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Temperatures from 4°C to 15°C are suitable for preserving the fertilizing capacity of stallion semen stored for 22 h or more in INRA96 extender

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## Abstract

This study tested whether variable temperatures (from -0.5 to 15°C) and air exposure could be used under laboratory and under field conditions to store stallion sperm diluted in extender INRA96 without loss of fertility. Experiment1 (laboratory conditions) measured the effects of two 72 h storage conditions (5°C with air versus 15°C without air). Experiment2 (fixed field conditions) measured the effects of 22 h of storage without air in disposable containers maintained at four ambient temperatures (7°C, 17°C, 27°C, 39°C with semen at -0.5°C to 3°C, 4°C to 7°C, 8°C to 10°C, 12°C to 15°C, respectively). Per cycle pregnancy rate (PC) was measured after one AI in uterine body of 200x10<sup>6</sup> total spermatozoa, 7 h (Experiment1) and 17 h (Experiment2) before ovulation. In Experiment1, PC was similar for both conditions (60% (n=40 cycles) vs 63% (n=40), respectively, 5 stallions x 8 cycles). Only velocity VCL and ALH were slightly higher at 15°C. In Experiment2, PC was reduced when ambient temperature was low (semen at -0.5°C to 3°C; PC=25%) rather than intermediate (semen at 4°C to 7°C; PC=53%) or high (semen at 8°C to 10°C; PC=50%) (4 stallions x 8 cycles) (P=0.008). Sperm stored at -0.5°C to 3°C had lower acrosome integrity/responsiveness, similar membrane integrity (HOS test) and motilities, and higher VCL and ALH, than semen stored between 4°C and 15°C. These results demonstrate a wide tolerance of equine sperm to variable positive temperatures and air exposure for 22 h storage and more. However temperatures close to 0°C are detrimental for fertility.

## 1. Introduction

Storage of chilled sperm is very important to optimize the management of reproduction in the equine species. However, better knowledge of this process is still needed in order to improve its efficiency in laboratory and also under field conditions. Temperature and duration of storage, extender, cooling curve, number of sperm per artificial insemination (AI) dose,

number of AI per cycle, interval between AI and ovulation affect the fertilizing capacity of stored equine semen.

Equine semen is usually stored for 12-48h at 4-5°C. Cooling to 4-5°C decreases the metabolic activity of spermatozoa, reduces microbial growth and consequently maintains the viability of sperm for extended periods of time [1]. Lower temperatures (0 and 2 °C) have been shown to reduce the motility of equine semen kept for 12 h in milk-based [2] or 24 h in milk-free extender [3]. In contrast, motility of semen stored at constant temperatures ranging from 4°C to 25°C can be maintained for short periods in most extenders. High variability of *in vitro* results has been observed for longer periods of storage depending on stallions and experimental conditions [2-7]. High variability in fertility was even more obvious [5,8,9].

The interactions between temperature, extender and air exposure could be important for the success of storage. Semen stored at 4°C in a milk-based extender (INRA82) exhibited higher motility under anaerobic or semi-anaerobic exposure compared to aerobic exposure and to semen stored in a modified Hanks' salts extender in any condition of air exposure [3]. In contrast, semen stored at 15°C exhibited higher motility under aerobic exposure compared to anaerobic exposure in this modified Hanks' salts extender. INRA96 extender (modified Hanks' salts solution supplemented with native phosphocaseinate) was very effective in maintaining the fertilizing capacity of equine sperm stored for 24 h at 4°C without air exposure or for 72 h at 15°C with air exposure in our standardized laboratory conditions [9,10]. In contrast, in another extender based on defined milk protein (Equipro), Price *et al.* [11] observed an effect of temperature on motility and membrane integrity of equine sperm (15°C being less efficient than 5°C) and no effect of air exposure after 72 h of storage or more. Therefore new information was needed to explore under fixed laboratory conditions the combined effects of temperature and exposure to air for a long storage time.

Optimized semen storage conditions are also a challenge in the field practice of AI. However field conditions of semen storage may not be strictly compared to laboratory conditions. As an example, semen transportation between distant sites is a current practice that affects the semen storage temperature [12]. During shipment of semen, storage conditions, and especially temperature, are not remained constant which introduces a new variable. Douglas-Hamilton [13] developed the reusable plastic container Equitainer II™ to be used with frozen cans, which is considered as the gold standard of storage containers. Different types of disposable container are now available for transportation of equine semen. They are relatively small and cheap with thermic performances that depend on ambient temperature and on the performance of the cooled or ice packs used [6,12,14,15]. Because of heat exchange between the ice packs, the container (and its contents) and the ambient air, the initial inner temperature of storage in the container decreases, then reaches a minimum before increasing slowly when the ice packs begin to warm up. So the inner temperature in these small containers is variable. *In vitro* studies made with stallion semen diluted in milk-based extenders and stored for 24 h in different types of disposable containers at different ambient temperature suggest that motility is affected if the inner temperature in the container is below 2°C or above 20 °C [6,15-19]. Consequently, Brinsko *et al.* [6] suggested that stallion semen may be able to tolerate a wider range of cooling rates and storage temperatures than previously thought. Although these small containers are used more and more in the equine industry [7,12], there is no study presenting standardized fertility data with the use of these containers at different ambient temperatures.

