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ABSTRACT For the past 50 yr, practices for ex situ preservation of endangered breeds have been extended. Semen and primordial germ cells, gonadic tissues have been frozen to create genetic stocks of chicken genetic diversity in cryobanks. Semen cryopreservation stays the preferred method since it is not invasive. Many protocols have been developed to cryopreserve chicken semen, but they give highly variable success rate. The aim of the present study was to standardize and prove the effectiveness of semen long-term storage for the restitution of lost families. We showed that semen straws stored for 18 yr in liquid nitrogen did not lose their fertilizing ability. We demonstrated the usefulness of cryopreserved semen stored in the French National Cryobank for the recovery of families of a subfertile experimental chicken line. In order to highlight the standardization of the cryopreserved method, different cryoprotectant protocols were also tested on a rare breed, freezing/thawing and insemination conditions were controlled. The best results were obtained using glycerol protocol, a sperm dilution of 1:4 (semen:extender). The insemination dose of 200 million sperm/female was as efficient as 400 million of sperm. Altogether, these results demonstrated the effectiveness of chicken semen long-term storage for the restoration of lost genetic resources and highlighted the importance of standardized chicken semen cryopreservation using procedures combining biophysical (cryoprotectants, freezing/thawing conditions) and zootechnical (artificial insemination) features.

Key words: cryopreservation, chicken, fertility, genetic resources

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INTRODUCTION

Farm Animal Genetic Resources conservation is an important challenge in order to maintain domestic biodiversity and adaptation of animal species to global changes or to breeding accidents or epidemics (Mara et al., 2013; Joost and Bruford, 2015). The genetic of many local or endangered breeds/species, with small population sizes, may be stored under the form of cryopreserved reproductive cells (FAO, 2013; Rakha et al., 2016; Svoradova et al., 2018). In breeding farming, reproductive cells cryopreservation represents also a security for the animal genetic and may be a useful tool to contribute to the measurement of genetic progress. Livestock conservation practices have evolved in the past 50 yr, allowing the development of new practices of conservation of reproductive cells in Cryobanks. In the past decade, many programs for ex situ in vitro preservation of endangered breeds through reproductive cells have been developed in many countries (Europe, North America, Africa or Asia) (Blesbois et al., 2007; Blesbois, 2011; Paiva et al., 2014; Biscarini et al., 2015; Purdy et al., 2015; Paiva et al., 2016; Alebachew et al., 2017; Liu et al., 2018). Different reproductive cell types are now stored in cryobanks: mainly semen and embryos in mammalian species, semen and primordial germ cells in birds, semen in fish, semen and larvae in shellfish. Some cryobanks include also somatic cells with the hope that, in the future, these cells will be able to be reprogrammed in efficient reproductive cells (Sekita et al., 2016; Canovas et al., 2017; Amini Mahabadi et al., 2018). In France, the National Cryobank of Domestic Animals contains reproductive cells and somatic tissues of avian, mammals, fish or shellfish conserved as semen, embryos or larvae depending on the species (http://www.cryobanque.org).

Progress in reproductive physiology knowledge and biotechnologies have made possible the extension of the range of species available in cryobanks. However, many cryobanks are used to store cells but not to distribute genetic resources. The reproductive potential of cells stored in cryobanks is also often not evaluated (Blesbois, 2012).

In poultry, semen cryopreservation represents the most popular method for the ex situ in vitro

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conservation of avian genetic resources and preservation of rare breeds since it is the only non-invasive and the less expensive in vitro method available up to day (Long, 2006; Blesbois, 2011; Ehling et al., 2012). It is actually not possible to conserve intact embryos in avian species because of the structure of the telolecithal bird eggs. Methodologies using primordial germ cells and gonadic tissues cryopreservation have been developed (Silversides et al., 2012; Liptoi et al., 2013; Nakamura et al., 2013; Nakamura, 2016; Sztan et al., 2017). Despite an increasing interest for these last methods and constant improvements in their developments (Nakamura, 2016; Nandi et al., 2016), these technologies stay still highly invasive and more expensive than semen methodologies.

