, and pairwise comparisons are presented in Table 1.

Table 1
Effect of osmolality and BSA during cold storage.

A. P-values[#] for interaction[$] and main effects[€]

	%Mot	%MI-DAPI	%KT
medium x day	0.000	0.7	0.32
Medium	0.000	0.008	0.000
Day	0.000	0.002	0.55

B. Means %Mot per medium per day[$,§]

medium	%Mot d0	%Mot d1	%Mot d2	%Mot d4
290	$65.4^{a,1}$	$53.7^{a,1}$	$56.3^{a,1}$	$48.6^{a,1}$
325	$62.9^{a,1,2}$	$54.7^{a,1}$	$57.2^{a,1}$	$52.2^{a,1}$
325-BSA	$63.6^{a,1,2}$	$55.5^{a,1}$	$56.3^{a,1}$	$52.3^{a,1}$
365	$56.5^{b,2}$	$49.0^{a,1}$	$43.3^{b,2}$	$33.7^{b,2}$
410	$35.7^{c,3}$	$24.7^{b,2}$	$14.3^{c,3}$	$10.6^{c,3}$
410–325*				$34.2^{b,2}$

C. Means of %MI-DAPI and %KT per medium or per day[$,§]

Averaged over days			Averaged over media		
Medium	%MI-DAPI	%KT	Day	%MI-DAPI	%KT
290	$85.6^{a,b,1,2}$	$13.1^{a,1}$	day 0	$86.0^{a,b,1,2}$	$8.9^{a,1}$
325	$84.4^{c,2}$	$12.3^{a,1}$	day 1	$84.8^{b,2}$	$9.7^{a,1}$
325-BSA	$86.5^{a,1}$	$8.5^{b,2}$	day 2	$86.9^{a,1}$	$10.3^{a,1}$
365	$84.8^{b,c,1,2}$	$6.3^{c,3}$	day 4	$82.7^{b,2}$	$7.8^{a,1}$
410	$84.3^{b,c,2}$	$5.6^{c,3}$			

[#] '0.000' indicates $P < 0.001$.
[§] Within a column, a common letter superscript indicates $P \geq 0.05$. A common number superscript indicates $P_{Bonf} \geq 0.05$.
[$] Model with interaction media x days.
[€]Model without interaction.
* 410-325 denotes semen in medium 410, but brought back gradually to 325 mOsm/kg on day 4.

Regarding days, the results suggest a slight decline of %MI-DAPI over time (Table 1C).

For the percentage of sperm with a kinked tail (%KT), the interaction of media x days was not significant. Main effects for days and pairwise comparisons between days were not significant. Main effects for medium were significant ($P = 0.001$). %KT was lower at higher osmolality (Table 1C), indicating that the inhibition of motility coincided with less appearance of kinks in the tails. Interestingly, BSA reduced the incidence of kinked tails, with significantly lower values in medium 325-BSA than in medium 325, which was upheld after Bonferroni correction (Table 1C).

3.2. ***Experiments 2, 3, 4.*** *Type of CPA and pre-freeze holding*

The effect of type of CPA and the effect of pre-freeze holding time were investigated in experiments 2-4. In experiment 2, CPAs DMA, DMF, MA, and MF were compared, and the effect of BSA was again tested. Before freezing (Table 2A), BSA had no effect on membrane integrity (% MI-DAPI). BSA appeared numerically to give an advantage for motility (%Mot higher) and tail normalcy (%KT lower), but the effects were not significant ($P = 0.15$ and 0.095, respectively).

For post-thaw sperm quality (Table 2B), the main effects for CPA were significant for %Mot, %MI-DAPI ($P < 0.001$), and %KT ($P = 0.006$). Post-thaw %Mot and %MI-DAPI were clearly lower for MA and MF than for DMA and DMF (significant after Bonferroni correction). In addition, the majority of all membrane-intact sperm cells had kinked tails after freezing with MA and MF. DMF gave higher %Mot than DMA, but had equal %MI-DAPI and higher %KT. DMA-BSA and DMA had no significant differences in %Mot, %MI-DAPI, or %KT, but BSA may have a small advantage, as it resulted in numerically higher %Mot and %MI-DAPI, and lower %KT.

In experiment 3, the effect of holding at 5 °C for 0h, 4h, and 20h (time between addition of DMA and freezing) was investigated. PE and PE-DMA contained BSA. Holding had no significant effect on pre-freeze motility (Table 3A, $P = 0.15$). However, post-thaw motility was much higher for 4h than for 0h holding (Table 3B; significant after Bonferroni correction). There was no effect of holding on %MI-DAPI. Holding had a small negative effect on tail normalcy, as %KT was higher for 4h than for 0h holding (Table 3B).

In experiment 4, the CPAs DMA and DMF were again compared, using 0h and 1h holding (Table 4). Since the better results obtained with DMA and DMF, compared with MA and MF (experiment 2), initially seemed to be explained by differences in the ratio of the melting point over the molecular mass (MP/MM), we now included two other CPAs, propylene glycol (PG) and diethylformamide (DEF), with even lower MP/MM values than DMA and DMF. Table 5 shows the MP/MM values as well as the lipophilicity (octane-water partitioning coefficient (K_{OW}) of the CPAs used.