Thus, we hypothesized that equine semen stored in INRA96 could withstand variable temperatures between 4 and 15°C and variable conditions of air exposure, without modifying

its fertilizing capacity. We chose two conditions to test this hypothesis: 1) optimized semen treatment with strictly controlled temperature and artificial insemination procedure (laboratory conditions), 2) conditions that mimic field conditions, using disposable containers and less favorable AI conditions but that are fixed to ensure interpretable results (fixed field conditions). In the laboratory conditions experiment, we measured the effects of long semen storage duration (72 h) with two fixed conditions (5°C without air exposure versus 15°C with air exposure) on semen quality measured *in vitro*, and *in vivo* by fertility of semen inseminated shortly (~ 6 h) before ovulation (Experiment 1). In the fixed field conditions experiment, we explored the effect of variable temperatures without air exposure, and used disposable containers for a shorter storage of 22 h (after this time, inner temperature is no more controlled) and a larger interval between insemination and ovulation (~ 17 h) (Experiment 2).

## 2. Materials and Methods

### 2.1. General procedures

#### 2.1.1. Reagents and chemicals

The extender INRA96, purchased from IMV (L'Aigle, France), is based on modified Hanks' salts, 67 mM glucose, 126 mM lactose, native phosphocaseinate (27 g/L, a milk purified fraction, composed of total micellar caseins) and is supplemented with penicillin (50 IU/mL) and gentamicin (50 µg/mL) [10]. Sperm washing medium (HH) was composed of Hank's salts. For the hypoosmotic test (HOS test), a hypoosmotic medium (50 mOsm/L) was prepared with the same composition as HH but without NaCl. For induction of the acrosome reaction, ionophore A23187 (reference C7522, Sigma, Saint Quentin Fallavier, France) was diluted in dimethyl sulfoxide at 1 mg/mL. Acrosome staining was carried out using fluoresceinated *Pisum Sativum* agglutinin (PSA-FITC, reference L0770, Sigma) diluted in HH. All the above solutions were stored at -20°C.

#### 2.1.2. Animals

Male Welsh ponies were housed individually at INRA Nouzilly. One hundred and twenty six female Welsh ponies (250-450 kg, 4-16 years old) were housed at INRA Nouzilly. Before ovulation, all mares were housed in pens of 4-5 and fed with the same concentrate and straw. After, they were kept in the same pasture and fed with grass and minerals until Day 14 post-ovulation. In this herd, overall per-cycle pregnancy rate per individual mare was higher than 50%. The authors of this study have read the policy of this journal relating to animal ethics and confirm that their study complies.

#### 2.1.3. Semen collection, dilution and storage

Semen of all stallions was routinely collected with a closed-ended vagina before and during all the experiments. After gel filtration, sperm concentration was estimated photometrically on 2 aliquots. Semen was diluted rapidly in INRA96 maintained at 37°C, at  $20 \times 10^6$  spermatozoa per mL. Doses for AI or for sperm evaluation consisted in 10 mL of diluted semen in 20-mL syringes. Syringes were always stored horizontally. Diluted semen was then cooled at different temperatures and under different air exposures, as fixed within each experiment. After storage, the doses were gently stirred, then either inseminated without rewarming or used for *in vitro* evaluation. Within each experiment, the Control group was the

storage condition closer to the routinely conditions used through the world : 4-5°C without air exposure.

#### 2.1.4. Insemination and evaluation of fertility

Follicular growth and ovulation of mares were monitored daily during estrus by transrectal ultrasonography. When mares presented growing follicles of 33 mm, induction of ovulation was performed with an intravenous injection of 15 mg of Crude Extract Equine Gonadotropin. This substance induces ovulation in 36 h in 80% of cycles [20]. Within an experiment, mares were randomly assigned to stallion and to group according to the time of induction of ovulation. A maximum of 4 mares (= 4 cycles) were inseminated per ejaculate and per group. Then, mean number of inseminated cycles per ejaculate per group within stallion was  $\approx 2$ . Uterine body inseminations were made with  $200 \times 10^6$  total sperm in 10 mL, once before ovulation. The time interval between induction of ovulation and AI was fixed within each experiment. All cycles during which mares ovulated too early, too late, more than once, or not at all were eliminated. Pregnancy was diagnosed ultrasonographically 14 to 16 days after ovulation. Fertility was measured according to the per-cycle pregnancy rate (PC), defined as the total number of pregnancies divided by the total number of estrus cycles. Most mares were inseminated on one cycle only. After pregnancy diagnosis, all mares received an injection of prostaglandin between the first and the second cycle. Some mares were inseminated on a second cycle to achieve the number of cycles, always with the sperm of another stallion, the group was assigned at random as for the first cycle.

#### 2.1.5. Evaluation of sperm

After storage, semen of AI doses was warmed in a water-bath at 37°C for 10 minutes prior to any evaluation. During *in vivo* part of Experiments 1 and 2, motility parameters were routinely evaluated. For Experiment 1, these results are presented in Results section. For Experiment 2, due to management constraint, *in vitro* tests (motility, HOS test and acrosome evaluation) were performed just after the *in vivo* part, on split ejaculates of the same males. Care was taken to check that motility parameters were equivalent during the *in vivo* and the *in vitro* parts of Experiment 2.

