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Abstract

Results on procedures for freezing stallion semen and the subsequent fertility during 20 years are presented. The present system applied in French National Stud includes 1) a freezing protocol (dilution in milk, centrifugation and addition of freezing extender (INRA82 + egg yolk (2%, v/v) + glycerol (2.5%, v/v) at 22°C, a moderate cooling rate to 4°C and freezing at -60°C/min in 0.5-ml straws); 2) selection of ejaculates showing post-thaw rapid motility >35%; and 3) an insemination protocol (mares examined once daily, 2 AI of 400 x 10^6 spermatozoa 24 h apart before ovulation, sufficient number of straws to have the possibility to perform 6 AI of 400 x 10^6 total spermatozoa, i.e. 2.4 x 10^9 total spermatozoa available per mare per season). This system was applied to > 110 stallions per year, the average post-thaw motility of ejaculates was 50% (>1800 ejaculates) before selection. The semen freezability was defined as the number of selected ejaculates divided by the total number of ejaculates frozen. Of the stallions, 5, 4, 5, 21 and 64% had semen freezability of 0 to 10, 10 to 33, 33 to 60, 60 to 90 and over 90%, respectively. Per-cycle pregnancy rate was 45 to 48% (>1500 mares per year, 1.8 cycles per mare) and foaling rate 64%. In comparison, per-cycle pregnancy rate and foaling rate of mares hand-mated to stallions were 57 to 59% and 64%, respectively. The average number of straws used was 32 to 35 (1.75 x 10^9 total spermatozoa) per mare per season. According to our results and the literature, the most important factors for improving fertility of frozen equine semen include: 1) a low concentration of glycerol (2 to 3.5% final concentration); 2) a suitable base extender for freezing like Lactose-glucose EDTA or INRA82; 3) a post-thaw motility >30 to 35%; and 4) a sufficient number of spermatozoa per mare per season (1.5 to 2 x 10^9 total spermatozoa for 2 to 3 cycles) divided into small units. Numbers of spermatozoa, lower than 750.10^6 total spermatozoa per cycle, could result in lower per-cycle pregnancy rate with higher additional costs for management of mares. Because there are no particular regulations on quality and quantity of equine semen in the European Community, there is a need for the uniformity of information about frozen semen. A codification is suggested, based on the number of spermatozoa available per mare per season, the post-thaw motility and the final glycerol concentration.

Keywords: Horse; Equine; Spermatozoa; Cryopreservation; Fertility; Freezability; Glycerol

1. Introduction

In France, the use of frozen semen is prominent in warm-blood horse breeds. In 2004, 6900 mares were inseminated with frozen semen (2200 with National Stud stallions and 4700 with private stallions), compared to 2150 mares in 1997 and only 90 mares in 1985. Within the main French breed of sport horses, Selle Français, 35% of the 7500 mares booked to private stallions are inseminated with frozen semen. Semen is mainly frozen in France, but comes also from abroad. In the breeding season of 2003, the foaling rates of mares inseminated with frozen semen were 64% for National Stud stallions, 62% for private French breed stallions and 57% for private foreign breed stallions (semen doses are mainly imported). In the same breeding season, the foaling rate was 64% for all the warm-blooded stallions in hand mating.

In this paper, a retrospective analysis of 20 years of practice in National Stud is presented, followed by a discussion on factors affecting fertility of frozen semen.

2. Material and methods

2.1. General procedures

Data were provided by the National Stud from 1985 to 2005. The stallions and mares were mainly of the "Selle-Français" warm-blooded breed. Stallions were selected according to criteria presented by Vidament et al. (2000b). Semen was frozen in autumn and winter according to different protocols (Palmer (1984) and derived procedures).

If not specified, milk in all these protocols was liquid UHT (Ultra Heat Treatment) skim milk. The INRA82 extender consisted of 0.5 L glucose-saline solution (138.73 mM glucose, 4.16 mM lactose, 2.52 mM raffinose, 0.85 mM sodium citrate dihydrate, 1.26 mM potassium citrate, 20 mM hepes) and 0.5 L milk with pH adjusted to 6.8 (Palmer, 1984). Extenders E1 (INRA 82 + centrifuged egg yolk 2%, v/v) and E2 (E1+ glycerol, 2.5%, v/v) were used. If not specified, all extenders contained 50,000 UI penicillin and 50 mg gentamycin per liter. In one protocol, glutamine (50 mM) was added to E2.

The semen was first diluted in E1, in E2 or in milk, centrifuged and frozen in E2 at a concentration of 75 x 10^6 spermatozoa/ml (no correction for sperm loss after centrifugation) to 100 x 10^6 spermatozoa/ml (correction of sperm loss since 1995). Straws (0.5 ml) were frozen at -60°C/min above liquid nitrogen vapor or in a programmable freezer (IMV, L'Aigle, France) at the same cooling rate.

At the first dilution and after the centrifugation, the temperature and extender differed among the protocols. Freezing protocols were named (Name XY/X'Y') according to the temperature (X) and extender (Y) at the first dilution and according to the temperature (X') and extender (Y') used at the second dilution. The main protocols used were 37 E1/4 E2 (Palmer, 1984) and 37 E1/22 E2 (Vidament et al., 2000a). At present, a new protocol 37 Milk/ 22 E2 is used (see below).

Selection of frozen ejaculates for insemination was based on 2 to 3 thawed straws, which were evaluated for motility either with a microscope (1985 to 1989) or with a computer-assisted motility analyzer (CASA) in a unique centre. Only ejaculates with post-thaw rapid motility higher than 35% were selected. Rapid motility is the percentage of spermatozoa moving above a certain average path velocity (VAP) and is provided by the CASA analyzer. The VAP threshold for determining rapid motility was 30 μm/sec with the CASA HTM version 2000 (Hamilton Thorne Research, Beverly, MA, USA) (used from 1990 to 2000) (Palmer and Magistrini, 1992),

but it was increased to 40 μm/sec once a new CASA analyzer (HTM-IVOS, version 10.9, Hamilton Thorn Research) was bought in 2001. More precisely for this IVOS system, parameter settings were: 30 frames acquired at 60 frames per second; minimum contrast 80, minimum cell size 4 pixels; lower VAP cut-off 20 μm/s; VAP cut-off for progressive and rapid cells 40 μm/s; Straightness cut-off for progressive cells 80.

Freezability of the semen of a stallion was calculated by the proportion of the number of ejaculates selected after freezing-thawing over the total number of ejaculates.

In the insemination centres, mares were inseminated at the end of estrus with various regimes of inseminations before ovulation (according to the number of AI, of spermatozoa, and of days between inseminations). After thawing at 37°C for 30 sec, 4 or 8 straws were inseminated into the uterine body close to the cervix. Each year, foaling rate results were recorded and published by S.I.R.E. (data base listing all French horses), the foaling rate is the percentage of mares which delivered a foal or a foetus / number of inseminated or mated mares.

