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Research Article

Development of a New Transport Box for Chilled Stallion Semen, ECOOL Box

Charlène Rouillon[1]*, Guy Delhomme[1]*, Frederic de Geoffroy[2], Clothilde Gourtay[2] and Eric Schmitt[1]

[1]IMV Technologies, Z.I. N°1 Est, Saint-Ouen-Sur-Iton, France

[2]Jumenterie du Haras du Pin, Institut Français du Cheval et de l'Equitation, Exmes, France

***Corresponding Author:** Charlène Rouillon and Guy Delhomme, IMV Technologies, Z.I. N°1 Est, Saint-Ouen-Sur-Iton, France.

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Abstract

Today, doses of refrigerated stallion semen are stored, sent, and transported in single-use polystyrene transport boxes. To address a growing concern for ecological and sustainable development, a new transport box made of recycled material, eco-responsible, called ECOOL Box, has been developed to ensure the transport and conservation of doses. Thermal performance to maintain the temperature between + 2°C and + 15°C for 48 hours, cooling kinetics during the first hour of storage, and biological parameters relating to motility, velocity and sperm viability were analyzed and compared to a control box. In total, up to 10 ejaculates were diluted, packaged in syringes (20.10^6 spermatozoa/mL, 10 mL per dose) and stored in transport boxes for 48 hours. The boxes were placed in a thermal chamber mimicking the daily temperature variations during a temperate winter or summer climate. Temperature readings were taken every 30 seconds using thermocouples. The quality of the semen was analyzed by a computer-assisted semen analyzer at the day of collection and after the 48 hours of storage. Sperm viability at the end of the 48 hours was analyzed using flow cytometry. No significant difference between the two boxes was observed on all thermal and biological parameters. The ECOOL Box allows the cooling of semen doses at a rate of - 0.13°C/min, with no detrimental effect. In winter conditions, the ECOOL Box allows to maintain the doses between + 2.7°C and + 4.0°C for 48 hours. In summer condition, the ECOOL Box maintains the doses between + 7.2°C and + 15°C for 42 hours. The total sperm motility is reduced by only 5% compared to the day of collection. However, it remains above 70% in both conditions at the end of the experiment, which is in line with the recommendations of the French National Studs. The ECOOL Box thus shows its thermal and biological efficiency and allows the transport of equine doses in a safe and eco-responsible way.

Keywords: *Stallion; Chilled Semen; Transport; Motility; Viability; Temperature*

Abbreviations

AI: Artificial Insemination; ECB: ECOOL Box; CB: Control transport Box; VAP: Velocity on Average Path; VCL: Curvilinear Velocity; VSL: Velocity on a Straight Line

Introduction

Since the 2000s, artificial insemination (AI) of mares with the use of fresh and chilled semen has been growing in the saddle race breeding industry [24]. Usually, the trend for AI technologies is linked to some common advantages with chilled and with frozen semen. They avoid animal transport and physical contact between mares and stallions, thus limiting transport costs and disease transmission. The use of chilled semen has extra advantages over frozen semen, which may explain the specific success of chilled semen in the AI technologies. Chilled semen is generally more resistant and requires less heavy and accurate insemination protocols. Indeed, using chilled semen at a time of AI results in better pregnancy rates than frozen semen [15,22,23]. This is mainly due to large differences in initial ejaculate quality [6,17,20] and ejaculate tolerance for chilled and cooled storage between stallions [5,8]. The composition of their seminal plasma does not

ensure the protection of the sperm membranes during the freezing process [1,27]. Therefore, the composition and quality of the sperm can help us to predict the success of fertility. Before chilling or freezing semen, semen quality must be evaluated on several discriminating parameters including motility, viability, or acrosome integrity [2].

Nevertheless, the success of artificial insemination after transportation of chilled semen depends also on other environmental factors. These factors are encountered at different stages of the equine fresh semen preparation and transport [11]. The first step involves the proper management of the stallion collection. The handling of semen must follow precise guidelines especially to avoid urine and bacterial contamination, or osmolarity shock [10]. Once properly collected, the semen quality, based on the normal morphology rate and the total motility of spermatozoa, must be validated to ensure the packaging and transport of good quality sample. This step allows to eliminate from the procedure the ejaculate that may be damaged during transport and whose quality is not maintained. Semen packaging, especially the dilution process and the dilution media, is necessary to decrease sperm concentration and maintain a good motility during the storage [26]. It can impact the quality of the semen and its storage overtime as well [3,18]. Based on purified milk proteins, the INRA96 (IMV Technologies, L'Aigle, France) is one of the media that can ensure optimal semen storage, conservation and maintain the fertility potential up to 48 hours post-collection [28].