##### 2.1.5.1 Motility (Experiments 1 and 2)

The sperm motility characteristics were measured by a computer-assisted motility analyzer (CASA) (HTM-IVOS, version 10.9, Hamilton Thorn Research, Beverly, MA, USA). Rapid motility was defined as the percentage of motile spermatozoa with average path velocity (VAP) higher than 40  $\mu\text{m}/\text{sec}$ . The parameter settings for this HTM-IVOS system were: 30 frames acquired at 60 frames per second; sorting on 16 frames/30 to define a track; minimum contrast 80; minimum cell size 4 pixels; lower VAP cut-off 20  $\mu\text{m}/\text{s}$ ; VAP cut-off for progressive and rapid cells 40  $\mu\text{m}/\text{s}$  and straightness cut-off for progressive cells 80.

##### 2.1.5.2. Hypoosmotic swelling test (HOS test) (Experiment 2)

For the HOS test, two aliquots of 1 mL semen was centrifuged at 600 g for 5 min and the pellet was resuspended in 1 mL of hypoosmotic medium (see 2.1. Reagents and chemicals) and incubated at 37°C for 15 min, then stored at 4°C. Two hundred spermatozoa per aliquot were observed under a phase-contrast microscope and were classified as non-swollen,

slightly swollen or highly swollen [21]. The results were expressed as percentage of swollen (including slightly and highly swollen) spermatozoa.

### 2.1.5.3. Acrosome evaluation (Experiment 2)

The acrosome reaction was performed according to an adapted method from Varner [22]. Briefly, four aliquots of 1 mL semen were incubated at 37°C for 2 hours, with (two aliquots) or without 6 µM ionophore A23187 (two aliquots) [23]. Acrosome staining with PSA-FITC, adapted from Morales & Cross [24] and validated for equine sperm on filters or in suspension [25-27], was then performed. After washing with HH solution, the spermatozoa were fixed with 1% paraformaldehyde (final v:v) (10 min at room temperature). After further washing, spermatozoa were diluted in 40 % ethanol (final v:v) for 30 min at 4°C. After another washing, the spermatozoa were then incubated with PSA-FITC (25µg/mL). One hundred spermatozoa per tube were observed under a fluorescence microscope and were scored according to the classification derived from Cummins [28]: 1) complete staining of the acrosome, 2) partial or patchy staining of the apical segment of the acrosome, with complete staining of the equatorial segment, 3) staining of the equatorial segment only, 4) others (e.g. faint staining of the whole sperm, bright staining all over the head, bright staining of the post-acrosome only). Pattern 1 was considered as intact acrosome. Patterns 2 and 3 were considered as acrosome reactions (AR). The difference between the percentages of AR for ionophore treatment and controls was called Diff. AR.

### 2.1.6. Measurements of temperature

Ambient temperatures were regularly monitored in chambers every 10 min by a thermocouple attached to a chart recorder (Tinytag Talk 2 Temperature Logger, Gemini, France). Semen temperature was determined by a very long thin thermocouple T (Kapton, diameter 1.3 mm, length 1.5 m, Fisher Bioblock Scientific, Illkirch, France) placed within the syringe dose and attached to an outside thermometer (Ecoscan JKT, Fisher, Bioblock Scientific, Illkirch, France).

## 2.2. Specific procedures

### 2.2.1. Fertility and *in vitro* aspects of semen cooled and stored for 72 h either at 5°C without air exposure or at 15°C under air exposure, in laboratory conditions (Experiment 1)

#### 2.2.1.1. Preparation of semen

In July, semen of 5 pony stallions was prepared and diluted in INRA 96 as described in 2.1.3. When maintained at 5°C, AI doses were stored in 20-mL syringes without air exposure: syringes were loaded with 10 mL diluted sperm and no air. Then these syringes were inserted in a foam cylinder before being stored at 5°C horizontally (Group 5 (A-) = Control). When maintained at 15°C, AI doses were stored in 20-mL syringes under air exposure; syringes were loaded with 10 mL extended sperm and 10 mL air. These syringes without protection were put directly in the 15°C chamber and stored horizontally ([9] (Group 15 (A+))). In this last condition, the sample has a large contact with air, but the oxygen pressure between the surface and the bottom of diluted sample could be different.

#### 2.2.1.2. Cooling curves of the semen

At 5°C storage, cooling down from 35 to 25°C was achieved in 20 minutes in a syringe containing 10 mL diluted semen surrounded by a foam cylinder. Then temperature of 20°C, 17°C, 16°C, 15°C, 11°C, 6°C and 5°C were reached in 10, 15, 20, 25, 40 min, 2 and 3 h respectively.

At 15°C storage, cooling down from 35 to 25°C was achieved in 7 minutes in a syringe, containing 10 mL diluted semen and 10 mL air, without insulation. Then temperature of 20°C, 17°C, 16°C and 15°C were reached in 5, 12, 22 and 40 min respectively.

### 2.2.1.3. Fertility trial

Semen was collected and stored in temperature chambers on Day 0, between 3.00 and 4.00 p.m. Ovulation was induced on Day 2 between 9.00 and 11.00 a.m. On Day 3, mares were checked for absence of ovulation between 9.00 and 11.00 a.m. and inseminated between 3 and 4.00 p.m. On Day 4, mares were checked for ovulation between 9.00 and 11.00 a.m. So the interval between AI and ovulation was assumed to be approximately 7 h. To achieve a total of 80 cycles, 66 mares were inseminated during 1 cycle, and 7 mares during 2 cycles.