2.2. Specific experiments or data analyses

2.2.1. First dilution in milk

Bedford et al. (1995) observed a lower motility when non-centrifuged fresh semen was stored 24 h at 4°C in extender containing egg yolk compared to centrifuged semen (without seminal plasma) in the same extender suggesting a negative interaction between stallion seminal plasma and egg yolk. In the French freezing protocol, first dilution is performed in E1 containing 2.5% egg yolk. The aim of the 3 following experiments (2 preliminary and 1 field trial) was mainly to compare the effect of performing the first dilution at 37°C either in extenders without egg yolk (INRA82 or milk) or in E1, with a freezing protocol 37 Various Extenders/22E2. Since UHT milk is not sold in all countries, some scientists and veterinarians have asked us if the base extender INRA82 could be made with non-fat dry milk.

The objective of experiment 1 was to compare post-thaw motility of sperm initially diluted in either E1, INRA82 or milk. Semen from 6 Merens breed stallions (2 ejaculates per stallion) was divided in 3 parts and frozen after the first dilution at 37°C either in E1, INRA82 or in milk without antibiotics. Post-thaw motion characteristics (rapid motility and VAP) on 3 straws per ejaculate x treatment were measured by CASA.

In experiment 2, the same treatments were investigated as in experiment 1 except that a second control was used. In the control, extenders E1 and E2 were derived from INRA82 in which milk was replaced by non-fat dry milk (Sanalac ND) and apyrogenic water (E1S/E2S). Semen from 5 Welsh ponies (2 ejaculates per stallion) was divided in four parts and frozen and post-thaw motion characteristics (CASA: rapid motility and VAP, 3 straws / ejaculate x treatment) and viability (carboxyfluorescein diacetate (CFDA) and propidium iodide (PI)) (Harrisson and Vickers, 1990), using flow cytometry (Flow cytometer Facstar Plus, Becton Dickinson, San Jose, USA) (3 straws x 2 tubes per ejaculate x treatment) were measured.

The aim of experiment 3 (major field trial) was to compare initially diluting in E1 or in milk without antibiotics, on post-thaw sperm motility of numerous field stallions (winters 2000 to 2001 and 2001 to 2002) and to measure the field fertility of some of these stallions during the 2001 and 2002 breeding seasons. For each stallion, three ejaculates were frozen per week and the two freezing protocols were alternated every other week. Stallions were included in the comparison of post-thaw motility, if more than 2 ejaculates per protocol were frozen in the subsequent week. Then, after freezing and post-thaw selection, semen was used in almost 90 different insemination centers without knowledge of the freezing protocol. Mares were

inseminated every 24 h until ovulation, most mares were inseminated 2 times during the same estrus with 400 x 10^6 total spermatozoa at each AI. They were examined for pregnancy between 13 and 35 days postovulation. Only stallions with fertility data for 6 or more cycles per freezing protocol were included in the fertility analysis.

2.2.2. Relationship between some characteristics of spermatozoa and fertility of frozen semen

Data were obtained from breeding records in 1999. Data on cycles (n=626) were included in the analysis if: (1) pregnancy result and ejaculate number were known, (2) only one ejaculate was used for all AIs of the cycle and (3) the ejaculate was frozen with the protocol 37E1/22E2 in 1999 or before. In a file, each cycle, positive or negative after a pregnancy diagnosis before 25 days, was associated with the values of 3 criteria measured on the corresponding ejaculate during the control analysis made at selection (rapid motility (Rapid %), velocity (VAP) and amplitude of lateral displacement (ALH)). Then, for each criterion, lines were ranked by increasing value of this criterion and assembled in classes with more or less the same number of cycles. Then fertility was calculated by class (positive cycles / total cycles of the class) and correlation between class value and fertility was calculated using the nonparametric coefficient of Spearman.

2.2.3. Insemination strategy

A comparison was conducted in field centres in 1994 (limited number of AI centres) and in 1995 (all centres) between the dose regimen “300 & 150 x 24 h” (first insemination with 300 x 10^6 spermatozoa followed at 24-h intervals by additional AI(s) with 150 x 10^6 until ovulation) and the dose regimen “300 x 24 h” (dose of 300 x 10^6 spermatozoa every 24 h until ovulation). Mares were inseminated during the first cycle with one dose regimen and during the second cycle (if non pregnant) with the other. Only cycles in which mares were inseminated with semen frozen with the same protocol (37E1/4E2) without correction of sperm losses were compared.

2.2.3. Statistical analyses

For Experiments 1 and 2, the model was balanced. General differences between means were tested by an ANOVA (GLM procedure of SAS; SAS Institute Inc., Cary, NC, USA) considering effects of protocol, stallion and ejaculate within stallion. For Experiment 3, the model was an unbalanced comparison of two protocols between ejaculates within stallions. General differences between means were tested by a factorial analysis for unbalanced data (GLM procedure of SAS) considering effects of protocol and stallion. Chi-square test was used to compare the percentages (per-cycle pregnancy rate) in Experiment 3. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Evolution in National Stud between 1985 and 2005

Changes in freezing protocol, semen production, insemination strategies and fertility of frozen semen in National Stud (1985-2005) are presented in Tables 1 and 2. Note for Table 2 that: 1) this table is not directly linked to Table 1, because at least half of the used semen in one year was frozen in the previous winter, the rest was stored for 1 or more years; 2) it is not known to which extent the AI recommendations were followed by veterinarians and technicians.

There has been a considerable development in the use of frozen semen in the National Stud that can be described in 4 steps:

— 1985-1988: beginning of commercial use of frozen semen with encouraging results (50% ejaculates selected after freezing, 33% per-cycle pregnancy rate, 60 to 62% foaling rate similar to fresh semen foaling rate), one freezing center, less than 10 AI centers and 90 to 300 inseminated mares per year
— 1989-1994: lowered results (≤40% ejaculates selected after freezing, ≤30% per-cycle pregnancy rate, ≤50% foaling rate) with the growing production (4 freezing centres) and geographic dispersion in the use of frozen semen (40 AI centers, 300 to 400 mares)
— 1995-1998: improved results due to modifications in the freezing protocol and insemination strategy (75% ejaculates selected, 50% per-cycle pregnancy rate, 70% foaling rate), which resulted in an increase in number of ejaculates frozen (>950) and of the number of inseminated mares (>700)
— 1999-2005: stabilization of the results (85% ejaculates selected after freezing, 46 to 48% pregnancy rate per cycle, 64% foaling rate) simultaneously with a high increase in number of ejaculates frozen (>1850) and of number of inseminated mares (up to 2000) in >90 national stud centres and in 65 private centres.

3.2. Progress in semen processing and in stallion management

From these data, proper comparisons between some freezing protocols have been published previously. The freezing protocol of 37E1/22E2, compared to 37E1/4E2, improved spermatozoal recovery after centrifugation, post-thaw motility (40% vs 36%) and pregnancy rate per cycle (56% vs 42%) (Vidament et al., 2000a). Freezing protocol 22E2/22E2 compared to 37E1/22E2 improved motility very slightly but not fertility, and freezing protocol 37E1/22E2Glu (E2 + 50 mM glutamine) consistently improved motility and velocity but not fertility as compared to 37D1/22D2 (Vidament et al., 2001).