Chilled semen is packaged into syringes doses, shipped the day of the semen collection, and received the following day for the insemination. A delay of up to 48 hours can thus elapse between the sending and the moment of insemination. Current transport methods tend to minimize the influence of transport conditions as much as possible. Indeed, several authors have shown that semen can be stored at temperatures from + 2°C to + 4°C, and up to + 15°C for 72 hours without significantly impacting the pregnancy rate of mares after insemination [3,4,18,23,25,28]. Indeed, the metabolism of spermatozoa stored at body temperature (+ 35°C) is high [21] and induces the formation of toxic products for the semen such as lactic acid and oxygen free radicals. This leads to a loss of sperm integrity, motility, and viability [9]. Therefore, it is essential to preserve semen in conditions that reduce their metabolic activities in order to better maintain semen quality. Indeed, it has been shown that by storing semen at + 4°C, sperm metabolism is reduced by only 7%. In the same manner, lowering the storage temperature below + 2°C may lead to the formation of crystals in the semen and damage the quality of the semen [9]. The kinetics of decrease temperature is also an important factor to consider, as well as the risk of a thermal shock. The temperature range in which stallion spermatozoa are most sensitive to a thermal shock induced by rapid cooling (greater than - 0.7°C/min) is between + 19°C and + 8°C. During this window, the authors recommend cooling the semen slowly, at a rate of - 0.05°C/min [19].

In this way, semen must be stored with sufficient thermal isolation to maintain a storage temperature of + 2°C to + 15°C for 48 hours. Consequently, the choice of semen container for cooling and shipment is critical. The cooling rate and isolation of chilled semen transport boxes must be maintained under rough external environmental conditions. Today, chilled semen is sent out in reusable boxes. However, these boxes are bulky, heavy, and expensive. They must be systematically returned to the semen collection center. This adds transport costs and brings sanitary risks as the boxes can carry pathogens. Some chilled semen is sent in disposable single-use boxes, which are cheaper. In addition, these boxes are made from polystyrene, an environmentally unfriendly material.

In the context of sustainable development, the objective of this study was to develop an eco-responsible disposable chilled semen transport box, called the ECOOL Box. The use of recycled polystyrene as a raw material appeared as a challenge. First, we studied the thermal performance of the box and compared it to single-use box already available to breeders on the market. Next, we characterized the semen conservation capacity of the box by assessing different sperm quality parameters. This study then allowed us to conclude on the effectiveness of this eco-responsible box and on its capacity to maintain the quality of doses.

Materials and Methods

Study location, animals, and semen packaging

The study was carried out at the Jumenterie des Haras du Pin (Exmes, France), and on IMV Technologies site (L'Aigle, France). Healthy sexually mature stallions (French Saddle and Trotter, Hanoverian) aged from 6 to 21 years old, originating from the French Horse and Riding Institute (IFCE), Jumenterie des Haras du Pin, were used as semen donor. All stallions were housed individually, under a natural

photoperiod during the reproductive season (May to October). The semen of five stallions of proved fertility, collected regularly for dose production (4 times a week) were collected at different moments of the study. A total of 10 to 12 ejaculates samples were collected on 5 to 6 different experiments.

After semen filtration, the concentration was measured in millions of spermatozoa per mL (M/mL) with a spectrophotometer (Accuread, IMV Technologies, L'Aigle, France). Then, the semen was diluted individually at a final concentration of 20.10^6 spermatozoa/mL in INRA96 previously heated at + 37°C (IMV Technologies, L'Aigle, France). Afterwards, the quality of the ejaculate was checked under the microscope. All ejaculates with more than 70% of motile spermatozoa after collection were used in this study.

Ten mL of diluted semen were packaged in 20 mL sterile syringes without air exposure. The syringes were placed in different environmental conditions: directly in a fridge at +4°C (Fridge), in a climatic chamber inside the eco-friendly transport box ECOOL Box (ECB) or in the non-eco-responsible Control transport Box (CB), or in the climatic chamber outside the boxes (Outside).

A programmable freezer (Mini Digitcool, IMV Technologies, L'Aigle, France), connected to a liquid nitrogen tank was used as a climatic chamber. The boxes were placed in the programmable freezer, and temperature programs mimicking the daily temperature variations during a temperate winter or summer climate, according to the AFNOR NF S99-700 standard (October 2007), were launched (Figure 1 and 2).