### 2.2.1.4. In vitro measurements

During the fertility trial, AI doses of 3 ejaculates per stallion were prepared and cooled according to the two conditions, then examined for motility.

## 2.2.2. Fertility and *in vitro* quality of semen cooled and stored 22 h without air exposure in disposable containers at different ambient temperatures reproducing field conditions (Experiment 2)

### 2.2.2.1. Preparation of semen and loading in disposable containers

Semen of 4 pony stallions was collected and diluted in INRA 96 as described in 2.1.3. Syringes were filled with 10 mL diluted sperm at 37°C, without air. Then syringes were loaded in the disposable containers. The disposable containers (in Neopor (Minitüb, Equiland, Ecausseville, France)) consisted of a styrofoam box with four compartments (two central compartments (for the two 20 mL syringes for storing the semen) and one external compartment on each side (one ice pack per external compartment)) and a cardboard cover. The disposable containers were loaded similarly at each use: with the two filled syringes and the two ice packs (maintained at -20°C for almost 24 h before use). Then, they were closed by the cardboard cover and placed at different ambient storage temperatures. For this experiment, we chose a storage time of 22 h since at elevated ambient temperatures, the semen temperature was controlled only for approximately 24 h by the containers.

### 2.2.2.2. The cooling and warming curves

Four ambient temperature (7°C, 17°C, 27°C, 39°C) that are commonly observed during semen transportation in France (outside or inside cars and vans), were chosen to obtain internal temperatures of 1) less than 0°C for a few hours for the lowest temperature, 2) ≈ 4°C (as a control), 3) ≈ 8 to 10°C and 4) ≈ 15°C (Figure I). At these 4 ambient temperatures, in a syringe with 10 mL diluted semen loaded in the disposable container, cooling down from 30 to 25°C was similar and was achieved in 10 min.

At an ambient temperature of 7°C, a minimal temperature of ~ -0.5°C was obtained in 2 h 50 min in the semen; the temperature was below 0°C for ~ 2 h 30 min and was ~ 3°C after 22 h

of storage (Group -0.5 to 3 (A-)) (Figure I). At ambient temperatures of 17°C, 27°C and 39°C, the minimal temperature in semen was ~ 4°C, 8°C and 12°C in 2 h 30 min, 2 h 20 min and 2 h 00, respectively, and temperatures after 22 h of storage were ~ 7°C, 10°C and 15°C, respectively (Groups 4 to 7 (A-), 8 to 10 (A-), 12 to 15 (A-)), (Figure I). Group 4 to 7 (A-) was considered as the Control.

During the fertility trial, the effects of the three lowest temperatures (the most frequent temperatures in field conditions in France) were evaluated. *In vitro* semen quality was measured for the four temperatures. Temperature of semen was always measured at loading and 2 and 22 h later, before opening.

### Place of Figure I

#### 2.2.2.3. Additional data: temperature measurements at the lowest temperature for practical application

At an ambient temperature of 7°C, when 2 syringes of semen were loaded in the container with 1 ice pack at - 20°C and 1 ice pack at 4°C, minimal temperatures of 1.6°C and 4.9°C, and temperatures at 22 h of 3.5°C and 5.2°C were recorded in the syringe near the ice pack at - 20°C and in the syringe near the ice pack at 4°C, respectively. At this same ambient temperature of 7°C, when 2 syringes of semen were loaded in the container with 2 ice packs at 4°C, minimal temperature and temperature at 22 h were similar: 6.6°C.

#### 2.2.2.4. Fertility trial

From mid June to end of July, extended semen was loaded into containers on Day 0, at 12.00 a.m. Ovulation was induced on Day 0, at 3.00 p.m. On Day 1, mares were checked for absence of ovulation and inseminated at 10.00 a.m. On Day 2, mares were checked for ovulation before 9.00 a.m. So the interval between AI and ovulation was assumed to be approximately 17 h. To achieve a total of 96 cycles, 52 mares were inseminated during 1 cycle, and 22 mares during 2 cycles.

#### 2.2.2.5. In vitro measurements

In August of the same year, two ejaculates per stallion were split, cooled and stored at the 4 temperatures. After 22 h of storage, motility, membrane integrity (HOS test), acrosome integrity and responsiveness to ionophore A23187 were evaluated.

### 2.3. Statistical analysis

Differences between fertility percentages (PC) were tested with a linear categorical data analysis, considering effects of treatment, stallion and treatment by stallion interaction (CATMOD, SAS Institute Inc., Cary, NC, USA). The procedure analyzed response functions (marginal probabilities) and the partitioning of variation into various sources, using weighed-least-square-estimation of parameters calculated on real values. If the residual and the treatment by stallion interaction were not significant, the model was considered as valid. Then the analysis determined whether there is a difference between all stallions and all treatments. In the case of significant difference between treatments, an estimation of each treatment and its significance compared to the overall mean of the file (named intercept) were also calculated.