For the experiments with the first dilution in milk, in experiment 1, rapid motility was similar when semen was frozen after the first dilution at 37°C in E1 (28%), INRA82 (25%) or in milk without antibiotics (29%) (P=0.27) (VAP for the three extenders was: 69, 67 and 69 μm/sec, respectively, P=0.49). In experiment 2, motility, velocity and viability were very similar for sperm in the various extenders (Table 3). In experiment 3, post-thaw rapid motility, velocity and per-cycle pregnancy rate were similar for the 2 extender protocols (Table 4). However, the number of straws per ejaculate was lower (P<0.05) with milk dilution than with E1 dilution. The stallions were ranked in 5 groups according to their mean post-thaw sperm motility after freezing with the 37E1/22E2 protocol. For stallions <35% post-thaw motility, milk dilution resulted in higher post-thaw motility than with E1 dilution (33% (69 ejaculates) versus 27% (70 ejaculates) (9 stallions, P<0.05)). There was no difference in post-thaw motility between the two freezing protocols in the other groups.

3.3. Evaluation of semen

A relationship was demonstrated between rapid motility and fertility (P<0.01) (Figure 1). Pregnancy rate per cycle was 43% (n=193), when motility was less than 45%, and 52% when motility was over this value (n=433) (Chi square = 4.33, P<0.05). Similarly, a relationship was found between VAP and fertility (P<0.01) (Figure 2). Pregnancy rate per cycle was 45% (n=355) when VAP was <66 μm/sec and 54% when VAP was over this value (n=271) (Chi square = 4.98, P<0.05). No relationship was found between ALH and fertility.

3.4. Progress in insemination strategy

During the first years of data collection, a dose of 300 x 10^6 every 48 h was decreased to 150 x 10^6 every 24 h; the AI dose for fresh semen was also reduced in the National Stud from 400 x 10^6 to 200 x 10^6 total sperm. Palmer and Magistrini (1992) observed no differences in fertility based on breeding records from 1986 to 1990 between the two doses but pointed out an improvement in fertility when 2 AI were used instead of 1 AI. A detailed analysis of breeding records from 1991 to 1995 confirmed this beneficial effect of multiple AI and showed a tendency for better results, when the last AI dose was 300 x 10^6 spermatozoa 24 h before ovulation (Vidament et al., 1997).

The comparison between the two dose regimens demonstrated that per-cycle pregnancy rates were higher ($P<0.05$) for mares inseminated twice with 300 x 10^6 sperm at 24h intervals ("300 x 24 h") than for those inseminated initially with 300 x 10^6 then with 150 x 10^6, 24h later ("300 & 150 x 24 h") (Table 5).

3.5. Results with the present system

The present French system is described in detail in Table 6.

In frozen semen, the mean final glycerol concentration was 2.1% and the average proportion of seminal plasma was 4.4%. Data on stallions whose semen was frozen in years 2003 to 2005 were pooled (freezing protocol: 37Milk/22E2). Among the population of stallions with ≥4 ejaculates frozen during one year, the distribution of the various mean rapid motility is shown in figure 2 (n= 344 stallions). The majority of stallions (80%) had a mean post-thaw motility higher than 40%. The distribution of the number of stallions with ejaculates of various freezability is depicted in Figure 3. Of the stallions, 5, 4, 5, 21 and 64% had a semen freezability of 0 to 10, 10 to 33, 33 to 60, 60 to 90 and over 90%, respectively. Thus, only 9% of the stallions had semen that could not be frozen (less than 33% ejaculates selected) with the present freezing protocol.

For ejaculates frozen in years 2003 to 2005 (Table 7), the mean post-thaw rapid motility was 50% and progressive motility approximately 38% in all ejaculates, and 55% and 42% in selected ejaculates, respectively. Around 80 to 85 straws were frozen from each ejaculate. As 48 straws were reserved for a mare per season, frozen spermatozoa for 1.4 to 1.5 mares per season were produced from each selected ejaculate.

When the recommended dose rate of AI changed in 1995 from "300&150 x 24 h" to "300 x 24 h", the number of straws per mare changed from 48 to 56 straws. Since the mean number of straws used was regularly 32 to 35 straws per mare in 1996 to 1998, the number of straws per mare was reduced to 48 in 1999. More spermatozoa were used per AI, but fewer cycles per pregnancy were used, since the insemination was more fertile.

4. Discussion

4.1. Semen processing and stallion management

During 1985-2005, the major modification that improved motility, freezability and fertility of stallions was the adoption of the 37E1/22E2 protocol (Vidament et al., 2000a). Fertility and motion characteristics of semen first diluted either in E1 or in milk were similar. During the first dilution of semen in E1 (the average proportion of seminal plasma was 25%), the negative interaction between egg yolk and seminal plasma described by Bedford et al. (1995) could have reduced the quality of spermatozoa after freezing. This was not the case for the majority of the stallions, since the sperm were in the first extender a short time (30 min).

Surprisingly, the number of straws was slightly reduced with milk. Constituents of E1 not present in milk (some sugars, salts or more likely egg yolk) could explain this effect. Moreover, the first dilution in milk is a simplification of the freezing protocol, since it saves time (fabrication and storage of extender) and money; the lower number of straws has been considered by the technicians as a minor drawback.

With the 37Milk/22E2 protocol, the post-thaw motility and the rate of selected ejaculates was very high, much higher than that described generally in the literature, since 64% of stallions had more than 90% of their ejaculates selected after freezing for use on mares. This is due to an efficient freezing protocol (high mean motility after thawing), but also to the management of stallions whose semen froze poorly. These stallions have to be rejected rapidly from production: rejection after six low quality ejaculates, especially if the results were the same the year before. This rule was insufficiently applied in the National Stud before 1994 and led to a catastrophic low mean rate of selected ejaculates. When the 37E1/22E2 and 37Milk/22E2 protocols were used, the proportion of stallions with unfreezable sperm (i.e. with less than 33% ejaculates selected after post-thawing control) was low (9 to 15%), much lower than with the 37E1/4E2 protocol (49%). Other authors have previously obtained high freezability results: 11 to 14% of stallions with unfreezable sperm and 83 to 90% of selected ejaculates post-thaw from the remaining stallions (Salazar-Valencia, 1983; Müller, 1982) but after a high selection of stallions and ejaculates before freezing (Müller, 1982).

Effect of some modifications of the freezing protocol on sperm quality cannot be estimated since no comparison was done before and after the modification. This was the case for the slight centrifugation of egg yolk (1992), correction for sperm loss (1995) and the use of apyrogenic water instead of charcoal demineralized or distilled water in the extenders (1995). Nevertheless, correction for sperm loss permitted a standardisation of the number of sperm per straw and an increase of the number of sperm per straw.