Semen motility was not affected by addition of the CPAs (not shown), except DEF, for which pre-freeze %Mot was lower and the motile sperm appeared to move in "slow motion". For all post-thaw sperm variables tested, it was clear that DEF is not a suitable CPA (Table 4). PG gave quite poor post-thaw results as well, with much lower values for %Mot and %MI-DAPI than obtained with DMA and DMF (Table 4). DMA and DMF both gave good post-thaw results. Thus it appears that the suitability for the CPAs DMA, DMF, PG, and DEF, MA, and MF (experiments 2-4) was not explained by their molecular mass, melting point, the ratio of MP/MM, or K_{OW}.

For DMA, holding had a positive effect on %Mot (62.2% vs 41.8%; $P_{Bonf} = 0.0001$). For DMA, holding also appeared to result in a bit higher %MI-DAPI ($P = 0.054$), and lower %KT ($P = 0.027$, $P_{Bonf} > 0.05$). Holding had no effect for the other CPAs for all sperm variables (Table 4).

3.3. ***Experiment 5 and 6.*** *Effects of [DMA] and cooling rate*

In experiment 5, semen from individual ejaculates/cocks was used,

Table 2
Effect of CPA and BSA on semen variables.

A. Pre-freeze[§]

	%Mot	%MI-DAPI	%KT
no BSA	$65.7^{a,1}$	$94.2^{a,1}$	$15.4^{a,1}$
with BSA	$69.1^{a,1}$	$94.2^{a,1}$	$12.5^{a,1}$

B. Post-thaw[§]

	%Mot	%MI-DAPI	%KT
DMA	$26.9^{b,2}$	$60.6^{a,1}$	$19.6^{b,c,1,2}$
DMA BSA	$28.2^{b,1,2}$	$60.6^{a,1}$	$16.6^{c,2}$
DMF	$32.5^{a,1}$	$60.0^{a,1}$	$21.6^{a,b,1,2}$
MA	$15.9^{d,4}$	$40.5^{b,2}$	$25.3^{a,1}$
MF	$20.7^{c,3}$	$39.0^{b,2}$	$21.3^{a,b,1,2}$

[§] Within a column, a common letter superscript indicates $P \geq 0.05$. A common number superscript indicates $P_{Bonf} \geq 0.05$.

Table 3
Effect of holding* on semen variables.

A. Pre-freeze[§]

Holding	%Mot	%MI-DAPI	%KT
0h	$77.4^{a,1}$	88.4	13.0
4h	$77.4^{a,1}$		
20h	$74.1^{a,1}$		

B. Post-thaw[§]

Holding	%Mot	%MI-DAPI	%KT
0h	$34.5^{b,2}$	$59.3^{a,1}$	$18.2^{c,3}$
4h	$52.8^{a,1}$	$60.0^{a,1}$	$25.2^{b,2}$
20h	$39.3^{b,2}$	$61.7^{a,1}$	$30.8^{a,1}$

* Time between adding DMA and freezing.
[§] Within a column, a common letter superscript indicates $P \geq 0.05$. A common number superscript indicates $P_{Bonf} \geq 0.05$.

Table 4
Effect of CPA[§] and holding* on post-thaw semen variables.

	%Mot			
	0h	1h	%MI-DAPI	%KT
DMA	41.8^{a,1}	62.2^{a,1}	60.7^{a,1}	16.9^{a,1,2}
DMF	48.4^{a,1}	52.2^{a,1}	60.2^{a,1}	17.6^{a,1}
PG	19.6^{b,2}	17.3^{b,2}	22.1^{b,2}	10.6^{b,2}
DEF	0.0^{c,3}	0.0^{c,3}	4.0^{c,3}	5.6^{c,3}

[§] Within a column, a common letter superscript indicates $P \geq 0.05$. A common number superscript indicates $P_{Bonf} \geq 0.05$. P-values from model with interaction CPA x holding in the case of %Mot.
* Time between adding CPAs and freezing.

Table 5
Characteristics of CPAs.

	MM[#]	MP[$,&]	MP/MM	Log $K_{ow}^{\%,&}$
MF	59.1	267.8	4.53	−0.97
MA	73.1	301.2	4.12	−1.05
DMF	73.1	212.9	2.91	−1.01
DMA	87.1	254.6	2.92	−0.77
PG	76.1	213.2	2.80	−0.92
DEF	101.1	<293	<2.9	0.48
Gly	92.1	291.3	3.16	−1.76

[#] Molar mass (g/mol).
[$] Melting point (K).
[%] [10]Log of the octane-water partitioning coefficient (K_{ow}).
[&] Values from https://ubchem.ncbi.nlm.nih.gov.