General differences between means of sperm characteristics were tested by a factorial analysis for balanced data (GLM procedure of SAS, SAS Institute Inc., Cary, NC, USA) considering the effects of sperm treatment, stallion and ejaculate within stallion. The interaction stallion by sperm treatment was used as an error term. Differences between means (stallions, sperm treatments) were further separated by a Student-Newman-Keuls procedure (in GLM) using the same error term. Statistical significance was assumed at  $P < 0.05$ .

### 3. Results

#### 3.1. Fertility and *in vitro* quality of semen cooled and stored for 72 h at 5°C without air or at 15°C under air exposure, in laboratory conditions (Experiment 1)

Fertility (PC) obtained with semen stored for 72 h was high (60 % and more) and no difference was evidenced between the two storage conditions, 5°C without air or 15°C with air (Table 1) (the statistical model was valid,  $P = 0.7$  on estimated values).

All the motility characteristics analyzed were similar between these two conditions (Table 2), except VCL and ALH which were significantly higher ( $P \leq 0.05$ ) when semen was stored at 15°C with air.

Place of tables 1 and 2

#### 3.2. Fertility and *in vitro* quality of semen cooled and stored for 22 h without air in disposable containers at different ambient temperatures reproducing field conditions (Experiment 2)

Fertility obtained with semen stored for 22h without air exposure, at variable temperatures ranging from 4 to 10°C (ambient temperatures 17°C or 27°C; semen at 4 to 7°C or at 8 to 10°C) was similar while lower temperature (ambient temperature 7°C; semen at -0.5 to 3°C) significantly altered fertility (Table 3) (the statistical model was valid,  $P = 0.002$  on estimated values).

As for fertility results, motility, membrane integrity (HOS test) and acrosome integrity /responsiveness (Diff. AR) were similar for semen stored between 4 and 10°C (4 to 7°C and 8 to 10°C) (Table 4). In addition, the higher semen temperature of 12 to 15°C (ambient temperature 39°C) gave also similar results. The temperature -0.5 to 3°C confirmed its harmful effect by lower levels of acrosome integrity and responsiveness (Diff. AR). More precisely, at this temperature, spermatozoa showed more spontaneous acrosome reactions ( $P = 0.02$ ) and more “other” acrosomes ( $P = 0.02$ ) in control tubes than in the other groups. Surprisingly, curvilinear velocity (VCL) and head amplitude (ALH) were higher after storage between -0.5 to 3°C ( $P < 0.01$ ) (Table 4).

Place of tables 3 and 4

### 4. Discussion

Despite the fact that some authors have demonstrated that stallion semen was better stored at 4°C than at higher temperatures after 24 h of storage or more [4,11], previous results obtained in our laboratory [9] suggested that equine semen stored in INRA96 could tolerate variable storage temperatures and degrees of air exposure. Recently, this extender has been shown to be also very efficient in maintaining fertility after freezing [29]. However, a fertility comparison of semen stored without freezing at different temperatures in the extender INRA96 either for a long time (72 h) or in disposable containers was still lacking. We clearly show in the present study that, under laboratory conditions, semen stored for 72 h either at 5°C without air exposure or at 15°C with air exposure gave similar fertility results. These

results confirmed our *in vitro* measurement (Table 2) with two exceptions: VCL and ALH were lower after 5°C without air exposure than at 15°C under air exposure. This difference of sperm motion could be possibly explained by differences in oxygen access for energetic metabolism. Although the respective effects of temperature and exposure to air could not be separated here, conservation of motility without air exposure is certainly dependent on glycolysis and possibly glycogen mobilization as suggested in other species [30-32] and in horses [3,33]. All these results suggest that equine sperm can use both aerobic and anaerobic pathways to survive, depending on the conditions of storage.

One of the main results of the present study concerns the adaptation of stallion semen storage to fixed field conditions. Experiment carried out in field conditions of semen transport and storage (22 h in disposable containers), involving large variations in temperature without air exposure and less favorable conditions of AI showed similar fertility results when the storage temperature ranged from 4°C to 10°C, and similar *in vitro* semen quality in the range 4°C to 15°C. In a recent field study on cooled semen of 67 stallions shipped in disposable containers to one center, Heckenbichler [12] found no effect of semen temperature upon arrival (4°C to 22°C, mean 9.8°C) on subsequent fertility, but number of sperm per AI, extender, storage time and AI-Ovulation interval were not fixed. In most disposable containers, after initial cooling, semen is usually maintained more or less at the same temperature up to 24 h when the ambient temperature is near 20°C [6,14]. This is also the case in our study but for a wider range of temperatures (Figure I). So the results of experiments with passive containers used during short time can, at least partly, be compared to those obtained in the laboratory at constant temperature. Thus we showed that, even in experimental field conditions, the tolerance to variable temperatures of stallion sperm stored without air ranged clearly between 4°C and 10°C as tested by fertility, or possibly more widely, as suggested by the successful *in vitro* tests made also on semen stored at 12°C to 15°C.