In the past 15 yr, many studies have been performed to improve methods for bird sperm reproductive potential conservation after freezing-thawing. Cryopreservation is known to be highly stressful for sperm that share a very specific function (fertilization). During poultry semen cryopreservation process (including the cooling-freezing and the thawing procedures), osmotic and thermic shocks may cause damages to cells structure and metabolism (Long, 2006). Many studies were performed to define the best conditions of freezing to avoid cell damages and preserve fertilization ability. Internal (glycerol, dimethyl-sulphoxide, dimethyl formamide (**DMF**), dimethyl acetamide (**DMA**), ethylene-glycol) and external (polyvinyl-pyrrolidone, sucrose, trehalose) cryoprotectants (**CPAs**); cooling and thawing rates; freezing methods and straw/pellet packaging are key points (Seigneurin and Blesbois, 1995; Chalah et al., 1999; Blesbois and Labbe, 2003; Massip et al., 2004; Blesbois et al., 2005; Partyka et al., 2012; Rakha et al., 2016; Seshoka et al., 2016; Abouelezz et al., 2017; Khiabani et al., 2017; Lotfi et al., 2017; Rakha et al., 2017a,b; Miranda et al., 2018). Addition of antioxidants such as vitamin E, selenium (Blesbois et al., 1993; Ebeid, 2012), or hyaluronic acid (Lotfi et al., 2017) to semen extenders was also reported to counteract the negative effect of reactive oxygen species produced during cryopreservation process (Surai et al., 1998a,b; Rui et al., 2017). A recent study demonstrated the positive impact of Metformin (**MET**), an antidiabetic inhibitor of complex 1 and activator of 5'-AMP-Activated Protein Kinase (AMPK), to increase the viability and the motility of frozen/thawed chicken sperm in vitro through an activation of antioxidant enzymes of the respiratory chain (Nguyen et al., 2015). In poultry species, sperm freezing has been mainly developed in chickens and was then applied to turkeys, drakes, ganders or guinea fowl (Tselutin et al., 1995; Tai et al., 2001; Blesbois et al., 2007; Blesbois, 2012; Seigneurin et al., 2013; Santiago-Moreno et al., 2017). The success of freezing procedures is also highly variable depending on genetic line, zootechnical conditions and mostly the know-how of each lab. This led to the development of many different methods. However, these multiple protocols are not routinely used in poultry breeding. They still need to be standardized and to prove their efficiency to restore lost genetic.

In this context, our aim was to improve the semen cryopreservation management for long-term conservation and for use to restore lost families. We standardized the chicken sperm cryopreservation procedure used for the storage of the genetic of very different lines and breeds. We tested different established protocols with their own internal CPA (glycerol, DMA, DMF, or ethylene glycol (EG) (Schramm, 1991; Seigneurin and Blesbois, 1995; Tselutin et al., 1999; Varadi et al., 2013)). We also tested added factors: semen dilution rate, MET addition to the extender, and artificial insemination (**AI**) dose for the use of chicken semen cryopreserved in straws. In order to evaluate the final use of cryopreserved semen, we tested the impact of long-term semen storage (18 yr) on the fertility potential. We also tested the use of semen straws from a subfertile genetic line (R+ experimental line with defective mitochondrial metabolism, low residual consummation (Bordas et al., 1992; Ladjali et al., 1995; Morisson et al., 1997)) in order to evaluate if semen freezing could be efficiently used to restore lost families of a subfertile line.

MATERIALS AND METHODS

Animal Housing and Management

Adult chickens were housed at the INRA experimental unit UE-PEAT (Nouzilly, France). Their breeding followed the European welfare and the French Direction of Veterinary Services regulations (agreement number C37-175-1). A total of 33- to 40-wk-old males were obtained from commercial pedigree stocks (free range T55 from SASSO, Sabres, France; meat type males I99 from Hubbard, Quintin, France), experimental line D+/D- (Peron et al., 2006) and R+ (Bordas et al., 1992; Ladjali et al., 1995; Morisson et al., 1997), and the rare local breed "Geline de Touraine" (Baeza et al., 2009). They were housed in individual battery cages under 14 L/10 D photoperiod and fed with a standard diet of 12.5 MJ/d. Females used for AI were 29- to 42-wk-old ISABROWN hens (ISA, Ploufragan, France) housed in battery cages by groups of 5 or 6 females or R+ subfertile line housed individually. All animals were under a 14 L/10 D photoperiod and fed a standard diet of 12.5 MJ/d. The females' diet was supplemented with calcium.