which was highly diluted, i.e. had a low sperm concentration. In experiment 6, pooled semen at a high sperm concentration (suitable for insemination) was used. Generally, post-thaw motility (%Mot) and membrane integrity (%MI-DAPI) had 'inverted U'-shaped curves when plotted against the CR (Fig. 3), showing CRs of 50–250 °C/min to be optimal, with minor differences between experiment 5 and 6. The interaction CR x [DMA] was significant for %MI-DAPI in experiments 5 and 6, and for %Mot in experiment 5. In a model without the interaction, main effects for CR and for [DMA] were highly significant for %MI-DAPI and %Mot in both experiments. Significance of pairwise comparisons between CRs per [DMA] for %Mot and %MI-DAPI are shown in Table 6. Post-thaw %Mot was generally higher at higher DMA concentration up to 1.0 mol/l DMA, where it peaked or levelled off (Table 6). It must be noted that hardly any motility was observed at the highest concentration of DMA (1.5 mol/l). However, semen frozen at 1.5 mol/l was motile after diluting the post-thaw semen with PE to lower the DMA concentration. The %MI-DAPI did not level off at higher [DMA] and highest values were reached at 1.5 mol/l DMA (Table 6).

Comparing experiments 5 and 6 (low and high sperm concentration), there appears to be a difference in the optimal cooling rate. In experiment 5, %Mot and %MI-DAPI tended higher at CR50, with several pairwise comparisons having P-values <0.05 (though not after Bonferroni correction). In contrast, in experiment 6, %Mot and %MI-DAPI were higher at CR 250 than CR 50, which was significant (after Bonferroni correction) for DMA 0.4, 1.0, and 1.5 mol/l (Table 6).

In experiment 6 (high sperm concentration), semen frozen in pellets (with 0.6 mol/l DMA and with a CR in the range of the highest CR used in straws) had significantly higher %Mot and %MI-DAPI than the straws frozen with CR 440 and 0.6 mol/l DMA (Table 6). In experiment 5, the differences between pellets and straws frozen with CR 440 and 0.6 mol/l DMA were very small and not significant.

Membrane integrity was also assessed by flow cytometry, measuring PI exclusion (%MI-PI, Table 7) in semen from straws produced from the same semen samples as −and frozen in parallel with− the straws used for assessing %Mot, %MI-DAPI, and %KT. Overall, the relationship of %MI-PI with CR was similar to that of %MI-DAPI (Fig. 3, Table 6). For %MI-PI (Table 7), the interaction [DMA] x CR was not significant. In a model without the interaction, the main effects for CR ($P = 0.08$) were also not significant. The main effects for [DMA] were significant ($P < 0.001$).

CASA motility and DNA integrity (TUNEL-negative sperm) were assessed in semen from straws produced from the same semen samples as −and frozen in parallel with− the straws used for assessing %Mot, %MI-DAPI, %KT, and MI-PI. The dependence of the percentage motile and progressively motile sperm cells (%Mot-CASA and %ProgMot-CASA) on CR (Table 8) were similar to these shown for %Mot and %MI-DAPI (Fig. 3; Table 6), i.e. 'inverted U'-shaped relationships, indicating CRs of 50–250 °C/min to be optimal. The interaction of [DMA] x CR was significant for %Mot-CASA and %ProgMot in experiments 5 and 6 (Table 8A). In a model without the interaction, main effects of [DMA] and CR were highly significant for both %MotCASA and %ProgMot ($P < 0.001$) in both experiments 5 and 6 (Table 8A). Highest %Mot was again seen at 1.0 and 1.5 mol/l DMA (over all CRs) (Table 8B). For %Mot-CASA, the optimal cooling rate in experiment 5 appeared to be 50 °C/min, as CR 50 gave significantly higher values than CR250 at 0.4, 0.6, and 1.0 mol/l DMA, whereas %Mot-CASA did not differ significantly between CR50 and CR250 in experiment 6.

Overall, the post-thaw %MotCASA and %ProgMot, and the sperm velocities (VCL, VAP, VSL) (Tables 8B and 8C), appeared to be lower in experiment 6 than in experiment 5. But then, also the pre-freeze semen motility had been higher in experiment 5 (76.4%) than in experiment 6 (66.5%). However, experiment 5 (low sperm concentration) and experiment 6 (high sperm concentration) also showed differences in the effect of [DMA] on velocity variables (Table 8C). In experiment 5, sperm velocities peaked at 0.6 mol/l DMA and declined at higher [DMA]. This decline appeared stronger at higher CR, especially at CR 440. But despite the significant interaction of [DMA] x CR, the negative effect of [DMA] was similar for all four CRs. In a model for VCL, VAP, and VSL without the interaction of [DMA] x CR, the main effect of [DMA] was highly significant, and pairwise comparisons showed significantly lower velocities at 1.0 and 1.5 mol/l DMA than at 0.4 and 0.6 mol/l DMA (Table 8C). The amplitude of sideward movements (ALH) also appeared to peak at 0.6 mol/l DMA and to show a decline at higher [DMA], whereas the frequency of sideward movements (beat cross frequency, BCF) steadily increased towards higher [DMA] (Table 8C). The main effects of [DMA] and CR were significant for both ALH and BCF. The same negative effect of higher [DMA] was seen in the derived ratio variables linearity (LIN = VSL/VCL) and wobble (WOB = VAP/VCL), and to a lesser extent also straightness (STR = VSL/VAP), indicating that higher [DMA] (especially at higher CR) was more negative for linear, progressive movement (VSL) than for average path (VAP) and track velocity (VCL). Interactions of [DMA] x CR were significant for LIN and WOB, and not for STR. In statistical analysis without this interaction, main effects of [DMA] and CR were significant for LIN, STR, and WOB (Table 8C), with declining values at higher cooling rates and higher [DMA], and with lowest values seen at 1.5 mol/l DMA combined with CR 440. In experiment 6, the effect of higher [DMA] on sperm velocities was different, as VCL, VAP, and VSL did not decline at higher [DMA] but rather appeared to level off at 1.0 mol/l DMA. But, LIN, STR and WOB did again decline at [DMA] above 0.6 mol/l (Table 8C), indicating that also in experiment 6, higher [DMA] was negative for effective progressive movement.