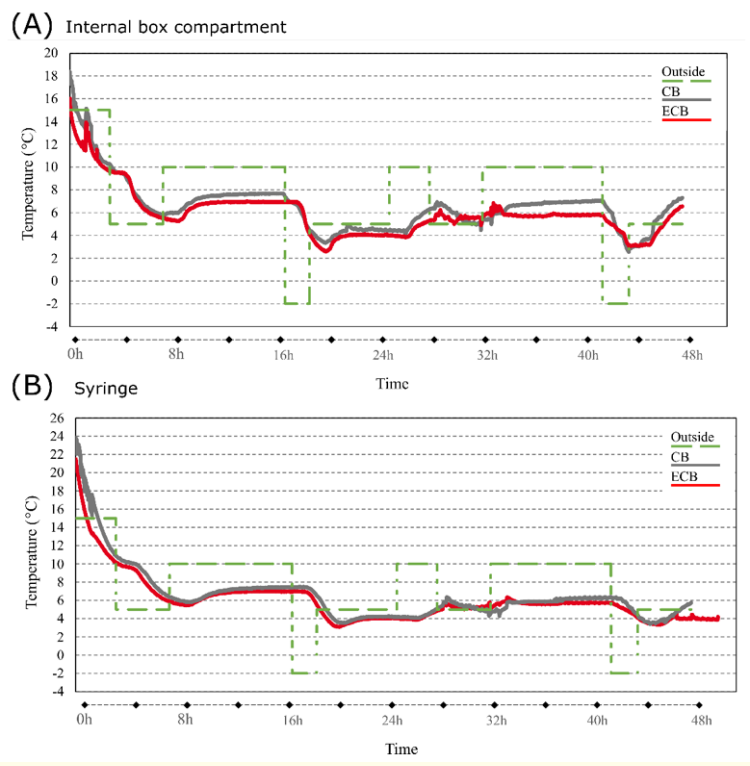

Figure 1: *Evolution of the outside temperature programmed in winter conditions according to the AFNOR standard (Outside, green dotted line), and evolution of the internal box compartment (A) and syringe (B) temperatures in the Control Box (CB, grey line) and in the ECOOL Box (ECB, red line), during 48 hours post-collection. Temperature curves of the CB and ECB represent the average of 5 experiments.*

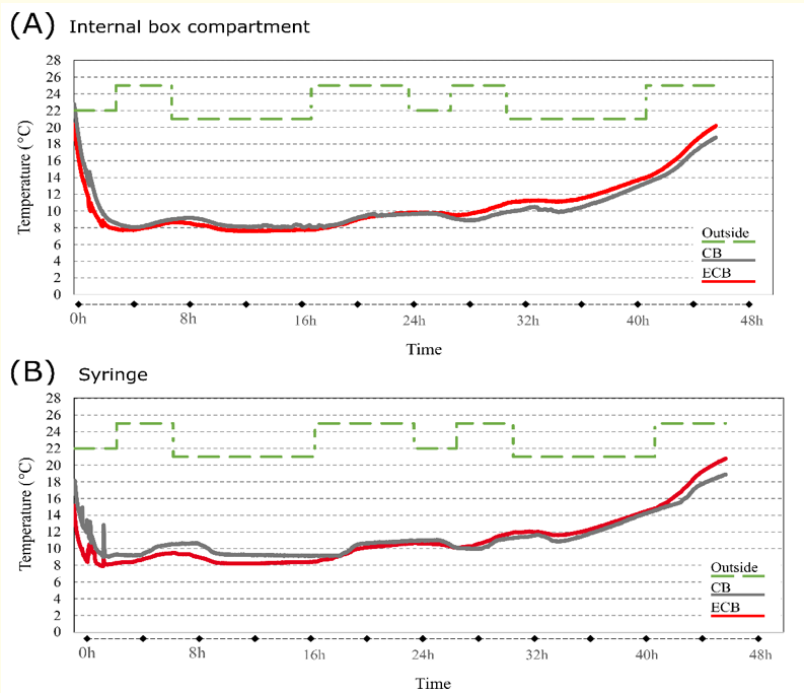

Figure 2: *Evolution of the outside temperature programmed in summer conditions according to the AFNOR standard (Outside, green dotted line), and evolution of the internal box compartment (A) and syringe (B) temperatures in the Control Box (CB, grey line) and in the ECOOL Box (ECB, red line), during 48 hours post-collection. Temperature curves of the CB and ECB represent the average of 5 experiments.*

Two syringes (for 2 ejaculates) were placed in each box. Furthermore, in dedicated compartment of the boxes, two eutectic plates were placed. For summer conditions, two eutectics were at - 20°C and for winter conditions, one eutectic was at - 20°C and another was at + 4°C.

The temperatures in the inner compartment of the boxes and in the syringes were measured to follow the temperature evolution. One measure every 30 seconds was recorded during the 48 hours of the experiment, using type K thermocouples (Mesurex, Saint-Arnoult-en-Yvelines, France) connected to a graphic recorder (mini LOGGER GL200A, GraphTec, Irvine, USA).