Semen Collection, Cryopreservation, and Thawing

Semen was collected by massage using the procedure described by Burrows (Burrows and Quinn, 1937). Care was taken to avoid semen contamination with cloacal products. Pools of semen were diluted with 1 or 2 mL of extender at room temperature depending on the experiment. Composition of the 3 extender employed,

Table 1. Different cryoprotectants and their methods of use for rare breed semen storage.

Internal cryoprotectant	GLY	EG	DMF	DMA
Extender	Lake PC	BHSV	BHSV	FEB
External cryoprotectant	PVP	Myo inositol	Myo inositol	PVP
Semen collection	1 mL semen in 1 mL extender	1 mL semen in 1 mL extender	1 mL semen in 1 mL extender	1 mL semen in 1 mL extender
Cooling	4°C—15 min	4°C—15 min	4°C—15 min	4°C—15 min
% Internal CPA	11%	10%	6%	6%
CPA addition	in 1 mL extender	in 1 mL extender	in 1 mL extender	in 1 mL extender
Equilibration time	10 min	10 min	4 min	2 min
Freezing rate	–7°C/min	–1/min	–15°C/min	–60°C/min
Thawing (°C -min)	4°C—3 min	4°C—3 min	4°C—3 min	40°C—5 s
Glycerol removal	Dilution 1/20 550 g—4°C—15 min			
No. female/treatment	20	20	20	20
IA dose	400 × 10^6 spz	400 × 10^6 spz	400 × 10^6 spz	400 × 10^6 spz
No. of AI	5	5	5	5
Fréquence d'AI	Every 4 d	Every 4 d	Every 4 d	Every 4 d
Egg collection (day post AI)	J2 to J5	J2 to J5	J2 to J5	J2 to J5

GLY = glycerol; EG = ethylene glycol; DMF = dimethyl formamide; DMA = dimethyl acetamide; PVP = polyvinylpyrolidone; AI = artificial insemination; CPA = cryoprotectant agent.

Lake PC, BHSV, FEB, was previously described (Lake and Ravie, 1981; Schramm, 1991; Tselutin et al., 1999; Seigneurin et al., 2013). Each extender corresponded to a specific internal CPA: glycerol with Lake PC, DMF with BHSV, DMA with FEB. Some extenders also contain an external CPA (PVP for Lake PC and FEB), or an antioxidant (Myo-inositol for BHSV).

Conditions to test different methods of cryopreservation with various CPA were described in Table 1. Briefly, semen and extender were gently mixed and cooled to +4°C for 15 min. At the same time, the extender containing the internal CPA agent (final concentration: 11% glycerol, 6% DMA or DMF, and 10% EG) was equilibrated at +4°C. Extender with CPA was added to samples and then equilibrated at +4°C (time linked to CPA used) with gentle agitation. After equilibration, semen was transferred in 0.5 mL plastic freezing straws (IMV Technologies, L'Aigle, France), which were sealed. The straws were placed in a biological freezer unit (MiniDigitCool, IMV Technologies) and frozen with different controlled freezing rate according to the experiment and the treatment (details in Table 1), and then the straws were plunged into liquid nitrogen.

For glycerol removal, straws were thawed in a water bath at +4°C for 3 min then were cut and semen was transferred to a glass beaker. Semen was progressively diluted (5 times each 2 min) with Lake C (Lake and Stewart, 1978) at +4°C with gentle agitation to a final dilution of 1:20. Glycerol was removed by centrifugation (15 min at 500 g at +4°C). The supernatants were discarded, and pellets were resuspended in 100 μL of Lake 7.1 (Lake et al., 1981).

To thaw straws prepared without glycerol as CPA, straws were plunged into a water bath with conditions (temperature and time) previously defined, according to the CPA used (Table 1).