4.2. Glycerol is essential for cryopreservation but limiting for fertility

Glycerol is the essential cryoprotectant in all conventional extenders for freezing stallion spermatozoa. During freezing, spermatozoa are protected since glycerol reduces concentration of extracellular salts during the dehydration time and raises the percentage of unfrozen water at any given temperature. Spermatozoa are apparently sequestered into narrow channels of unfrozen water between large ice crystals (Amann and Pickett, 1987).

The concentration of glycerol varies strongly (from 1- to 3-fold) in the equine freezing extenders: 2.5% (Palmer, 1984), 3% (Wilhelm et al., 1996), 3.5% (Tischner, 1979; Müller, 1987; Burns, 1992), 4% (Cochran et al., 1984; Heitland et al., 1996), 5% (Nishikawa, 1975; Martin et al., 1979) and 7% (Pace and Sullivan, 1975). The optimum glycerol concentration for maximal post-thaw motility of equine spermatozoa has not been clearly evidenced, and could differ according to the extender composition (Ecot et al., 2000). Another confusing point is that concentration of glycerol in papers is very often indicated in the freezing extender and not in the processed semen (final concentration). This final concentration depends on the dilution rate of the freezing extender by the pellet, and it is not always possible to calculate the level with the information given in the paper.

In INRA82 extender, the optimal glycerol concentration for maximal post-thaw motility is around 2-3% (final concentration) (Ecot et al., 2000; Vidament et al., 2001; Vidament et al., 2002). In the most commonly used extender, Lactose-Glucose EDTA (Martin et al., 1979), this optimal concentration could be 4% (extender concentration) (Cochran et al., 1984; Cristanelli et

al., 1985). In Kenney freezing extender, Burns and Reasner (1995) reported that the maximal post-thaw motility improvement was obtained between 0 and 2% glycerol concentration (extender concentration).

Compared to fresh semen without glycerol, lower fertility in mares has been observed when equine fresh semen was prepared with glycerol at concentrations of 4% (final concentration, Bedford et al., 1995), 5% (extender concentration, Loomis et al. 1983), 4.8% (final concentration, Vidament et al., 2005) and 7% (extender concentration, Pace and Sullivan, 1975; Demick et al., 1976). However, based on our 1985 to 2005 data with frozen semen, 2.5% glycerol (in freezing extender) routinely provided satisfactory per-cycle pregnancy rate. In limited studies, we observe a similar per-cycle pregnancy rate when semen was frozen with 2 or 3% glycerol (final concentration) (Vidament et al., 2002) and when fresh semen was prepared and cooled with 2.2 (53%) or 3.5 (53%) glycerol (final concentration) (Vidament et al., 2005).

In extensive studies where fertility per cycle was reported to be over 40% in more than 100 cycles and in which characteristics of freezing processing are known, final glycerol concentration was low in most cases: less than 2.5% (Sieme et al., 2003 and our data), less than 3.5% (Müller, 1987), less than 4% (Salazar-Valencia, 1983, calculated data) and less than 5% (Nishikawa, 1975). A contraceptive effect of glycerol is well documented in hens, even though glycerol is the most successful cryoprotectant for the spermatozoa in this species (reviewed by Hammerstedt and Graham, 1992). Glycerol has also a certain contraceptive effect in equine species, this effect seems to be stronger in asine species (Vidament et al., 2005).

Glycerol is difficult to measure precisely because of its high viscosity, so mistakes in the measuring of glycerol volumes are possible. Osmotic pressure measurements during preparation of extender could help to adjust correctly this concentration.

A compromise should be found between the cryoprotective effect and the negative effect on fertility. A low level of glycerol in frozen semen should be considered as a quality factor for fertility if a minimum post-thaw motility is obtained. Finally, considering both motility and fertility, the recommended glycerol final concentration is 2.5 to 3.5%.

Other cryoprotectants like ethylene glycol and dimethylformamide (DMF) have been proposed recently for freezing equine semen (Medeiros et al., 2002; Vidament et al., 2002; Mantovani et al., 2002; Squires et al., 2004). However, more extensive studies including fertility trials are needed before considering replacing glycerol.

4.3. Base extender

Comparisons between freezing extenders for stallion semen are poorly documented. Mainly 3 extenders are presently used: Lactose-Glucose EDTA Freezing Extender (FE) (Lactose - 25% glucose EDTA solution - 20% egg yolk - 5% glycerol; Martin et al., 1979) modified by Cochran et al. (1984) (4% glycerol), INRA82 FE (INRA82 + 2% egg yolk + 2.5% glycerol = E2 in our experiments; Palmer, 1984) sometimes also named SMEY Extender by other authors (Heitland et al., 1996) and Kenney FE (Kenney extender + 4% clarified egg yolk + 3.5% glycerol; Burns, 1992). Only a few comparisons have been made between these extenders and only one comparison of fertility has been done.

Post-thaw motility was higher when semen was processed in INRA82 FE than in Lactose-Glucose EDTA FE (the two extenders contained 4% egg yolk and 4% glycerol) (Heitland et al., 1996). Post-thaw motility was higher when semen was processed in a modified Lactose-Glucose EDTA in which the 25% glucose EDTA solution was replaced by 25% of Kenney extender than in the original Lactose-Glucose EDTA FE (Braun et al., 1995; Alghamdi

et al. 2002; Schembri et al, 2003). In the above-modified Lactose-Glucose EDTA FE, the percentages of acrosome reaction and capacitated sperm were higher if Kenney extender contained bicarbonate (Schembri et al., 2003). In our laboratory, post-thaw motility was higher when semen was frozen in Kenney FE (with bicarbonate, 3.5% glycerol, 4% egg yolk) than in INRA82 FE (2.5% glycerol) (Ecot et al., 2000). However, the percentage of intact acrosomes was lowered for sperm frozen in Kenney FE and per-cycle pregnancy rate was clearly reduced with sperm prepared in Kenney FE (38%) compared to INRA82 FE (63%) (Ecot et al., 2001).

In conclusion, although Kenney FE alone or mixed with Lactose-Glucose EDTA FE results regularly in higher motility compared to the other two extenders (Lactose-Glucose EDTA FE and INRA82 FE), there is indication of lowered fertility in some conditions. This could be due to the presence of bicarbonate in some formulae of Kenney; bicarbonate induces capacitation in spermatozoa of different species including the horse (for review: Gadella et al., 2001). Capacitation occurred rapidly in Kenney extender during storage of fresh semen (Pommer et al., 2002).

4.4. Estimation of quality of frozen semen: value of motility

Numerous investigators have attempted to find different criteria post-thaw that could best predict fertility of frozen semen, but very few studies are available with a sufficient number of stallions or mares (Samper et al., 1991; Kirk et al., 2005). Motility is the simplest to perform. In the analysis reported here, a relationship was found between the two sperm motion measurements, rapid motility and velocity, and the fertility of corresponding cycles. However, for velocity, the slope of the curve was so flat that this relationship cannot be used for selecting ejaculates for insemination. For rapid motility, the relationship to fertility was clearer.

In 1998, the analysis was done in a similar manner, but only rapid motility was used. The same type of result was found but it was non-significant: 45% fertility (n=359 cycles) when motility was less than 45% and 50% (n=488) when motility was higher than 45% (P=0.15).