Motility parameters analysis

The quality of the ejaculate was analyzed, following semen dilution and at the end of the 48 hours of storage, with a computer-assisted semen analyzer (IVOS II, Hamilton Thorne, Beverly, USA). Diluted semen from the doses was incubated for 10 min at + 37°C. Then, 3 µl of semen were placed in a Leja slide (Leja, IMV Technologies group, GN Nieuw-Vennep, Netherlands). Sperm motility and concentration were checked. For each sample, the percentage of total motile spermatozoa, the percentage of progressive spermatozoa (rapid sperm with straightness > 80%), the velocity on average path (VAP > 40 µm/sec), the curvilinear velocity (VCL), and the velocity on a straight line (VSL) were calculated. Two chambers per samples were analyzed (frame rate = 60, frame acquired = 30, with a minimum of 8 frames per chamber).

Viability and acrosomal integrity analysis

Viability assay was performed with the EasyKit: Viability and Concentration (IMV Technologies, L'Aigle, France) and an EasyCyte flow cytometer (Luminex Corporation, Austin, USA). This kit allows to mark cells with a defect in membrane integrity. In the EasyKit 96 wells plate, a total of 57 000 spermatozoa had been dispensed in each analysis wells, and diluted with EasyBuffer B (023862, IMV Technologies) (qsp 200 µL). After incubation of the samples at + 37°C, during 10 min, the plate was analyzed on a flow cytometer. The parameters measured were the percentage of viable and non-viable (dead) spermatozoa.

In addition, acrosome integrity assay was performed with the EasyKit:Viability and Acrosome integrity (IMV Technologies, L'Aigle, France) and an EasyCyte flow cytometer (Luminex Corporation, Austin, USA). This kit allows to mark cells with a rupture of the acrosome's integrity in addition to marking the membrane integrity of the spermatozoa. In the appropriate EasyKit 96 wells plate, a total of 40 000 spermatozoa were dispensed in each analysis wells, and diluted with EasyBuffer B (023862, IMV Technologies) (qsp 200 µL). After incubation of the samples at + 37°C, during 15 min, the plate was analyzed on a flow cytometer. The parameters measured were the percentages of viable or non-viable spermatozoa, and intact or disrupted acrosome.

Statistical analysis

Statistical analysis was performed on R software, version 1.1.463 (R Core Team, 2014). Results were presented as bar plots, with the mean and standard deviation (SD). For multiple comparison analysis, Kruskall Wallis non-parametric and Tukey post-hoc test was done. Significant difference in the results was consider when p-value < 0.05 ($p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***).

Results

Temperature evolution in the boxes and syringes

The temperature variation in the internal compartment in the boxes and in the syringes were studied in both Control Box (CB) and ECOOL Box (ECB). In winter condition, the internal temperature curve of the ECOOL Box was on average slightly below the CB (- 0.62°C) for the 48 hours of storage. Nevertheless, the difference was not significant (CB: 6.57°C ± 0.78; ECB: 5.95°C ± 0.62; $p = 0.1649$) (Figure 1A). The temperature of both boxes remains within the adequate range of temperature for semen storage during the 48 hours of experiment between + 2.0°C and + 7.3°C (CB: +2.10°C ± 0.79 to +7.30°C ± 5.06; and ECB: + 2.01°C ± 0.42 to +6.97°C ± 4.83) (Figure 1A). There was not any significant difference on the minimal and maximal temperature reached in both boxes (minimal temperature $p = 0.9071$; maximal temperature $p = 0.4003$) (Figure 2). Regarding the temperature of the semen inside the syringes, no significant difference was observed during all experiment (CB: 6.57°C ± 1.05; ECB: 6.08°C ± 1.36; $p = 0.4649$) (Figure 1B). The two curves overlap and remain in the correct range as well, between + 2.5°C and + 5.8°C (CB: +2.51°C ± 1.08 to +5.79°C ± 3.07; ECB: 2.72°C ± 1.18 to +3.98°C ± 0.98) (Figure 1B). No significant difference was observed in the minimal and maximal temperature reached in both boxes (minimal temperature $p = 0.744$; maximum temperature $p = 0.2948$).

In summer condition, the internal temperature curves of the CB and ECB overlapped and no significant difference was detected (CB: 11.26°C ± 0.57; ECB: 11.03°C ± 0.76; $p = 0.7949$) (Figure 2A). The minimum temperature recorded for the 48 hours of storage in the ECB was + 7.16°C ± 1.11 and is significantly lower from the minimum recorded in the CB (+ 8.28°C ± 0.21) (by a factor of 0.8; $p = 0.01381$). Despite this difference of about 1°C, the minimum temperature reached in ECB remains above the minimum temperature of + 2°C required for semen conservation. Regarding the maximum temperature recorded in both boxes, it was reached at the end of the 48 hours of experiment. They exceeded the maximum temperature of + 15°C required for semen conservation (CB: +18.88°C ± 0.87; ECB: +20.8°C ± 3.77; $p = 0.1742$). The same pattern was observed on the temperature syringe curve with no significant difference observed between the average temperature of both boxes (CB: 10.30°C ± 0.77; ECB: 10.42°C ± 0.76; $p = 0.9079$) (Figure 2B). The minimum temperature recorded in the ECB was + 7.24°C ± 0.71 and was not significantly different from the minimum recorded in the CB (+ 7.73°C ± 0.41) ($p = 0.2222$) The + 15°C critical temperature was reached in the ECB syringes after 41h48 ± 2h14 and in the CB after 42h34 ± 2h04 of the experiment, without significant difference between boxes ($p = 0.5241$).