In addition to the CASA assessment, a subjective 'score' of sperm quality was obtained by phase contrast microscopy (not shown). While this variable was not included in the statistical analysis, it is interesting to see that the relations of this subjective microscopy score with [DMA] and with CR in experiments 5 and 6 were similar to those of the CASA-derived VCL, VAP, and VSL.

For the post-thaw percentage sperm with not-fragmented DNA (TUNEL negative, Table 8C), there appeared to be no appreciable effect of [DMA] or CR. The interaction [DMA] x CR was not significant, and in a model without this interaction, main effects for [DMA] and CR were not significant either, nor was any of the pairwise comparisons.

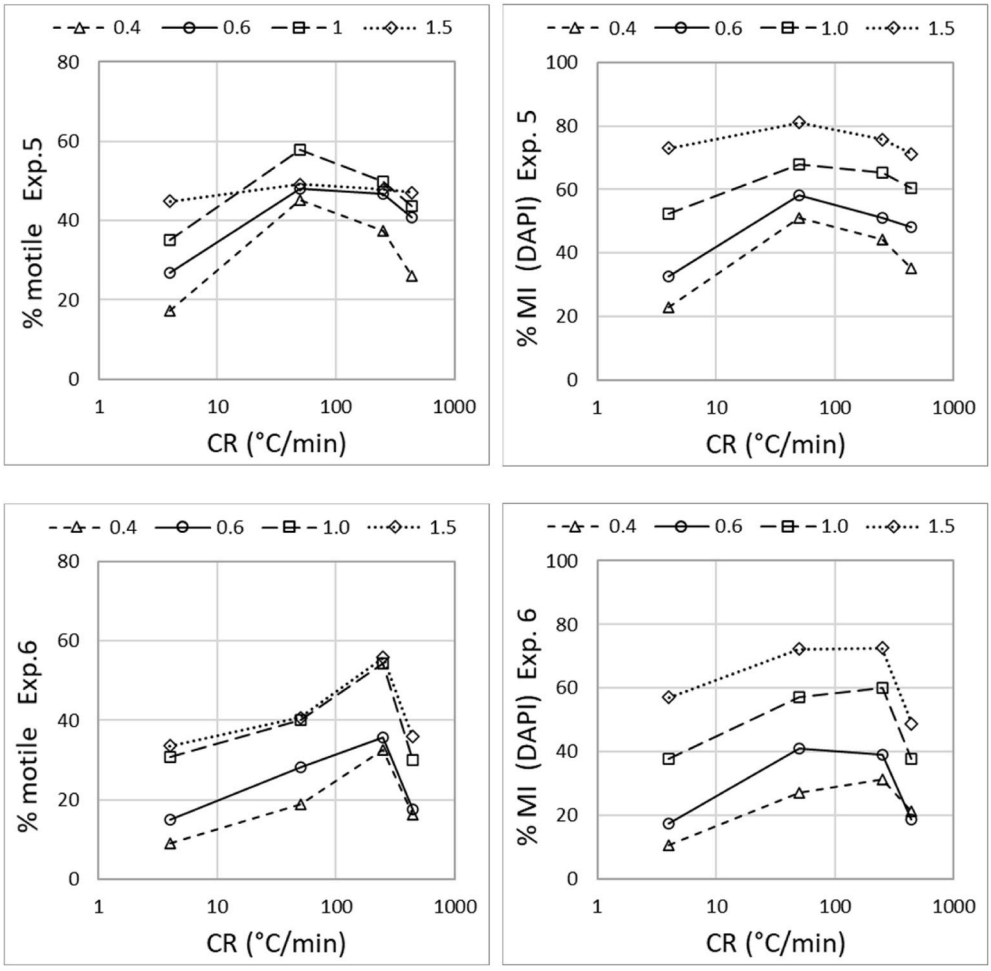

Fig. 3. Effect of [DMA] and CR on post-thaw motility and membrane integrity (%MI-DAPI) in experiments 5 (upper panels) and 6 (lower panels). Significance of pairwise comparisons between cooling rates is shown in Table 6.