Nevertheless, all these data permit us to conclude that there is a relationship between post-thaw motility and fertility of frozen semen, at least among ejaculates frozen by the same technique and used in a similar manner. So the selection by this criterion is justified. However, when semen is frozen using different protocols, this relationship may no longer exist because other components of spermatozoa can react differently to various extenders, or various cooling curves.

4.5. Insemination strategy

In some countries, mares bred with frozen semen are examined 4 to 6 times per day and inseminated immediately before or within 6 h post-ovulation. This is based on the idea that frozen-thawed sperm do not survive long in the mare's reproductive tract. Also, stallion owners provide only limited doses of frozen semen for each mare, with sometimes no precise indication of the number of sperm and the quality of semen in the straws. Moreover, there is no international agreement on the minimum number of frozen sperm that should be sold for a cycle or for a mare per season.

Because sufficient numbers of straws are available in the French system (6 doses of 400 x 10^6 total spermatozoa, i.e. 48 straws), mares are examined once a day, when a preovulatory follicle is present, and inseminated every 24 h until ovulation. Fertility is improved when the mares are inseminated twice 24 h apart and before ovulation compared to once (Palmer and Magistrini, 1992; Vidament et al., 1997).

In extensive studies where fertility per cycle was reported to be over 40% in more than 100 cycles, either mares were intensively monitored around ovulation and inseminated once (Salazar-Valencia, 1983; Samper et al., 1991; Darenius, 1999; Barbacini et al., 2003) or mares were monitored and inseminated every 24 h until ovulation (our data and those of Müller (1987)). The major drawback of post-ovulation AI is the increased rate of embryonic loss, as it has been demonstrated with fresh semen (Woods et al., 1990).

These two insemination strategies have been compared recently. Squires et al. (2003) reported similar per-cycle pregnancy rates where mares received either 2 AI of frozen sperm 24 and 40 h after administration of hCG (mares examined once a day) or 1 AI after ovulation (mares examined 3-6 times a day). Sieme et al. (2003) analyzed the effect of multiple AI versus single AI on fertility of frozen semen and concluded that 1) high fertility could be obtained if mares are inseminated once 12 h before to 12 h after ovulation, 2) multiple insemination increases the probability to inseminate during this optimal window and 3) the choice between the two AI regimes (one AI, frequent mare examinations versus multiple AI, examination once per 24 h) depends largely on the balance between the cost of the semen per dose versus the costs of insemination and veterinary attention.

The means of improving fertility with the multiple insemination are: increasing the total number of spermatozoa available for the mare, increasing stimulation of the genital tract, and increasing the variability of the spermatozoa population within the female genital tract by frequent insemination of freshly thawed sperm. However, 4 inseminations of 100 x 10^6 total spermatozoa each, during 24 h, with the last AI 5 h before ovulation, resulted in lower fertility than only 1 insemination with 400 x 10^6 12 h before ovulation (Clément et al., 2005).

Another confusing factor when comparing different studies is that most authors express sperm numbers in progressively motile spermatozoa (pms) without giving the motility value, whereas other workers use the total number. The tendency for higher fertility observed when the mare has received an insemination dose of 300 x 10^6 total sperm per AI than 150 x 10^6, 24 h before ovulation (Vidament et al., 1997) has been confirmed (trial 1994 and 1995). As most mares in our data received 2 AI, one can consider that fertility was higher when mares received 600 x 10^6 (2 x 300) than 450 x 10^6 (300 then 150) total spermatozoa. So until the definitive adoption of the dose of 300 x 10^6 every day in 1996, and subsequently 400 x 10^6 every day after correction of sperm losses, the mares in our studies received regularly an insufficient number of spermatozoa. This could explain a part of the very low results observed from 1989 to 1994.

Amann and Pickett (1987) have suggested that maximum fertility for the stallion is a function of number of spermatozoa inseminated until a critical number is reached at which point fertility would not increased. For equine frozen semen inseminated into the uterine body, this critical value has not been determined yet: ranges between 175 x 10^6 motile spermatozoa (Volkman and Zyl, 1987) and 320 x 10^6 motile spermatozoa (Leipold et al., 1998), or between 450 x 10^6 total spermatozoa (our present data) and 800 x 10^6 total spermatozoa (Samper, 1995). So this number could be around 250 x 10^6 motile i.e. 750 x 10^6 total spermatozoa since most often post-thaw motility is 33 to 40% in most papers.

In extensive studies where fertility per cycle was reported to be over 40% in more than 100 cycles and in which characteristics of semen are known: the number of spermatozoa per AI have been equal or greater than 400 x 10^6 total (Salazar-Valencia, 1983), 240 x 10^6 motile (Loomis, 2001), 2 times 400 x 10^6 total (our data) and 700 x 10^6 total spermatozoa (Nishikawa, 1975; Müller, 1987; Samper et al., 1991; Darenius, 1999; Sieme et al., 2003).

In conclusion, the number of frozen sperm usually used per cycle seems very near this critical number. It is important for the mare owners to have the possibility to purchase a number of spermatoza above this value rather than below. Nevertheless, this critical number of spermatozoa is dependent on the stallion, the post-thaw quality of sperm, the extender (glycerol concentration), but certainly dependent also on the time between AI and ovulation and on the insemination site.

To insure a proper number of spermatozoa for the mares, it is necessary to produce straws with a fixed and known concentration. It is, therefore, necessary to evaluate and correct sperm losses after centrifugation. These losses are reported to be 10 to 40%, depending on temperature, extender, volume, centrifugation time, centrifugation force and volume of supernatant left above pellet (Pickett et al., 1975; Cochran et al., 1984; Volkman and van Zyl, 1987, Heitland et al., 1996; Vidament et al., 2000). In milk, the recovery was less than in extender E1 in the experiments presented in this paper. Different techniques of cushion under the pellet have been proposed but they have never permitted a total recovery of spermatozoa (Cochran et al., 1984; Volkman et al., 1987). Recently, a dense medium was proposed as a cushion and resulted in a total recovery of sperm if clear extender is used (Ecot et al., 2005).

Numerous studies with low doses of spermatozoa and/or deep uterine AI performed either with an endoscope or by a rectally-guided approach have been conducted recently with fresh or frozen equine semen and have demonstrated that pregnancies can be obtained with very low doses in very strict conditions (reviews: Ball, 2004; Morris, 2004). Unfortunately, some of them are inconclusive because: 1) the comparison between groups is confounded with too many factors, 2) numbers of cycles and stallions are insufficient, 3) freezing technique is unspecified or invalid. There is a lack of comparison between these techniques and well established standard procedures (Sieme et al., 2004). We have observed that per-cycle pregnancy rate was reduced when 50 x 10^6 total frozen spermatozoa were deposited 12 h before ovulation either into the uterine body (26%) or into the tip of the horn ipsilateral to the preovulatory follicle by a rectally-guided approach (25%), compared to 400 x 10^6 total frozen spermatozoa into the uterine body (46%) (Clément et al., 2005). Sieme et al. (2004) found no difference in fertility between 100 x 10^6 total spermatozoa (frozen in modified INRA82 + 2% egg yolk + 2.5% glycerol) inseminated into the uterine body (per-cycle pregnancy rate 43%), into the tip of the horn by a rectally-guided approach (45%) or by endoscopy (46%) and 800 x 10^6 total spermatozoa inseminated into the uterine body (43%), in all cases, the unique AI was performed 6 hours before ovulation. In these latter two papers, insemination into the tip of the horn did not improved fertility, compared to similar numbers of spermatozoa inseminated into the uterine body.