Kinetics of temperature lowering showed that the temperature in syringes inside the ECB dropped during the first hour of the experiment at a rate of - 0.14°C/min ± 0.09 in summer conditions without any significant difference with the CB (- 0.15°C ± 0.10; $p = 0.6852$). In winter condition, the temperature inside the ECB syringes fell at a rate of - 0.12°C/min (± 0.06), similarly to the CB pattern (- 0.10°C ± 0.08; $p = 0.8065$).

Semen motility evaluation

At collection day, semen used for the winter condition experiment had a percentage of total motility of 79.9% ± 7.98 and a percentage of progressive sperm of 46.5% ± 16.3 (Figure 3A and 3B).

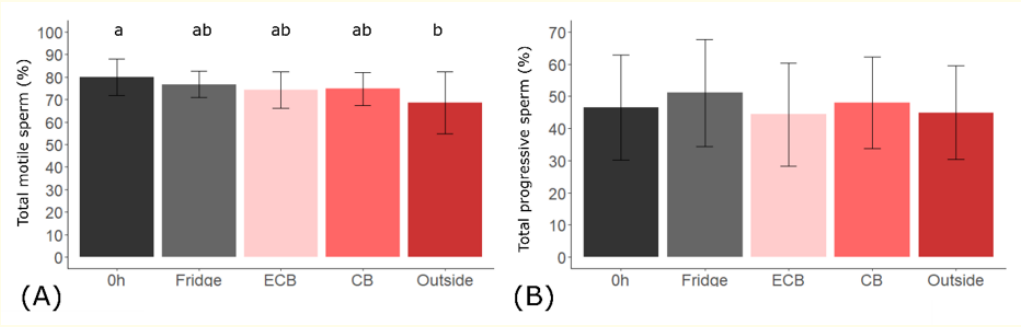

Figure 3: *Motility parameters analysis after semen collection (0h) and after 48 hours of storage at +4°C (Fridge), in the ECOOL box (ECB), in the Control Box (CB) or at external temperature (Outside) during winter condition. (A) Percentage of motile sperm and (B) percentage of progressive sperm were analyzed using an IVOS II, after incubation of the semen samples at +37°C for 10 min (n = 10 semen samples, 5 experiments).*

After 48 hours of experiment in winter condition, no difference in total motility was observed between doses stored in ECB (74.2% ± 8.11), CB (74.7% ± 7.28) nor at + 4°C (Fridge: 76.7% ± 5.92) after 48 hours of storage (Figure 3A). Nevertheless, the total motility was significantly lower by 11% when it was placed in a cold external environment, compared to the day of collection (Outside: 68.4% ± 13.8; $p < 0.001$) (Figure 3A). Regarding the proportion of sperm cell swimming mostly in straight line (progressive sperm), no significant difference was observed between the day of collection and the end of the 48 hours of experiment in all conditions (ECB: 44.4% ± 16.0; CB: 48.0% ± 14.3; Fridge: 51.0% ± 16.6). No significant difference was observed in the velocity parameters as well, between all five conditions (Table 1).

Winter conditions		**48h**				
	0h	**Fridge**	**ECB**	**CB**	**Outside**	**P-value**
Motile VAP (µm/sec)						
Mean (SD)	92.6 (19.5)	100 (11.8)	98.0 (17.2)	92.8 (12.9)	90.2 (11.2)	0,392
Motile VSL (µm/sec)						
Mean (SD)	72.3 (16.7)	81.2 (11.0)	77.0 (14.7)	74.8 (10.9)	73.6 (5.44)	0,314
Motile VCL (µm/sec)						
Mean (SD)	195 (29.6)	199 (24.5)	201 (28.3)	189 (24.4)	185 (23.0)	0,240
Progressive VAP (µm/sec)						
Mean (SD)	92.2 (19.2)	103 (11.1)	99.2 (17.1)	94.9 (13.6)	94.4 (10.7)	0,328
Progressive VSL (µm/sec)						
Mean (SD)	82.1 (18.1)	93.9 (10.1)	90.1 (16.1)	86.6 (12.8)	86.0 (9.22)	0,260
Progressive VCL (µm/sec)						
Mean (SD)	183 (27.8)	190 (18.5)	190 (24.3)	181 (19.0)	182 (18.4)	0,541