However, we demonstrated that there are clear lower limits for the temperatures inside the containers. Temperatures near or below 0°C were not previously recommended [2,3,14,19,34]. But Katila [14] and Brinsko [6] demonstrated that storage below 0°C in transport containers for 4 to 8 h was not so detrimental for motility characteristics measured after 24 h of storage. However, we showed that such storage (2.5 h just below 0°C) is very deleterious because it affects fertility. Fertility, in this case, was strongly decreased for all stallions tested, even the most fertile. Another interesting point of our study was to combine fertility results with a wide range of *in vitro* tests. After storage at the lowest temperature, motility characteristics were not modified, except VCL and ALH that were higher. However, acrosome quality was clearly decreased in our study, thus confirming the sensitivity of stallion acrosomes to thermic stress. It is important to notice that this reduction of sperm quality may usually not be detected in field conditions where only motility evaluation by microscopy is performed upon arrival. Thus motility control after transport-could give false information about the fertility potential of sperm cells. This also means that the measurement of storage temperature not only on arrival but also throughout transport would be very important for the successful use of the transport containers.

Finally, all these results can help to manage semen storage in such containers. In France, external temperatures during the breeding season can be very variable, especially in March and April (range: -5°C to 25°C). Moreover, at the same time of the year, temperature during semen transport in vans and cars may occasionally be much higher (more than 40 °C, up to 50°C). McKinnon & Walker [16] showed that there is no disposable container able to maintain a correct temperature at ambient temperature of 50°C and so high temperatures

should simply be avoided (vans and cars should not be parked in the sun). In our study, the manufacturer's recommendation for using the disposable container was to freeze 2 ice packs at  $-20^{\circ}\text{C}$  before loading them and the sperm into the container. But when ambient temperature is below  $7^{\circ}\text{C}$ , we suggest to use 2 ice packs at  $4^{\circ}\text{C}$  to avoid too low storage temperature. Finally, as ambient temperatures during transportation are often not known, and in view of the above discussion, it is certainly better to manage the storage conditions so that the sperm is stored at temperatures higher than  $2^{\circ}\text{C}$  inside the container.

Motility and velocity of equine sperm are often impaired progressively along storage, this has been confirmed in extenders as INRA96 and equivalent ([3,11,35]. Although motility characteristics are often considered in equine to be predictive of the fertility capacity of the stallions at collection or after semen cooling [12,36,37], some authors estimated that conventional laboratory tests for semen evaluation are insufficient [38-40]. In the two experiments, we observed the same phenomenon for one group (Group  $15^{\circ}\text{C}$ (A+) in Experiment 1 and Group  $-0.5$  to  $3^{\circ}\text{C}$ (A-) in Experiment 2) : VCL and ALH were increased whereas RAPID\_PCT, PROG\_PCT and STR were similar, suggesting strong lateral movement of the head and less pronounced forward movement. As the group with increased VCL and ALH showed similar fertility (Experiment 1) or lower fertility (Experiment 2) as the Control group, we could postulate that these motility characteristics could not be considered as the sign of a better quality after storage. Higher VCL was also observed by Brinsko [6] and by Katila [14] (tendency) after 24 h storage in some of their containers, when internal temperature was below or very close to  $0^{\circ}\text{C}$ . LeFrappier *et al.* [41] also observed higher VCL values in addition to decreased PROG\_PCT compared to 0 h values, during storage at temperature less than  $7^{\circ}\text{C}$ , in different extenders including INRA96. The major subpopulation of equine spermatozoa identified by high linearity and low VAP and ALH values at 0 h decreased during storage at  $4^{\circ}\text{C}$  [42]. In conclusion, during storage, high velocities and ALH, that were not associated with high percentage of motile sperm cells (total and progressive motility) and high straightness, could be considered as doubtful criteria of quality of equine semen after storage.

All these observations could suggest the following sequence of modification of the motion characteristics of equine sperm during storage and aging : after a first phase of motion characteristics considered as an adaptation of the sperm cells to their environmental conditions, a subpopulation of spermatozoa (the less fertile?) could have their motility parameters modified from coordinated to non-coordinated movements (strong lateral and less forward movements), leading to increased ALH and VCL and unchanged RAPID\_PCT and VAP. Then in a third phase, this subpopulation presents a very strong reduction of motility, leading to reduced motility characteristics. We may question whether these changes could be attributable either to "capacitation like" events during storage or to hyperactivation. Capacitation includes successive physiological events leading to membrane reorganization then to modification of its permeability to ions. When the spermatozoa reach the fertilization site, the zona pellucida of the oocyte primes the sperm to initiate the acrosome reaction [43] and motility hyperactivation may be observed. In Experiment 2, spontaneous acrosome reaction rate was higher at the lower temperature ( $-0.5$  to  $3^{\circ}\text{C}$ ) and was associated with the motility variations discussed above. However, movements observed after storage were quite different from those observed during hyperactivation of equine spermatozoa incubated in capacitation medium at  $37^{\circ}\text{C}$  (star-shaped movement pattern, VSL and STR decrease associated with ALH increase) [44]. So whether the observed changes are related to "capacitation like" process, or to membrane or intracellular alterations, energetic or mitochondrial abnormalities has still to be explored.

Another interesting result is the alteration of acrosome integrity observed with the less fertile treatment in the second experiment. “Other” patterns of acrosomes were observed after *in vitro* storage. Whether these “other” patterns of acrosomes, observed by fluorescent microscopy, are specific to the storage conditions, or whether they represent normal degenerative forms, should now be explored. In addition, a higher level of spontaneous acrosome reactions was observed with this treatment, suggesting that the acrosome is especially sensitive to storage temperature injury.