Sperm concentrations were immediately determined after thawing by light absorption of semen with a photometer (Accucell photometer, IMV Technologies) at a wavelength of 530 nm (Brillard and McDaniel, 1985) in order to standardize the insemination semen dose.

For dilution rate, insemination dose, MET assay, length time of storage assay, R+ families restoration experiments, only freezing using glycerol protocol was used.

Artificial Inseminations

Intravaginal AI (4 cm depth) were done with fresh or frozen-thawed semen. The AI number, sperm doses are shown in results section. Eggs were collected from days 2 to 5 after each insemination. Fertilization rates (fertile eggs/incubated eggs × 100) were determined by candling the eggs at 7 d after the start of incubation.

For the R+ families restoration, hatchability (hatched/fertile eggs) was measured at hatching.

For AI using fresh semen, semen was collected onto 200 μL of BPSE extender (Sexton, 1977). Sperm concentration was determined as previously described before AI.

Statistical Analysis

Statistical analysis for multiple comparisons was performed using analysis of variance followed by Fisher's LSD. Percentages were transformed to arcsine square-root before analysis. Pairwise comparisons were performed using paired *t*-tests. Data are represented as means ± SEM. The level of significance of the differences between experimental conditions (fertility means) was set at a P value of 0.05.

Table 2. Long-term storage in liquid nitrogen.

Freezing/AI	AI 1	AI 2	AI 3	AI 4	AI 5	Mean fertility
1995/1996	36.2 (50/138)	62.3 (81/130)	56.8 (71/125)	64.1 (83/135)	52.5 (73/139)	53.7 ± 4.7[a] (358/667)
1995/2013	49.1 (58/118)	60.0 (66/110)	51.6 (66/125)	51.7 (62/120)	47.8 (55/115)	52.2 ± 2.1[a] (307/588)

AI = artificial insemination. Eggs collected from days 2 to 4 after each AI.
Different superscripts indicate significant difference between treatments ($P < 0.05$).

Table 3. Restoring genetic diversity in the R+ experimental line.

	Family 1	Family 2	Mean
Fertility (%)	16.2%	1.8%	8.7%
No. of fertile/incubated eggs	25/154	3/169	28/323
Hatchability (%)	84%	67%	92%
No. of hatched/fertile eggs	21/25	2/3	23/25
No. of male/female	8/13	2/0	10/13

Eggs collected from days 2 to 15 after the first AI.

Table 4. Effect of methods with different internal cryoprotectant on the fertility ability of frozen chicken sperm.

	AI 1 and 2	AI 3	AI 4	AI 5	Fertility mean
GLY	84.6 (66/78)	83.8 (57/68)	88.9 (56/63)	75.8 (50/66)	83.3 ± 2.8[a]
EG	1.3 (1/75)	3.2 (2/62)	0 (0/63)	1.6 (1/63)	1.5 ± 0.7[d]
DMF	44 (37/84)	65.7 (44/67)	71.2 (47/66)	78.1 (50/64)	64.8 ± 7.4[b]
DMA	26.5 (22/83)	40.6 (26/64)	43.5 (30/69)	31.2 (20/64)	35.3 ± 3.9[c]

GLY = glycerol; EG = ethylene glycol; DMF = dimethyl formamide; DMA = dimethyl acetamide; AI = artificial insemination. Eggs collected from days 2 to 5 after the first AI.
Different superscripts indicate significant differences between treatments ($P < 0.05$).

RESULTS

Effect of Long-term Storage of Chicken Semen Straws in Liquid Nitrogen

Semen from I99 commercial males were collected and cryopreserved in 1995 using the glycerol protocol and then thawed and inseminated in 1996 or in 2013 in Isabrown females. Insemination conditions were the same in 1996 and in 2013 (i.e., same sperm dose of 400 million of sperm/female). Interestingly, the fertility obtained with semen frozen in 1995 was not significantly affected by 18 yr of storage in liquid nitrogen (Table 2; 52.2% in 2013 and 53.7% in 1996; $P < 0.05$).