Table 1: *Table of velocity parameters evaluated on motile and progressive sperm after semen collection (0h) and after 48 hours of storage at +4°C (Fridge), in the ECOOL box (ECB), in the Control Box (CB) or at external temperature (Outside) in winter condition. Three different velocities were analyzed using an IVOS II, after incubation of the semen samples at +37°C for 10 min (n = 10 semen samples, 5 experiments) : VAP means velocity average pathway, VSL means straight- line velocity, and VCL means curvilinear velocity. The P-value indicates the difference between all the groups.*

At collection day, semen used for the summer condition experiment had a percentage of total motility of 77.6% (± 9.32) and a percentage of progressive sperm of 40.9% (± 16.8) (Figure 4A and 4B). After 48 hours of storage, no significant difference was observed, in the total motility between doses stored for 48 hours in ECB (72.2% ± 7.44), in the CB (75.5% ± 6.28), nor at + 4°C (75.0% ± 6.55) (Figure 4A and 4B). Nevertheless, the motility was 30% lower compared to doses stored in boxes or at +4°C, when it was placed in a summer external environment (Outside: 42.7% ± 31.2; p = 0.001) (Figure 4A). The progressive motility of semen was unchanged for all conditions (ECB: 41.7% ± 20.2; CB: 42.8% ± 18.5; Fridge: 38.0 ± 17.2), except doses stored in a summer external environment which had a progressive motility 20% lower (Outside: 17.2% ± 15.3; p = 0.001). Regarding the velocity parameters, no difference was observed in motile sperm velocity parameters (Table 2). General significant difference was observed on average straight-line and curvilinear velocity of progressive sperm between all five groups (Table 2). Nevertheless, no significant difference between groups was detected by the multicomparison Tukey test.

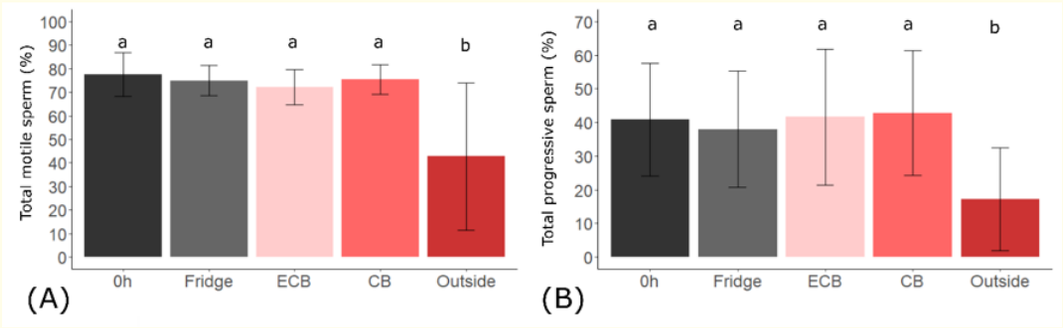

Figure 4: *Motility parameters analysis after semen collection (0h) and after 48 hours of storage at +4°C (Fridge), in the ECOOL box (ECB), in the Control Box (CB) or at external temperature (Outside) during summer condition. (A) Percentage of motile sperm and (B) percentage of progressive sperm were analyzed using an IVOS II, after incubation of the semen samples at +37°C for 10 min (n = 12 semen samples, 6 experiments).*

Summer conditions		48h				
	0h	Fridge	ECB	CB	Outside	P-value
Motile VAP (µm/sec)						
Mean (SD)	85.3 (24.2)	98.9 (18.3)	90.5 (13.1)	90.2 (14.0)	87.4 (17.8)	0,172
Motile VSL (µm/sec)						
Mean (SD)	64.2 (18.0)	70.6 (10.9)	68.4 (15.4)	67.4 (14.4)	59.1 (17.1)	0,232
Motile VCL (µm/sec)						
Mean (SD)	179 (44.0)	209 (37.2)	188 (20.6)	186 (21.8)	194 (33.6)	0,051
Progressive VAP (µm/sec)						
Mean (SD)	84.7 (19.9)	98.1 (14.5)	91.1 (11.9)	89.0 (14.9)	84.4 (33.3)	0,035*
Progressive VSL (µm/sec)						
Mean (SD)	75.0 (18.5)	88.0 (12.5)	82.1 (12.0)	80.4 (14.1)	74.6 (29.8)	0,022*
Progressive VCL (µm/sec)						
Mean (SD)	167 (31.8)	191 (24.3)	178 (15.8)	172 (19.5)	175 (72.5)	0,024*

Table 2: *Table of velocity parameters evaluated on motile and progressive sperm after semen collection (0h) and after 48 hours of storage at +4°C (Fridge), in the ECOOL box (ECB), in the Control Box (CB) or at external temperature (Outside) in summer condition. Three different velocities were analyzed using an IVOS II, after incubation of the semen samples at +37°C for 10 min (n = 12 semen samples, 6 experiments): VAP means velocity average pathway, VSL means straight- line velocity, and VCL means curvilinear velocity. The P-value indicates the difference between all the groups.*

Viability and acrosomal integrity tests

In winter condition, sperm viability results following 48 hours of storage under all three conditions were similar, both after analysis with the Easykit viability (ECB: 74.2% ± 10.9; CB: 72.9% ± 10.0; Outside: 73.9% ± 11.3, p = 0.9439) (Figure 5), and Easykit acrosome integrity (viable intact + disrupted spermatozoa: ECB: 77.4% ± 11.3; CB: 78.0% ± 6.0; Outside: 74.7% ± 8.1, p = 0.304) (Table 3).