4.6. Proposal for uniform information about frozen semen

In European Community, there are no particular regulations on quality and quantity of equine frozen semen, except for a sanitary point of view. So mare owners need technical information on the frozen semen they want to purchase. The above discussion has focussed on some important points that affect fertility: the total number of sperm available for a mare per season (number of spermatozoa per AI dose x number of AI doses), the post-thawing motility and the glycerol concentration in the straw. Unfortunately, the idea of using low doses of frozen semen has spread widely and could reduce fertility. With the French system, a stallion on the average produces a mean of 82 straws (50 x 10^6 sperm/straw) per ejaculate. During one week (3 ejaculates produced, 85% ejaculates selected), the production is 209 straws (82 x 3 x 0.85). When 48 straws (6 doses of 8 straws) are reserved for a mare per season, 3 ejaculates provide

enough sperm for insemination of 4.35 mares. If a stallion owner sells two straws of 50 x 10^6 total sperm per mare per season, in 3 ejaculates, a stallion can provide enough semen for 105 mares. It seems to be a rather misleading practice. To keep 2.4 x 10^9 total sperm (6 x 8 straws (number of straws per insemination)) for a mare per season does not seem exaggerated, since for most stallions, it represents only 33% or less of an ejaculate.

I suggest a codification for equine frozen offered for sale on the breeding market: the first letter describes the number of spermatozoa available per mare per season, the second letter describes the post-thaw motility and the third letter describes the final glycerol concentration (Table 8). For instance, all the semen sold by French National Stud is A***AA. This codification would indicate to the owners the easiness to obtain pregnancy with the frozen semen they purchase. A form like the one presented in Table 9 should come with the straws. I hope that this codification could be discussed between equine semen experts and be modified, if necessary, and could help in equine semen exchanges.

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Table 1
Equine frozen semen: evolution of freezing protocols and frozen semen production in the French National Stud (1985-2005).

Year	Basic protocol [a]	in compari-son with	Correction for sperm loss	No of freezing centres	No of stallions	No of frozen ejaculates	Rate of selected ejaculates (%)
1985	37E1/4E2		no	1	6	93	58
1986	37E1/4E2		no	1	10	109	52
1987	37E1/4E2		no	1	10	156	40
1988	37E1/4E2		no	1	10	223	48
1989	37E1/4E2		no	2	15	224	56
1990	37E1/4E2		no	2	41	363	35
1991	37E1/4E2		no	4	42	608	32
1992	37E1/4E2		no	4	47	635	46
1993	37E1/4E2		no	4	65	772	26
1994	37E1/4E2		no	4	58	696	44
1995	37E1/4E2	37E1/22E2	yes	4	64	521	46
1996	37E1/4E2	37E1/22E2	yes	4	70	703	67
1997	37E1/22E2	22E2/22E2	yes	4	87	988	61
1998	37E1/22E2	22E2/22E2	yes	4	84	974	79
1999	37E1/22E2	37E1/22E2Glu	yes	5	124	1622	83
2000	37E1/22E2	37E1/22E2Glu	yes	5	141	2051	78
2001	37E1/22E2	37Milk/22E2	yes	5	123	1821	83
2002	37E1/22E2	37Milk/22E2	yes	5	127	1810	87
2003	37Milk/22E2		yes	5	133	1931	86
2004	37Milk/22E2		yes	5	117	1873	88
2005	37Milk/22E2		yes	4	114	1802	81

[a] Combination of temperatures and extenders before freezing: the temperature (°C) and extender of the first dilution / the temperature (°C) and extender of the second dilution after the centrifugation; example : 37E1/4E2 : first dilution at 37°C in E1, cooling to 4°C and second dilution after centrifugation at 4°C in E2.; E1 : INRA82 + egg yolk (2%, v/v); E2 : E1 + glycerol (2.5%, v/v); Glu : 50 mM glutamine; Milk: UHT (Ultra Heat Temperature) skim milk.

Table 2
Equine frozen semen: evolution of insemination strategies, number of inseminated mares and fertility (per-cycle pregnancy rate and foaling rate) in the French National Stud (1985-2004)[a].

Year	Frozen semen							Hand mating	Fresh semen
	Insemination recommendations			Number AI centres [d]	Number insemi-nated mares	Per-cycle pregnancy rate (%)	Foa-ling rate (%)	Foa-ling rate (%)	Foa-ling rate (%)
	Basic protocol [bc]	in comparison with [bc]	Num-ber AI/ cycle						
1985	300 x 48h		1	6	93	29	. [e]	.	.
1986	300 x 48h		1	6	125	37	.	.	.
1987	300 x 48h		1	10	200	36	60	60	61
1988	300 x 48h	150 x 24h	1	10	311	32	62	62	66
1989	300 x 48h	150 x 24h	2	12	327	30	56	62	66
1990	300 x 48h	150 x 24h	2	16	248	28	45	56	.
1991	300 x 48h	300 & 150 x 24h	2	29	273	31	48	57	63
1992	300 x 48h	300 & 150 x 24h	2	37	499	27	49	61	63
1993	300 x 48h	300 & 150 x 24h	2	40	372	29	48	62	62
1994	300 x 48h	300 & 150 x 24h	2	40	292	34	52	60	64
1995	400 x 48h	400 & 200 x 24h	2	41	307	43	.	.	.
1996	400 x 24h		2	44	408	44	61	.	.
1997	400 x 24h		2	48	481	53	72	63	63
1998	400 x 24h		2	54	704	49	69	64	66
1999	400 x 24h		2	66	1032	47	.	.	.
2000	400 x 24h		2	74	1326	49	66	63	63
2001	400 x 24h		2	92+24	1390	49	66	63	68
2002	400 x 24h		2	93+32	1503	48	64	64	69
2003	400 x 24h		2	95+46	1723	45	64	64	65
2004	400 x 24h		2	97+65	1990	46			

[a] This table is not directly linked to the table 1 because at least half of the used semen in one year was frozen in the previous winter, the rest was stored for 1 or more years, so modification of the freezing protocol and correction of sperm loss could have a moderate effect the first year on the fertility.
[b] Total number of spermatozoa ($x10^6$) per AI x interval between AI. Example: 300 & 150 x 24 h: first AI with $300x10^6$ total sperm, then $150x10^6$ 24 h later and every 24 h until ovulation.
[c] It is not known to which extent the recommendations were followed by veterinarians and technicians.
[d] Number of National Stud insemination centres+ number of private insemination centres
[e] Missing data

Table 3
Post-thaw motion characteristics of spermatozoa frozen after 3 protocols of the first dilution (Experiment 2)[a].