Restoration of Genetic Families of The R+ line With Cryopreserved Semen

Semen of subfertile R+ chicken males were cryopreserved in 2003 using the glycerol protocol and stored in the French National Cryobank of Domestic Animals (Blesbois et al., 2007). After the death in 2016 of chicken males from 2 different genetic families, the chicken line was highly at risk. We used cryopreserved semen straws to restore animals. Semen straws of 6 males from these 2 lost families were selected to inseminate 5 to 6 sister females each (300 million sperm/female) at the end of the reproductive period (low laying rate 50%), 4 AI were performed. We obtained at least 23 chicks (10 males and 13 females) corresponding to the 2 genetic families. The fertility rates varied between 1.8% and 16.2% with a fertility mean of 8.7% for the 2 families (Table 3).

Comparison of Different CPA Protocols for Chicken Semen Cryopreservation

In this experiment, made on a local endangered breed "Geline de Touraine" (conditions described in Table 1), we compared the use of 4 protocols with different internal CPAs: glycerol, EG, DMF, and DMA combined with their original associated extender applied to chicken semen frozen in straws (Table 4). The highest fertility results (83%, $P < 0.05$) were obtained with the glycerol protocol despite the need of removal of this CPA at thawing. The second best method was the one using DMF (65%, $P < 0.05$). The DMA protocol showed low results (35%, $P < 0.05$) and the EG protocol was clearly not effective (1.5%, $P < 0.05$).

Comparison of 2 Sperm Dilution Rate During Freezing

In this experiment, achieved on free-range T55 breeder males, we tested the impact of the semen dilution rate (final dilution of 1:3 or 1:4) on fertility rate after AI with a constant sperm dose (400 million per female). The fertility rate (Table 5) was significantly higher (93%) when semen was cryopreserved at the 1:4 dilution rate compared to 1:3 (80%), and was very close to the fertility rate obtained with fresh semen (98.3%) ($P < 0.05$).

Evolution of Fertility Rate According to the Insemination Dose

In this experiment, tested on a different flock of T55 breeder males (semen with lower in vitro quality, i.e., mean motility, than the previous flock), we inseminated females with different doses of spermatozoa (400 or 200 million per female). The fertility rate was not significantly different when females were inseminated with

Table 5. Effect of different semen dilution rate on the fertility ability of chicken sperm frozen with glycerol method.

	Dilution rate	AI dose ($\times 10^6$ spz)	AI 1	AI 2	AI 3	AI 4	Mean fertility
Fresh semen							
Fertility (%)/	/	200	95.5	100.0	100.0	97.1	98.3 ± 1.1[a]
No. of fertile/incubated eggs			(21/22)	(31/31)	(29/29)	(33/34)	(114/116)
Frozen-thawed semen							
Fertility (%)	1:3	400	61.8	75.5	91.7	86.5	80.2 ± 6.6[b]
No. of fertile/incubated eggs			(21/34)	(40/53)	(44/48)	(45/52)	(150/187)
Frozen-thawed semen							
Fertility (%)	1:4	400	85.0	100.0	93.3	90.6	93.0 ± 3.1[a,b]
No. of fertile/incubated eggs			(17/20)	(32/32)	(28/30)	(29/32)	(106/114)

AI = artificial insemination. Eggs collected from days 2 to 4 after each AI.
Different superscripts indicate significant differences between treatments ($P < 0.05$).

Table 6. Comparison of semen insemination dose on fertility rate.

	Dilution rate	AI dose ($\times 10^6$ spz)	AI 1	AI 2	Mean fertility
Fresh semen					
Fertility(%)/	/	200	97.1	97.1	97.1 ± 0.0[a]
No. of fertile/incubated eggs			(33/34)	(34/35)	(67/69)
Frozen-thawed semen					
Fertility (%)	1:4	400	81.6	80.4	81.0 ± 0.6[b]
No. of fertile/incubated eggs			(40/49)	(41/51)	(81/100)
Frozen-thawed semen					
Fertility (%)	1:4	200	77.0	81.3	79.2 ± 2.1[b]
No. of fertile/incubated eggs			(47/61)	(52/64)	(99/125)

AI = artificial insemination. Eggs collected from days 2 to 4 after each AI.
Different superscripts indicate significant differences between treatments ($P < 0.05$).