Table 6
Effect of Cooling rate[§] and [DMA] on post-thaw motility (%Mot) and membrane integrity (%MI-DAPI).

	%Mot					%MI-DAPI				
	CR (°C/min)					CR (°C/min)				
	4	50	250	440	600*	4	50	250	440	600*
[DMA] (mol/l)	Experiment 5[a]					Experiment 5				
0.4	$17.2^{d,3}$	$45.3^{a,1}$	$37.5^{b,1,2}$	$26.2^{c,2,3}$		$22.9^{d,3}$	$51.0^{a,1}$	$44.3^{b,1,2}$	$35.2^{c,2}$	
0.6	$26.8^{c,2}$	$48.0^{a,1}$	$46.9^{a,b,1}$	$40.8^{b,1}$		$32.8^{c,2}$	$58.3^{a,1}$	$51.1^{b,1}$	$48.2^{b,1}$	
0.6				$40.8^{a,1}$	$41.1^{a,1}$				$48.2^{a,1}$	$49.5^{a,1}$
1.0	$35.2^{c,3}$	$57.9^{a,1}$	$50.0^{b,1,2}$	$43.7^{b,2,3}$		$52.5^{c,3}$	$67.9^{a,1}$	$65.4^{a,b,1,2}$	$60.4^{b,2,3}$	
1.5	$44.9^{a,1}$	$49.2^{a,1}$	$47.9^{a,1}$	$47.0^{a,1}$		$73.1^{b,1}$	$81.2^{a,1}$	$75.7^{a,b,1}$	$71.2^{b,1}$	
	Experiment 6					Experiment 6				
0.4	$9.2^{c,3}$	$18.9^{b,2}$	$32.5^{a,1}$	$16.2^{b,2,3}$		$10.8^{c,3}$	$27.1^{a,1,2}$	$31.1^{a,1}$	$21.1^{b,2}$	
0.6	$15.0^{b,2}$	$28.3^{a,1}$	$35.6^{a,1}$	$17.5^{b,2}$		$17.4^{b,2}$	$40.9^{a,1}$	$39.1^{a,1}$	$18.8^{b,2}$	
0.6				$17.5^{b,2,}$	$29.9^{a,1}$				$18.8^{b,2,}$	$46.1^{a,1}$
1.0	$30.9^{c,3}$	$40.1^{b,2}$	$54.3^{a,1}$	$30.1^{c,2,3}$		$37.6^{b,2}$	$57.1^{a,1}$	$59.9^{a,1}$	$37.6^{b,2}$	
1.5	$33.6^{b,2}$	$40.8^{b,2}$	$55.9^{a,1}$	$36.0^{b,2}$		$57.0^{c,2}$	$72.3^{a,1}$	$72.6^{a,1}$	$48.9^{b,2}$	

[§] Within a row, a common letter superscript indicates $P \geq 0.05$. A common number superscript indicates $P_{Bonf} \geq 0.05$.
* Semen frozen in pellets.

4. Discussion

The current study investigated the effect of osmolality on in vitro sperm quality after 0, 1, 2, and 4 days of cold storage of chicken semen. Secondly, this study investigated the suitability of various CPAs, other than the (contraceptive) glycerol, for freezing chicken semen, and the influence of cooling rate (CR) and DMA concentration ([DMA]).

In an earlier study we measured seminal plasma osmolality to be in the range of 325 mOsm/kg water (unpublished), which is in line with the values given in Refs. [15,38], and with the concentrations of the solutes in seminal plasma reviewed in Ref. [14], although also somewhat lower values (295 mOsm/kg) have been reported [1]. The current results confirmed our earlier observation that hyperosmotic diluent inhibits motility. The inhibition of motility is not seen, or is less apparent,

Table 7
Post-thaw membrane integrity measured by flow cytometry (%MI-PI)*.

	Cooling rate (°C/min)			
	4	50	250	440
[DMA] (mol/l)	Experiment 5			
0.4	81.2	81.4	75.1	83.0
0.6	78.7	83.1	83.9	80.6
1.0	81.5	87.7	88.3	87.5
1.5	86.5	89.4	89.9	89.6
	Experiment 6			
0.4	65.9	83.7	86.2	79.7
0.6	80.2	87.2	85.9	82.8
1.0	83.8	92.5	90.5	90.3
1.5	91.2	93.8	92.4	91.2

* P-values for main effects and interaction of [DMA] and CR are presented in the main text.

when semen is only lightly diluted with a hypertonic diluent, for instance as was done by Lake and Ravie [19], where semen was mixed with an equal volume of the extender, giving a final osmolality half way that of the neat semen and the extender. Sperm cells generally behave as 'perfect osmometers', and have a substantial reduction of intracellular water at hyperosmotic conditions [52]. The tighter 'packing' of the flagellum may cause friction, inhibiting the sliding of the microtubule filaments or other structural elements in the flagellum. The inhibition of motility was not completely reversed when physiological osmolality was restored in a step-wise fashion prior to motility assessment, suggesting a permanent damage of the sperm cells.