First extender Freezing extender	E1 [bc] E2 [bc]	E1S [bd] E2S [bd]	INRA82 E2 [c]	Milk [e] E2 [c]	Pooled S.E.M.
Motility (%)	46	45	48	48	2
VAP (µm/sec) [f]	76	78	77	79	0.9
Viability CFDA/IP (%) [g]	56	58	54	59	15

[a] Freezing protocol: first dilution at 37°C in various centrifugation extenders, cooling to 22°C and second dilution after centrifugation in freezing extender at 22°C before moderate cooling to 4°C and freezing. Values are means of 10 split ejaculates (5 stallions x 2 ejaculates)
[b] E1 : INRA82 + egg yolk, 2%, v/v; E2 : E1 + glycerol, 2.5%, v/v.
[c] E1 and E2 made like usually with liquid UHT (Ultra Heat Temperature) skim milk.
[d] E1S et E2S: E1 and E2 made with non fat dry milk (Sanalac TM).
[e] Milk: UHT (Ultra Heat Temperature) skim milk.
[f] VAP: average path velocity.
[g] Viability was determined with flow cytometry after staining with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI).

Table 4
Post-thaw motion characteristics and fertility of spermatozoa frozen after 2 protocols of the first dilution (in either E1 or in milk) (Experiment 3) [a].

	E1 [b]			Milk [c]			Pooled SEM
	No. of ejaculates	No. of cycles	Means	No. of ejaculates	No. of cycles	Means	
Winter 2001: 21 stallions							
Motility (%)	102		54	127		55	1
VAP (µm/sec)			93			92	0.9
Winter 2002: 104 stallions							
Motility (%)	867		52	922		52	1
VAP (µm/sec)			84			84	0.5
No. straws / ejaculate			91 a			84 b	2
Breeding seasons 2001 & 2002: (23 stallions x breeding seasons)							
Per-cycle pregnancy rate (%)		353	49		335	48	

[a] Different letters within a row denote differences ($P<0.05$). Either E1 or milk was used as centrifugation extenders. Freezing protocol was first dilution at 37°C in centrifugation extender, cooling to 22°C and second dilution after centrifugation in freezing extender E2 at 22°C before moderate cooling to 4°C and freezing.
[b] E1: INRA82 + egg yolk, 2%, v/v; E2 : E1 + glycerol, 2.5%, v/v.
[c] Milk: UHT (Ultra Heat Temperature) skim milk.
[d] VAP: average path velocity.

Table 5

Per-cycle pregnancy rates with frozen semen after two protocols of insemination[a].

	Protocol of insemination	
Number of total spermatozoa (10^6)		
First AI	300	300
Subsequent AIs	150	300
Interval between AIs until ovulation	24h	24h
Per-cycle pregnancy rate (%)		
Breeding season 1994	33 (n=40)	47 (n=36)
Breeding season 1995	33 (n=131)	45 (n=129)
Total	33 b (n=171)	45 a (n=165)

[a] Different letters within a row denote differences ($P<0.05$). Freezing protocol was 37E1/4E2: first dilution at 37°C in E1, moderate cooling to 4°C and second dilution after centrifugation in freezing extender E2 at 4°C before freezing. E1 : INRA82 + egg yolk, 2%, v/v; E2 : E1 + glycerol, 2.5%, v/v.

Table 6
Present French system: freezing protocol 37Milk/22E2 and insemination strategy.

Semen collection	Frequency	3 times / week
	Months	October to February
Centri-fugation	Centrifugation extender	UHT (Ultra Heat Temperature) skim milk at 37°C
	Sperm numbers	in general $2.5x10^9$ spermatozoa / 50 ml-tube
	Total volume (semen + milk)	45 ml (maximum 15 ml semen)
	Cooling until centrifugation	in water-bath at 22°C for 10 min
	Centrifugation	600 g x 10 min at ambient temperature
	Pellet volume	3.5 ml (until corner of the conical tube)
Addition of freezing extender	Freezing extender	INRA82 + egg yolk (2%, v/v) + glycerol (2.5%, v/v)
	1st addition of extender at 22°C	about half of the calculated volume
	Estimation of spz concentration	with haemocytometer
	2nd addition of extender at 22°C	adjustment to $100x10^6$ spermatozoa/ml
	Final glycerol concentration	1.2 to 2.3 (mean 2.1%)
	Final seminal plasma concentration	1 to 15 (mean 4.4%)
Cooling and freezing	Cooling to 4°C	in 50-ml tubes (containing variable volume) for 1h 15 min
	Straws volume	0.5 ml, loaded at 4°C
	Stay at 4°C	3 to 4 ejaculates are frozen together in the same machine, so sperm could wait at 4°C 0 to 60 min
	Freezing	- 40°C/min to - 60°C/min, until -140°C, then in liquid N2
Selection of ejaculates	Selection of ejaculates	if rapid motility ≥ 35% on ≥ 2 separated straws diluted in INRA82 (1v/5v)
Straws and Mare management	No. of straws per mare per season	48 straws ($2\ 400x10^6$ total sperm)
	Frequency of scanning mares	every day when follicle ≥ 30 mm
	Thawing	30 sec in water bath at 37°C, no further preparation of semen
	Site of insemination	into the uterine body
	Number of total spz / AI	400×10^6 (8 straws)
	Number of AI / cycle	2
	AI frequency	every day until ovulation, no AI post-ovulation

Table 7

Production of frozen semen in the French National Stud (2003-2005)[a].

	2003	2004	2005
Number of stallions	133	117	114
Frozen ejaculates:	1931	1873	1802
Rapid motility (%)	51	51	49
Progressive motility (%)	39	40	37
Selected ejaculates after thawing :			
Rapid motility (%)	55	55	55
Progressive motility (%)	42	42	42
Rate of selected ejaculates (%)	86	88	81
Number of straws / frozen ejaculates	84	86	78
Number of mares per season (48 straws)	2857	/	2375
Ratio: mares per season / frozen ejaculates	1.5	/	1.4
Stallions with unfreezable semen [b] (%)	9%	6%	15%

[a] Freezing protocol: 37Milk/22E2, first dilution at 37°C in milk (UHT (Ultra Heat Temperature) skim milk), cooling to 22°C and second dilution after centrifugation in freezing extender E2 at 22°C before moderate cooling to 4°C and freezing. E2: INRA82 + egg yolk (2%, v/v) + glycerol (2.5%, v/v).

[b] Stallions with less than 33% post-thawing selected ejaculates.

Table 8

Proposition of codes associated with frozen semen offer for a mare per season.