400 and 200 million of spermatozoa (Table 6: 81.0% and 79.2%, respectively). We thus recommend the use of 200 million sperm for individual insemination of cryopreserved semen.

Effect of MET Supplementation and AI Dose on Fertility Rate

In this experiment, made with free range T55 males (same flock as in the last paragraph), we tested the influence of addition of 1 mM MET, an AMPK activator, to the freezing medium. We also estimated fertility after AI with 2 insemination doses, the optimal one (200 million of sperm/female) and a smaller dose (100 million of spermatozoa/female); in order to evaluate if 100 million sperm could finally be a sufficient insemination dose. The presence of MET 1 mM in the freezing medium had no significant effect on fertility (Table 7), but there was a clear tendency of higher efficiency of in vivo semen storage, seen between 7 and 11 d after a single insemination (Figure1). The insemination dose of 100 million sperm showed clearly lower results than 200 million, irrespective of the presence or lack of MET during semen freezing.

DISCUSSION

In the present study, we showed that a long-term storage of chicken semen cryopreserved with glycerol was efficient to keep fertility and restore lost families of a subfertile chicken line. We also highlighted different parts of the freeze-thaw process and of the management of cryopreserved sperm allowing optimization and standardization of it.

Since Polge et al. (1949) showed that chicken sperm could stay motile after freezing, a number of studies have been made to develop cryopreservation methods. In our lab, we started in 1995 and showed the efficiency of an improved adaptation of the freezing method using glycerol (Lake et al., 1981) in order to keep the highest fertilizing ability of chicken frozen-thawed sperm (Seigneurin and Blesbois, 1995). The same year we cryopreserved an experimental meat line (D+) with mean fertility in order to test the long-term efficiency of our method. In the present study, we show for the first time in the chicken that this efficiency is kept many years since inseminations made in the same conditions and on the same kind of females 18 yr later showed equivalent fertility rates. These results were expected since metabolic activity of the cells is very close to 0 in the liquid nitrogen at –196°C. The main risk during long years of storage is most often lost by accident of liquid nitrogen in the tank where semen straws are stored, increasing suddenly the temperature inside the containers that protect the sperm cells from increasing temperatures and sample loss. Shared programs of cryobanking with secured storage of cells avoid this problem. In our conditions of semen storage in the French National

Table 7. Metformin effect on chicken fertility after AI with cryopreserved semen.

	AI dose ($\times 10^6$ spz)	AI 1	AI 2	Mean fertility
Without metformin 1mM				
Fertility (%)	200	77.0	81.3	79.2 ± 2.1[a]
No. of fertile/incubated eggs		(47/61)	(52/64)	(99/125)
Fertility (%)	100	63.8	64.6	64.2 ± 0.4[b]
No. of fertile/incubated eggs		(44/69)	(42/65)	(86/134)
With metformin 1 mM				
Fertility (%)	200	78.8	86.6	82.7 ± 3.9[a]
No. of fertile/incubated eggs		(52/66)	(58/67)	(110/133)
Fertility (%)	100	61.4	70.8	65.9 ± 4.7[b]
No. of fertile/incubated eggs		(43/70)	(46/65)	(89/135)

AI = artificial insemination; Eggs collected from days 2 to 4 after each AI.
Different superscripts indicate significant differences between treatments ($P < 0.05$).

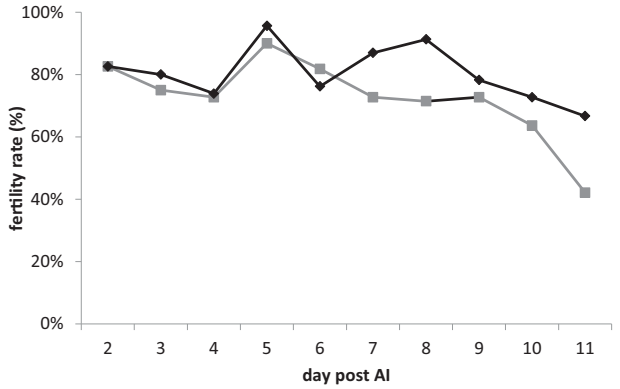

Figure 1. Metformin effect on chicken fertility after AI with cryopreserved semen fertility rate (%) obtained after a single artificial insemination with 200 million spermatozoa per female using frozen/thawed semen frozen with (♦) or without (■) Metformin 1 mM.