However, at higher osmolality, there were fewer sperm with a kinked tail. Instead of 'protecting' the sperm tail against bending, the effect of hyperosmolality may be a side effect of, or have the same cause as, the inhibition of motility. Kinked tails appeared to relax after cells died. For instance, after freezing with diethylformamide (DEF), post-thaw %MI sperm was extremely low. All few remaining MI sperm had kinked tails, but almost all dead sperm did not have kinked tails, and the total %KT had become clearly lower than that seen in the same semen before freezing. Post-thaw %KT must therefore be interpreted with care; after freezing, the %KT as a proportion of all cells may be lower than before freezing, but the proportion of membrane-intact sperm with KT may have increased, indicating freezing/thawing-induced tail damage.

We observed that the percentage sperm cells with kinked tail in cold-stored semen was significantly lower when using extender with BSA (added to semen that had already been prediluted with the same extender without BSA). BSA in the freezing medium may also have a small beneficial effect for post-thaw semen (lower %KT and higher % Mot and %MI-DAPI, experiment 2), but this was not significant in the analysis over all replicates. Larger effects of BSA on the post-thaw results

Table 8
Effect of [DMA] and CR on post-thaw CASA motility variables and DNA integrity (TUNEL).

A. P-values[#] for interaction[$] and for main effects [DMA] and CR[€]

	%Mot Casa	%Prog Mot	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	LIN	STR	WOB	ALH (μm)	BCF (Hz)	%TUNEL-negative
	Experiment 5										
[DMA] x CR	0.000	0.000	0.000	0.000	0.000	0.003	0.18	0.000	0.55	0.31	0.98
[DMA]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.099	0.000	0.45
CR	0.000	0.000	0.18	0.23	0.27	0.001	0.006	0.002	0.005	0.012	0.25
	Experiment 6										
[DMA] x CR	0.000	0.000	0.89	0.74	0.75	0.74	0.94	0.27	0.000	0.12	0.25
[DMA]	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.13	0.000	0.015	0.55
CR	0.000	0.000	0.000	0.000	0.001	0.16	0.09	0.14	0.024	0.98	0.70

B. Post-thaw means per CR per [DMA][$,§]

	% motile sperm (CASA)				% Progr. motile sperm (CASA)			
	CR (°C/min)				CR (°C/min)			
	4	50	250	440	4	50	250	440
[DMA] (mol/l)	Experiment 5				Experiment 5			
0.4	$25.7^{c,3}$	$55.4^{a,1}$	$42.9^{b,2}$	$34.7^{b,1,2,3}$	$17.5^{c,3}$	$40.2^{a,1}$	$31.7^{b,1,2}$	$25.7^{b,2,3}$
0.6	$38.6^{c,2}$	$62.5^{a,1}$	$52.0^{b,1}$	$55.6^{a,b,1}$	$26.6^{c,1}$	$46.9^{a,1}$	$37.9^{b,1}$	$42.7^{b,1}$
1.0	$56.1^{b,1,2}$	$69.3^{a,1}$	$58.5^{b,1,2}$	$53.3^{b,2}$	$42.8^{b,c,1,2}$	$52.3^{a,1}$	$42.6^{b,1,2}$	$34.6^{c,2}$
1.5	$68.7^{a,1}$	$61.9^{a,1,2}$	$68.3^{a,1}$	$53.7^{b,2}$	$46.8^{a,1}$	$36.5^{b,1,2}$	$40.1^{a,b,1}$	$25.9^{c,2}$
	Experiment 6				Experiment 6			
0.4	$6.7^{c,3}$	$30.3^{a,1}$	$30.7^{a,1,2}$	$18.0^{b,2}$	$2.3^{c,2}$	$12.2^{a,1}$	$15.0^{a,1}$	$7.7^{b,1}$
0.6	$17.1^{b,2}$	$36.1^{a,1}$	$37.5^{a,1}$	$15.9^{b,2}$	$6.4^{b,2}$	$19.2^{a,1}$	$20.0^{a,1}$	$6.6^{b,2}$
1.0	$38.6^{b,1}$	$49.9^{a,1}$	$49.2^{a,1}$	$26.1^{c,2}$	$17.5^{b,2,3}$	$24.1^{a,1,2}$	$25.4^{a,1}$	$11.0^{c,3}$
1.5	$38.5^{b,3}$	$54.6^{a,1}$	$51.7^{a,1,2}$	$40.5^{b,2,3}$	$18.5^{b,2}$	$25.5^{a,1,2}$	$28.4^{a,1}$	$18.8^{b,1,2}$