Taken together, these results suggest the need to be aware of two possible increasing defects visible after storage: 1) excessive individual sperm head movements and 2) acrosome injuries.

The fertility results obtained in our study were high in the laboratory conditions experiment (PC ~ 60% after 72 h storage) and also in the fixed field conditions experiment (PC ~ 50% after 22 h storage at 4 to 10°C). This is consistent with previous results with the same extender [9,10] and higher than recent field results observed after shipping semen in disposable containers (PC ~ 40%, storage time around 24 h, various extenders, 67 stallions) [12]. Despite the difference in storage time between our 2 experiments, we suggest that these high fertility levels could be due to 1) the AI-Ovulation interval, more favorable in the longer storage experiment (~ 7 h) and 2) the set of experimental mares. However, one has to have in mind that in “traditional” field conditions, the fertility rate could be lower because of more variable and less favourable conditions.

## 5. Conclusion

The present study shows that stallion semen may tolerate various air exposure and a wide temperature range (4°C to 15°C) during *in vitro* storage. Temperatures close to 0°C are detrimental, even for short periods of time (2 to 3 hours). The use of disposable containers for the transportation of stallion sperm may be encouraged for 22 h storage, provided that the temperature inside the container does not fall below 4°C.

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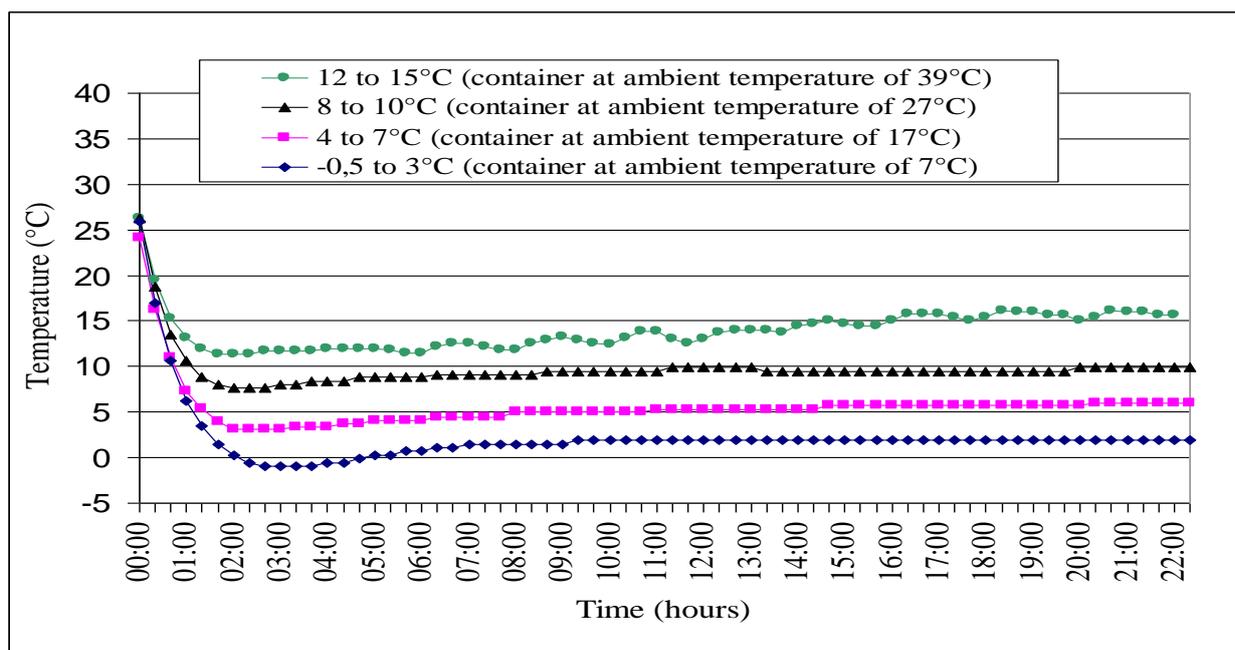


Figure I : Temperature curves in the sperm dose loaded in the disposable container placed at different ambient temperatures, after samples reached 25°C (Experiment 2). Each bar on x axis represents 20 min. Lowest temperature was obtained in 2 h 50 min, 2 h 30 min, 2 h 20 min and 2 h 00 min, in container stored at 7, 17, 27 and 39°C, respectively.

Table 1 : Per-cycle pregnancy rate (PC) after one insemination with semen stored for 72 h either at 5°C without air exposure (5(A-)) or at 15°C with air exposure (15(A+)) (interval IA-ovulation: 7 h) (Experiment 1)

Stallion	Group 5(A-)	Group 15(A+)
A	3 / 8	1 / 8
B	4 / 8	3 / 8
C	5 / 8	7 / 8
D	5 / 8	7 / 8
E	7 / 8	7 / 8
Total	24 / 40	25 / 40
Observed PC (%)	60	63
Estimated PC <sup>a</sup> (%)	60	63

<sup>a</sup> Weighted least square estimates

Table 2 : Spermatozoal motion characteristics (CASA) after storage for 72 h either at 5°C without air exposure (5(A-)) or at 15°C with air exposure (15(A+)) (Experiment 1)