Cryobank of the Domestic animals, we have such security (Blesbois et al., 2007).

During the last 20 yr, many studies have been made through the world in order to develop chicken semen cryopreservation. Many attempts to simplify the methods and to adapt them to different breeds or subspecies were investigated. They gave rise to the development of a number of protocols that are still difficult to compare and to evaluate for a given breed. One of the main factors of change between these different methods is the internal CPA (Blanco et al., 2000, 2012; Seigneurin et al., 2013; Mosca et al., 2016). The main CPAs used to protect chicken sperm during the freeze-thaw process are DMA, DMF, EG, and glycerol. Specific extenders and freeze-thaw processes were developed for each CPA. In order to evaluate the best procedure for endangered breeds/lines that do not ever show the optimal fertility, we tested these different methods on a local endangered breed showing mean fertility, the "Géline de Touraine". Clearly, in the present study, the "glycerol" protocol shows the best conservation of sperm fertilizing ability, up to more than 90% fertility with highly fertile males. If these results comfort previous suggestions of our lab (Tselutin et al., 1999; Blesbois et al., 2007), they may differ from the results found by Abouelezz's lab (Abouelezz et al., 2015, 2017) and Rakha's lab (Rakha et al., 2016, 2017b). The team of Abouelezz found better results with DMA. However, they used a lower glycerol amount (8% vs.11% in our present study), different freezing conditions (N_2 vapors vs. standardization with programmable freezer with known and real-time controlled freezing temperature in our case), and pellets packaging (that cannot actually be employed in large cryobank programs for problems of sample identification and standardization). Thus, we cannot really compare our results with these obtained by Abouelezz's lab. The case of the studies made by Rakha's lab is different since they studied a wild subspecies of chicken, the Indian red jungle fowl in extensive conditions of use, and still without a programmable freezer. The reproduction of this last fowl seems very specific and the sperm of Indian Red Jungle Fowls show original reactions to different CPAs. They seem to adapt quite well to most of them (Rakha et al., 2016) with mean fertility after semen cryopreservation between 50 and 70%. In our study, we definitively cannot recommend DMA for a large use in domestic chicken semen freezing except in the cases of highly fertile males which semen is expected to resist to most of the freezing-thawing damages whatever the method employed. EG also is "out of the game" with a too low fertility rate with straws packaging. The case of DMF is different since we observed with the "Gélines de Touraine" males a potential acceptable degradation of the fertility results when compared to glycerol protocol (65% for DMF vs. 83% for glycerol). The protocol of semen freezing with DMF is simpler than the one using glycerol. Indeed, the glycerol needs to be removed after thawing and before insemination in order to avoid any contraceptive effect (Neville et al., 1971; Hammerstedt and Graham, 1992). In contrast, DMF removal is not necessary. However, for breeds of unknown fertility or for low fertility breeds and lines, we highly recommend the use of glycerol method. The experiment of restoration of the R+ experimental subfertile line is an illustration of this point. In 2007, mean fertility after AI with frozen semen from 8 different males of this line was 14% (Blesbois et al., 2007). In the present trial, with other

males of the same line and semen cryopreserved in 2003, we obtained at least 23 chicks. The mean fertility after insemination of "sisters" at the end of their reproductive period (low laying rate) was 8.7% that is still very low and not significantly different from our previous results ($P \leq 0.01$). These results show that it is possible to use frozen semen in order to restore families and to reintroduce genetic diversity even in an extreme case of low reproductive potential of the males and the females. The number of sperm straws stored in cryobanks for a given breed/line must take into account the reproductive potential (higher number of straws must be stored for low fertility lines/breeds).

In order to optimize the glycerol protocol that is expected to "save" the most difficult cases, we tested 2 other points of the freeze/thaw process: the semen dilution rate and the addition of MET to the freezing extender.