Codes for minimal number of spermatozoa for the breeding season	
A***	≥3 x 750.10^6 total sperm i.e. ≥2.25 x 10^9 total sperm in ≥6 straws (for 3 cycles)
A**	≥2 x 750.10^6 total sperm i.e. ≥1.5 x 10^9 total sperm in ≥4 straws (for 2 cycles)
A*	≥1 x 750.10^6 total sperm in ≥2 straws (for 1 cycle)
B	Other offers or unspecified number
Codes for minimal post-thawing motility (total or rapid)	
A	≥35 %
B	< 35% or unspecified
Codes for maximal final glycerol concentration	
A	< 4 % (v/v)
B	≥4 % (v/v) or unspecified

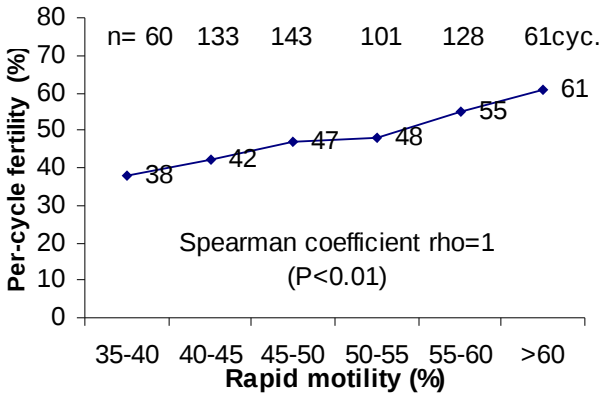
Frozen semen offer is coded with 3 letters, based on total sperm number available per mare and per season (first letter), on post-thaw total or rapid motility (second letter) and on final glycerol concentration (third letter). For instance, a frozen semen offer A***AA is very good technically (in quantity (sufficient number for 3 cycles) and in quality), an offer BAA indicates that sperm is very good in quality but that the number of spermatozoa are lower than the minimum number of spermatozoa required for one cycle and an offer A***AB indicates that enough spermatozoa are provided for 3 cycles, with good motility but with unspecified or too high glycerol concentration.

Table 9

Information form on frozen semen.

Name and address of the collection and the freezing centre:
Name and identification (registration no.) of the stallion:
Date of collection (and number if more than one collect/day):
Identification of the straws:
Volume per straw (ml):
Concentration of sperm (millions of spermatozoa (10^6) per ml):
Total number of sperm per straw (millions of spermatozoa (10^6):
Motility of the ejaculate after freezing and thawing:
Total number of motile sperm (≥35%) per straw:
Final concentration of glycerol in the straw:
Thawing protocol for the straws:
Location of frozen semen within the container:

Figure 1



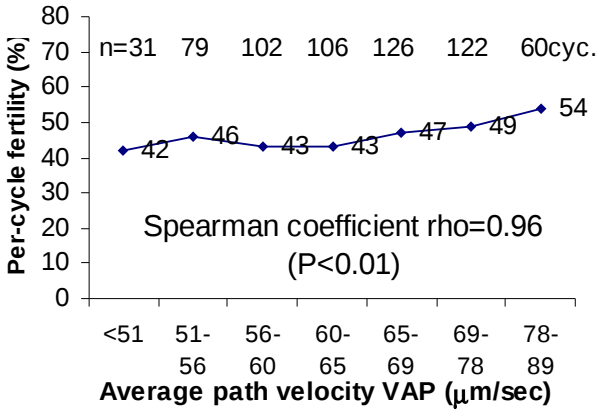


Figure 1
Per-cycle pregnancy rates with equine frozen semen categorized by motion characteristics of spermatozoa after thawing (rapid motility and average path velocity (VAP)).

[a] Each cycle of breeding season 1999, positive or negative after a pregnancy diagnosis, was associated with the values of 2 criteria measured on the corresponding ejaculate during the control analysis made at selection. Then, for each criteria, lines of the file were ranked by increasing value of this criterion and assembled in classes with more or less the same number of cycles. Then fertility was calculated by class (positive cycles / total cycles of the class) and correlation between class value and fertility was calculated using the non parametric coefficient of Spearman between center of the class and fertility. The number of cycles within each class is indicated above each point (n, cyc.).

Figure 2

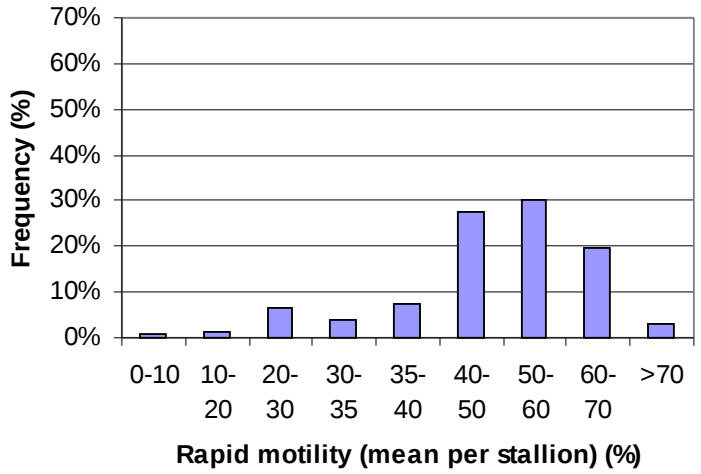


Figure 2
Distribution of the mean post-thaw rapid motility per year of 344 stallions (2003 to 2005)
Freezing protocol 37Milk/22E2: first dilution at 37°C in UHT (Ultra Heat Treatment) skim milk, cooling to 22°C and second dilution after centrifugation in freezing extender E2 at 22°C before moderate cooling to 4°C and freezing. E2: INRA82 + egg yolk (2%, v/v) + glycerol (2.5%, v/v).

Figure 3

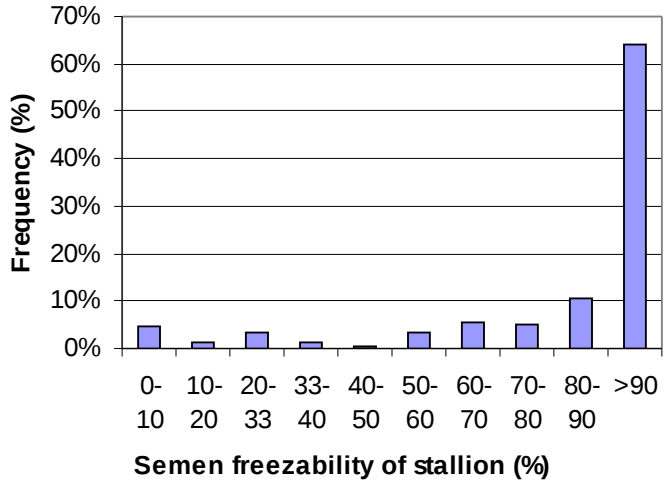


Figure 3
Distribution of semen freezability of 344 stallions (number of selected ejaculates / number of frozen ejaculates for a given stallion per year) (2003 to 2005).
Freezing protocol 37Milk/22E2: first dilution at 37°C in UHT (Ultra Heat Treatment) skim milk, cooling to 22°C and second dilution after centrifugation in freezing extender E2 at 22°C before moderate cooling to 4°C and freezing. E2: INRA82 + egg yolk (2%, v/v) + glycerol (2.5%, v/v).