Long name	Short name	Group 5(A-)	Group 15(A+)	Pooled S.E.M	P
Rapid motility (%)	RAPID_PCT (%)	63	66	3	0.5
Progressive motility (%)	PROG_PCT (%)	29	23	4	0.3
Average path velocity (µm/s)	VAP (µm/s)	92	98	3	0.11
Straight-line velocity (µm/s)	VSL (µm/s)	64	63	2	0.8
Curvilinear velocity (µm/s)	VCL (µm/s)	200 <sup>B</sup>	217 <sup>A</sup>	4	0.04
Amplitude of lateral head displacement (µm)	ALH (µm)	7.63 <sup>B</sup>	8.11 <sup>A</sup>	0.09	0.02
Beat cross frequency (nb/s)	BCF (nb/s)	36	34	0.6	0.11
Straightness (%)	STR (%)	72	67	3	0.2

Values are means of 15 split ejaculates (5 stallions x 3 ejaculates)

<sup>A,B</sup> Different letters within a line denote significant differences (P<0.05).

Table 3 : Per-cycle pregnancy rate (PC) after one insemination with semen stored without air exposure (A-) for 22 h in a disposable transport container exposed to three ambient temperatures leading to three cooling/rewarming curves for the sperm (interval IA-ovulation: 17 h) (Experiment 2)

Stallion	Group -0.5 to 3 <sup>a</sup> (A-)	Group 4 to 7 <sup>a</sup> (A-)	Group 8 to 10 <sup>a</sup> (A-)
F	2/8	1/8	2/8
G	1/8	5/8	6/8
H	3/8	5/8	4/8
I	2/8	6/8	4/8
Total	8/32	17/32	16/32
Observed PC (%)	25	53	50
Estimated PC (%) <sup>b</sup>	22 <sup>§</sup>	53	51

<sup>a</sup> Temperatures (°C) of the semen in the disposable container : lowest temperature and temperature after 22 h storage. Cooling curves are presented in Figure I.

<sup>b</sup> Weighted least square estimates

<sup>§</sup>: Within the row, significant difference from the estimate of the mean percentage of all treatments (intercept): 41%, P< 0.002

Table 4 : Spermatozoal motion characteristics (CASA), membrane integrity (measured by HOS test) and acrosome integrity and responsiveness (measured by an ionophore test) after storage for 22 h without air exposure (A-) in a disposable transport container exposed to four ambient temperatures resulting in four cooling/rewarming curves for the sperm (Experiment 2)

	Group -0.5 to 3 <sup>a</sup> (A-)	Group 4 to 7 <sup>a</sup> (A-)	Group 8 to 10 <sup>a</sup> (A-)	Group 12 to 15 <sup>a</sup> (A-)	Pooled S.E.M.	P
RAPID_PCT (%)	76	78	80	78	1.3	0.2
PROG_PCT (%)	38	41	43	41	1.3	0.16
VAP (µm/s)	90	88	83	84	2	0.1
VSL (µm/s)	67	66	63	63	1.5	0.35
VCL (µm/s)	199 <sup>A</sup>	190 <sup>AB</sup>	180 <sup>B</sup>	183 <sup>B</sup>	3	0.007
ALH (µm)	8.5 <sup>A</sup>	7.9 <sup>B</sup>	7.6 <sup>B</sup>	7.7 <sup>B</sup>	0.11	0.008
BCF (nb/s)	32	33	33	33	0.7	0.4
STR (%)	74	75	76	75	0.7	0.51
Sperm with intact membrane (HOS test) (%)	37	40	41	43	0.02	0.16
Sperm in control tubes: with intact acrosome (%)	65 <sup>B</sup>	77 <sup>A</sup>	78 <sup>A</sup>	80 <sup>A</sup>	2.1	0.004
with reacted acrosome (%)	22 <sup>A</sup>	16 <sup>B</sup>	14 <sup>B</sup>	13 <sup>B</sup>	1.8	0.02
others <sup>b</sup> (%)	12 <sup>A</sup>	7 <sup>B</sup>	8 <sup>B</sup>	8 <sup>B</sup>	0.8	0.02
Sperm in ionophore tubes: with intact acrosome (%)	13	15	12	13	1.3	0.60
with reacted acrosome (%)	75 <sup>A</sup>	76 <sup>A</sup>	81 <sup>A</sup>	81 <sup>A</sup>	1.7	0.03
others <sup>b</sup> (%)	12 <sup>A</sup>	9 <sup>AB</sup>	7 <sup>AB</sup>	6 <sup>B</sup>	1.4	0.04
Diff. AR <sup>c</sup>	53 <sup>B</sup>	60 <sup>AB</sup>	68 <sup>A</sup>	69 <sup>A</sup>	2.5	0.005

Values are means of 8 split ejaculates (4 stallions x 2 ejaculates)

<sup>a</sup> Temperatures (°C) of the semen in the disposable container : lowest temperature and temperature after 22 h storage. Cooling curves are presented in Figure I.

Explanation of CASA parameters : cf table 2

<sup>b</sup> others: with acrosome neither intact, nor reacted (see text : section 2.1.5.3)

<sup>c</sup> Diff. AR: % of acrosome reaction (AR) in ionophore tubes minus % of AR in control tubes

<sup>A,B</sup> Different letters within a line denote significant differences (P<0.05).