In the routine practice of semen freezing, we often noticed that a reduction of the dilution rate of the semen, from 1:4 to 1:3, was expected by the users for practical reasons (decrease of the number of straws, thawing of smaller number of straws for AI, decrease of the cost of freeze/thaw process). Since the difference between these 2 dilution rates was low and since reducing the dilution rate could decrease an expected unnecessary use of extra-extender, we decided to test it. However, the results of our study clearly showed that the higher dilution was better to obtain high fertility rate (93.0% and 80.2%, respectively), very close to fertility rate obtained with fresh semen (98.3%) in the presence case. Clearly, this difference of results between the 2 dilution rates was not expected since 1:3 and 1:4 did not seem to us to be so different. Our results indicate a high sensitivity of chicken sperm to the contact with the components of their surrounding environment. It is known that the glycogalyx that surrounds and protects the sperm at ejaculation is in lower amount in birds than in mammals (Froman and Engel, 1989; Tecle and Gagneux, 2015). This could contribute to explain the high sensibility of chicken sperm. An added suggestion is that the availability of CPA is higher with the 1:4 than with the 1:3 dilution rate allowing a higher protection level of sperm during the freezing process.

Cryopreservation is a non-physiological process that could alter structures and metabolism of spermatozoa and have consequences on motility, membrane integrity, and ATP content (Long, 2006). In order to improve the freezing process, we tested the addition of MET, an antidiabetic, also inhibitor of the complex I of the electron transport chain in cell respiration, and activator of AMPK (Zhou et al., 2001). This modulator was demonstrated to increase viability and motility of frozen chicken sperm (Nguyen et al., 2015). In our experiment, we used the same amount of MET (1 mM) and the conditions previously defined as optimal to obtain the best fertility rate. We showed no significant effect of the presence of MET in the freezing medium but a clear tendency to higher semen in vivo storage in the female tract (sperm storage tubules), as seen within the better maintaining of fertility after 6 d and farer from the single insemination. This result needs to be confirmed by the insemination of a larger number of females.

Finally, we re-examined the number of sperm to be inseminated in order to get the best fertility results after AI with cryopreserved chicken sperm. It has been demonstrated a long time ago that the number of sperm inseminated affects the number of sperm stored in the female sperm storage tubules and thus the level of fertility and the duration of the fertile period (Taneja and Gowe, 1962; Blesbois and Brillard, 2007). Optimal fertility was routinely obtained in the chicken with 100 to 200 million of spermatozoa per female using fresh semen (Brillard, 1986; Brillard et al., 1989). It is expected that half of the sperm die during the freeze-thaw process, and Lotfi et al. (2017) demonstrated that the AI dose of 100 million applied to cryopreserved semen induced low fertility rate (40%). On the other hand, we noticed that high insemination doses (500 to 700 million sperm, unpublished observations) were sometimes problematic with partial rejection of the semen by the female after insemination and by unexpected low fertility rate. The usual dose suggested for inseminating cryopreserved chicken sperm is 300 to 400 million per female. In order to rationalize the use of frozen-thawed sperm, we decided to test 200 and 100 million in our experimental conditions. We concluded that the fertility rate was not reduced when the dose decreased by half from 400 to 200 million, maybe due to the capacities of storage in the sperm storage tubules (Brillard, 1993), while 100 million was a suboptimal dose, but not so bad as expected. The low AI dose of 100 million should be an advantage for small chicken breeds for which only few semen straws would be available for AI but should not be a hindrance to get offspring. However, in all the other cases, we now recommend the AI dose of 200 million sperm for cryopreserved samples.

To conclude, we demonstrate here that semen straws stored for 18 yr in liquid nitrogen conserved their fertilizing ability. We also prove the usefulness of cryopreserved semen stored in the French National Cryobank to restore chicken families and increase genetic diversity of a highly subfertile chicken breed. Finally, we present an efficient standardization of the chicken freeze-thaw process. The method involves glycerol protocol, a dilution rate of 1:4, and an insemination rate of 200 million sperm/female.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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