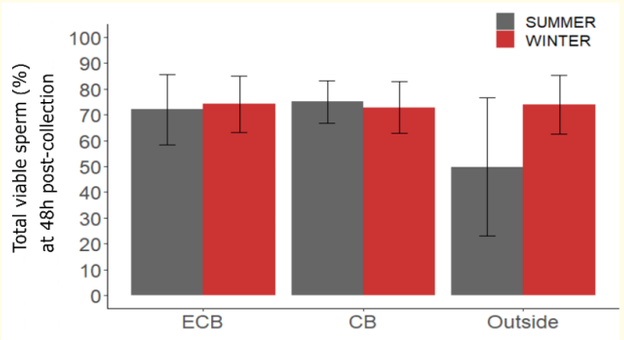

Figure 5: *Viability analysis after 48 hours of storage at in the ECOOL box (ECB), in the Control Box (CB) or at external temperature (Outside) during summer and winter conditions. Percentage of viable sperm was analyzed using the EasyKit Viability and concentration and the EasyCyte flow cytometer (n = 4 semen samples, 2 experiments).*

(A) Winter Conditions	ECB	CB	Outside	P-value
Viable (intact + disrupted) (%)				
Mean (SD)	77.4 (11.3)	78.0 (6.05)	74.7 (8.15)	0,304
Viable intact (%)				
Mean (SD)	74.1 (10.7)	74.7 (4.98)	71.4 (7.48)	0,683
Viable disrupted (%)				
Mean (SD)	3.28 (1.23)	3.30 (1.54)	3.28 (1.71)	0,981
Dead intact (%)				
Mean (SD)	19.4 (9.74)	19.6 (6.27)	21.9 (7.26)	0,276
Dead disrupted (%)				
Mean (SD)	3.20 (1.70)	2.43 (0.618)	3.43 (1.48)	0,469
(B) Summer Conditions	**ECB**	**CB**	**Outside**	**P-value**
Viable (intact + disrupted) (%)				
Mean (SD)	73.9 (13.9)	77.1 (9.51)	46.9 (28.6)	0,077
Viable intact (%)				
Mean (SD)	71.6 (13.6)	73.2 (10.3)	45.2 (27.9)	0,174
Viable disrupted (%)				
Mean (SD)	2.33 (0.846)	3.85 (2.45)	1.68 (1.11)	0,242
Dead intact (%)				
Mean (SD)	23.0 (11.3)	19.0 (6.96)	50.5 (27.5)	0,043*
Dead disrupted (%)				
Mean (SD)	3.15 (2.82)	3.90 (2.97)	2.63 (1.23)	0,779

Table 3: *Acrosome integrity and viability analysis after 48 hours of storage in the ECOOL box (ECB), in the Control Box (CB) or at external temperature (Outside) during winter (A) and summer (B) conditions. Percentage of viable or dead sperm with intact or disrupted acrosome were analyzed using the EasyKit Viability and EasyCyte flow cytometer (n = 4 semen samples, 2 experiments). The P-value indicates the difference between all the groups.*

In summer condition, the sperm viability following 48 hours of storage in the ECB, CB or outside the boxes were identical (ECB: 72.1% ± 13.5%; CB: 75.1% ± 8.2). However, the sperm viability was 25% lower when doses were stored outside the boxes with a large standard deviation caused by the drop to 11% of viability of one ejaculate state (49.8% ± 26.8%) (Figure 5). Therefore, the difference was not significant ($p = 0.1988$). Same results in the percentage of viable spermatozoa (with intact + disrupted acrosome) were observed when using the acrosome integrity assay without any significant difference (ECB: 66.6% ± 18.4, CB: 76.9% ± 9.6, Outside: 54.4% ± 32.7; $p = 0.077$) (Table 3). Nevertheless, a significant difference in the percentage of dead spermatozoa with intact acrosome was observed between all three conditions. The multicomparison test showed this percentage tended to be higher when the doses were placed outside the boxes, than in the boxes, especially in the CB ($p = 0.076$).

The acrosomal integrity analysis table also shows no trend between the three conditions in either summer or winter. The proportion of semen with damaged acrosome (viable + non-viable sperm) changed between 4.3% and 7.8% (Table 3).