C. Means per [DMA] (mol/l)[€,§]

	%Mot Casa	%Prog Mot	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	LIN	STR	WOB	ALH (μm)	BCF (Hz)	% Tunel-Negative
[DMA] (mol/l)	Experiment 5										
0.4	$39.7^{d,4}$	$28.8^{c,2}$	$118.1^{a,1}$	$109.2^{a,1}$	$102.0^{a,1}$	$0.86^{a,1}$	$0.93^{a,1}$	$0.92^{a,1}$	$2.36^{b,1}$	$8.8^{c,3}$	$43.4^{a,1}$
0.6	$52.2^{c,3}$	$38.5^{a,b,1}$	$122.2^{a,1}$	$111.9^{a,1}$	$102.8^{a,1}$	$0.84^{b,1,2}$	$0.92^{b,2}$	$0.91^{a,1}$	$2.46^{a,1}$	$8.9^{c,3}$	$45.3^{a,1}$
1.0	$59.3^{b,2}$	$43.1^{a,1}$	$103.9^{b,2}$	$93.5^{b,2}$	$85.4^{b,2}$	$0.82^{c,2}$	$0.91^{b,2,3}$	$0.90^{b,2}$	$2.40^{a,b,1}$	$9.3^{b,2}$	$45.7^{a,1}$
1.5	$63.2^{a,1}$	$37.3^{b,1}$	$70.4^{c,3}$	$60.3^{c,3}$	$54.7^{c,3}$	$0.76^{d,3}$	$0.90^{c,3}$	$0.84^{c,3}$	$2.35^{b,1}$	$9.9^{a,1}$	$39.5^{a,1}$
	Experiment 6										
0.4	$21.4^{d,3}$	$9.3^{d,3}$	$62.7^{c,3}$	$53.1^{b,2}$	$46.4^{b,2}$	$0.71^{a,b,1,2}$	$0.85^{a,1}$	$0.83^{a,b,1}$	$2.28^{c,3}$	$8.2^{b,2}$	$65.5^{a,1}$
0.6	$26.7^{c,2}$	$13.0^{c,2}$	$71.2^{b,2}$	$61.1^{a,1}$	$54.1^{a,1,2}$	$0.75^{a,1}$	$0.88^{a,1}$	$0.85^{a,1}$	$2.53^{b,2}$	$8.7^{a,1,2}$	$65.2^{a,1}$
1.0	$41.0^{b,1}$	$19.5^{b,1}$	$80.6^{a,1}$	$67.7^{a,1}$	$56.1^{a,1}$	$0.68^{b,c,2}$	$0.82^{b,2}$	$0.83^{a,b,1}$	$2.90^{a,1}$	$8.6^{a,b,1,2}$	$60.2^{a,1}$
1.5	$46.3^{a,1}$	$22.8^{a,1}$	$83.0^{a,1}$	$68.9^{a,1}$	$55.9^{a,1}$	$0.67^{c,2}$	$0.81^{b,2}$	$0.82^{b,1}$	$2.96^{a,1}$	$9.0^{a,1}$	$56.7^{a,1}$

[#] '0.000' indicates $P < 0.001$.
[$] Model with interaction CR x [DMA].
[€] Model without interaction.
[§] Within a row (B), or within a column (C), a common letter superscript indicates $P \geq 0.05$. A common number superscript indicates $P_{Bonf} \geq 0.05$.

for chicken semen frozen with glycerol were reported elsewhere [4], but it seemed that the presence of BSA was not a huge factor in our study.

A number of amides have been used as CPA for freezing semen. For chicken semen, the amides used are mainly DMA and DMF (e.g. Refs. [11,20,29,33,36,42,45,46], but also MA has been used [29,31], or a combination of MA and DMF [13]. In the current study, MA and MF resulted in lower post-thaw %Mot and %MI-DAPI than DMA and DMF, and in higher %KT than DMA. It was especially striking that the majority of all membrane-intact sperm cells had kinked tails after freezing with MA and MF. Therefore, we did not include MA and MF in our further experiments. DMF appeared to have an advantage over DMA in the % Mot, although the difference was not significant. However, pre-freeze holding of the semen at 5 °C after adding DMA improved the post-thaw expression of motility (experiments 3 and 4), as the percentage motile sperm approached the percentage membrane-intact sperm, i.e. most 'viable' (membrane-intact) sperm expressed motility. In fact, 1h holding after adding DMA (experiment 4) was sufficient, as the post-thaw percentage motile sperm after 1 h holding was equal to the percentage membrane-intact sperm (Table 4). Holding did not have a similar effect for DMF, DEF, and PG.

The suitability of the CPAs DMA, DMF, MA, MF, PG, and DEF does not seem to be readily explained, at least not over the full range of these CPAs, by their molecular mass (MM), melting point (MP), the ratio of MP/MM, or their lipophilicity (octane-water partitioning coefficient (K_{OW})), which determines the ability of partitioning of the CPA into, and the diffusion through, the membrane lipid bilayer. However, the clear unsuitability of DEF (at 0.6 mol/l) may be caused by toxicity that was already apparent in the pre-freeze motility, and that may be related to its very high partitioning coefficient. Assuming that the partitioning for lipid bilayer/water is similar to that of octane/water, the presence of DEF inside the cell membranes of sperm at a medium DEF concentration of 0.6 mol/l, could approach 20 vol%, which could possibly disturb membrane structure and function. The partition coefficient of DMA is lower than that of DEF, but it is still quite high compared with that of the other tested CPAs, and especially high relative to that of glycerol, which could be the reason for the negative effects of DMA on motility, that we have seen especially at the highest concentration of 1.5 mol/l.