Discussion

Despite the complexity of the development of a fresh semen transport box in recycled material, the challenge of combining thermal performance and biological requirements was attempted with the ECOOL Box. This box has demonstrated its thermal insulation capacity in all temperate seasonal conditions to maintain semen doses in the adequate range of + 2°C to + 15°C for 48 hours of storage.

In cold conditions, semen doses are largely stored at the adequate temperature for 48 hours, between + 2.72°C to + 3.98°C. It is possible that these thermal conditions may be maintained for several more hours before exceeding the recommended maximum + 15°C. Quality was assessed based on several parameters of motility and viability. Although the proportion of total and progressive sperm has been reduced by 2 to 5% after 48 hours of storage, the ECOOL Box allows to maintain a high sperm quality with more than 70% of motile spermatozoa. According to the standard protocol applied by the French National Studs [27], with doses at a minimum concentration of 120.106 spermatozoa/ml and a motility at collection greater than 70%, the motility after 24 hours of storage at + 4°C must be greater than 40% to ensure good fertility results. The proportion of progressive sperm in the refrigerated and transported doses of semen was also shown to affect the pregnancy rate of mares [13]. The ECOOL Box has been shown to maintain the progressive sperm content during long storage. Semen quality (motility and viability) was preserved in the ECOOL Box as well as in a constant temperature of + 4°C. These results suggest that the box ensures good conservation of sperm quality in temperate winter conditions. Although the box continued to be in the correct temperature range beyond the 48 hours of storage, and the effects of prolonged storage on the semen was not investigated, it is not recommended to store the semen in the box for any longer period of time. It was reported that the stress induced by storing chilled semen beyond 48 hours induced the activation of apoptotic pathways in the nucleus of sperm cells and led to DNA fragmentation. This is deleterious to the semen and leads to a reduction in fertility in artificial insemination [14,16,17].

In summer conditions, the box maintains the required temperature window for up to 42 hours. Beyond this time, the temperature of + 15°C is exceeded and can damage the sperm. Indeed, although the conservation of stallion semen at + 15°C, diluted in INRA96 medium, and conditioned anaerobically, has shown that the parameters of sperm motility and gestation rates were as good as a conservation at + 4°C, we do not know the effects of conservation beyond this temperature [28]. It was shown that an incubation of only 1 hour at + 37°C of stallion semen, previously chilled at + 4°C for an hour, induces DNA fragmentation [16]. We already demonstrate in our results that the semen doses stored in warm outside condition induce a significant degradation of the semen quality. An increase in the storage temperature can thus induce a resumption of the metabolic activity of the cells. This would generate oxidative stress through the production of ROS and acidification of the dilution medium by cellular release of lactic acid, with deleterious consequences for the viability of the cells [7]. However, even though the semen was stored for 48 hours, i.e. 6 hours at a temperature above the recommended + 15°C (from 42h to 48h), the quality of the doses was not affected (motility, velocity, viability). The total motility was lower by 5.4% in the ECOOL Box after 48 hours of storage compared to the day of collection, but still follows the standard protocol of the French National Studs [27]. Therefore, the percentage of progressive motile sperm was not affected by the storage. The eco-designed transport box therefore presents no difference with the control box and remains just as effective, as if the semen was stored diluted at constant + 4°C for 48 hours.

The quality of the ECOOL Box is also based on its cooling kinetics. Although it is recommended to have a maximum temperature drop of 0.05°C/min, especially between + 19°C and + 8°C [19]. We have shown in this study that a faster temperature drop of 0.13°C/min in the ECOOL Box did not induce a thermal shock of the semen. As our results showed no impact of storage on semen quality, we can conclude that this kinetics was not deleterious to the semen.

Beyond the thermal and biological performances that the ECOOL Box brings, we must also underline the important environmental benefits of the use of this box for the transport of the equine semen doses. The ECOOL Box is made of 100% recycled polystyrene (rEPS). The integration of recycled plastics like this one, brings a real environmental gain. It allows, per ton of recycled plastics, a reduction of the greenhouse effect with less 3 000 kg of CO_2 equivalent produced, a reduction of energy and water consumption (13,000 kWh and 5.2 m^3 respectively saved), a reduction of air acidification (- 5.2 mol H+ eq.) and of eutrophication of fresh and marine waters (- 48 g P-eq. and -970 g N-eq.) [12].

Conclusion

In conclusion, this study showed the thermal performance of the ECOOL Box despite the use of recycled material. We also showed the efficiency of this box for the conservation of equine semen for 42 to 48 hours. However, because multiple environmental factors can impact the quality of semen, our study was conducted using a standardized protocol including the dilution of semen in a validated conservation medium, INRA96. The transport of doses in the ECOOL Box, as well as the respect of the protocol associated with its use, thus makes it possible to safely send doses of standard semen in an eco-responsible way.

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Conflict of Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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