For DMA, the higher concentrations were beneficial for the percentage membrane-intact sperm. However, concentrations above 1.0 mol/l were negative for motility, as 1.5 mol/l almost completely blocked post-thaw motility (although this could be restored by post-thaw dilution of the DMA concentration). Also, the post-thaw sperm velocities peaked at 0.6 mol/l or 1.0 mol/l DMA in experiments 5 (low sperm concentration) and 6 (high sperm concentration), respectively, and linearity of movement decreased at [DMA] higher than 0.6 mol/l. Post-thaw velocities were generally lower in experiment 5 (low sperm concentration) than in experiment 6 (high sperm concentration), while the clear negative effect of high [DMA] on the sperm velocity seen in experiment 5 was absent or less clear in experiment 6. The latter may be due to the fact that higher CPA concentrations lead to a larger volume of unfrozen fraction [28,51], which may be necessary to comfortably accommodate all sperm cells in the unfrozen fraction at the very high sperm concentration used in experiment 6. This beneficial effect of high [DMA] at high sperm concentration may cancel out the negative effect of high [DMA] seen at low sperm concentration in experiment 5. Earlier [53], we obtained good fertility with semen frozen with 0.6 mol/l DMA and a CR of 200 °C/min. But the current results suggest that perhaps a higher [DMA] (between 0.6 and 1.0 mol/l) may be optimal and could be tested in an insemination trial.

Regarding the influence of CR, post-thaw sperm variables had generally 'inverted U'-shaped curves when plotted against CR, in line with the 'two-factor hypothesis' [27], with best results seen at CR 50 and CR250. This is a bit different from what we reported earlier, where 200 °C/min was found superior to 50 °C/min [53].

The highest cooling rates (CR 440 in straws, or freezing in pellets) provide still relatively good post-thaw results with DMA as CPA, which does not seem possible with glycerol [53]. Woelders and Chaveiro [51] showed that the optimal or maximal permissible CR is proportional to the hydraulic conductivity (L_p) of the sperm membrane. Therefore, the relatively good survival in semen frozen with DMA in pellets or in straws at CR440 would suggest that L_p in the presence of DMA is higher than L_p in the presence of glycerol. Indeed, it has been reported that glycerol, unlike various other CPAs (PG, EG, DMSO) decreased the hydraulic conductivity of the sperm membrane, for instance in dog [23] and bull sperm [12].There appear to be no values in the literature for L_p in the presence of DMA for sperm cells of chicken, or even other species.

The CR in pellets (approximately 600 °C/min averaged over the entire cooling trajectory) was higher than the highest CR we employed for straws. While post-thaw %Mot and %MI-DAPI in pellets was lower than that in straws frozen at the optimal CR range (50–250), pellets performed better than, or equal to, straws frozen at CR440, despite the very high CR in pellets. The core of the 4.6 mm diameter pellets will also have this same very high CR when averaged over the cooling trajectory, but it could be that the CR of the core was lower in the most important range between 0 to −30 °C because of insulation and heat of fusion provided by the peripheral layer of the droplets. But, importantly, the bulk of the droplets will also not be exposed to supercooling prior to ice formation, as ice nucleation will occur on the surface directly exposed to LN_2, when the bulk of the droplet would still be at or above the freezing point. Thermocouple measurements (unpublished) showed that the supercooling in the bulk of a wider (0.5-ml) straw, or in an ampoule, is absent or at least much smaller than that in the narrow 0.25-ml straws. Therefore, supercooling may also play a smaller role in the 4.6 mm wide pellets than in 0.25-ml (1.7 mm wide) straws.

In experiment 5, effects of CR and [DMA] were studied using semen from individual cocks. Cocks, obviously, differed in pre-freeze and post-thaw semen quality, but plots per cock (not shown) do not indicate that optimal CR or [DMA] differed between cocks.

5. Conclusion

Higher than physiological osmolality of the base medium (non-permeant solutes) inhibited motility, which, after 4 days of cold storage, was only partly reversible. The modified L&R7.1 medium with a physiological osmolality of 325 mOsm allowed cold storage of 'fresh' semen (diluted to 200 million sperm/ml) for several days with hardly any loss of membrane integrity and little loss of motility. Chicken semen frozen in straws had best post-thaw quality when using fairly high cooling rates of 50–250 °C/min. The current results suggest that somewhat higher [DMA] than the 0.6 mol/l DMA we currently use may be beneficial, but concentrations above 1.0 mol/l can decrease sperm velocity or even inhibit sperm motility.

Declaration of competing interest

None.

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