Cryopreservation of semen from genetic resource chicken lines

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Abstract

The objective of the present study was to establish an effective semen freezing procedure to set up a cryobank for chicken breeds and genetic lines of special interest. The fowls used in the experiments originated from White Leghorn lines maintained as conservation flocks. In this study, different protocols were evaluated in terms of differences in the fertilization success. 1 A) Comparison of slow and fast freezing procedures using a cryoprotectant containing Mix 2 (1 part 6.5 % Dimethyl formamide + 2 parts 6.5 % Methyl acetamide). The slow freezing procedure in a programmable freezer included two steps from 0 °C to -35 °C with a freezing rate of -3 °C/min, and from -35 °C to -130 °C with a freezing rate of -50 °C/min. Fast freezing was carried out in a box by exposing the straws to liquid nitrogen vapour at 4 to 4.5 cm above liquid nitrogen surface (-40 °C/min). 1 B) Test of insemination procedures with one or two straws with semen frozen slowly with Mix 2, and 2) comparing three cryoprotectants Mix 1 (1 part 6.5 % DMF + 1 part 6.5 % MA), Mix 2 and MA only (6.5 %). The base extender was the trehalose containing HS1-diluent to Hanzawa et al. (2006). The cryoprotectant-diluted semen was packaged in 0.25 ml plastic straws after dilution to a standardized number of spermatozoa (300 million/straw). All samples were thawed in a water bath at 4 °C. The females were inseminated intravaginal with a dose of 300 million (1 straw) or 600 million sperm (2 straws). The respective results were: the slow freezing procedure was superior to fast freezing and resulted in 49.3 % compared to 11.5 % fertility. There was no significant fertility difference between inseminations with one (43.6 %) and two straws (52.1 %). The fertility of both Mix 1 (77.3 %) and Mix 2 (81.1 %) showed higher fertilization rate than MA only (39.8 %). In conclusion, the slow freezing procedure with Mix 2 media is an applicable method for establishing a sperm cryobank in chicken genetic resource populations.

Keywords: Chicken, semen, cryopreservation, insemination, cryobank, ex situ conservation

Zusammenfassung

Kryokonservierung von Hahnensperma von Genreservelinien

Ziel der vorliegenden Untersuchungen war die Etablierung einer Einfriermethode für Hahnensperma, um eine Spermabank für genetisch wertvolle Linien aufbauen zu können. Für die Experimente wurden eigene Genreservelinien verwendet, die seit Jahren am Institut in situ gehalten werden. Folgende Versuche wurden durchgeführt: 1 A) Vergleich von langsamer und schneller Einfrierrate unter Verwendung des Gefrierschutzmittels Mix 2 (1 Teil 6,5 % Dimethylformamid + 2 Teile 6,5 % Methylacetamid); 1 B) Vergleich von zwei Besamungsdosen mit Mix 2 tiefgefrorenem Sperma; 2) Vergleich der Kryoprotektiva Mix 1 (1 Teil 6,5 % DMF + 1 Teil 6,5 % MA), Mix 2 und MA (6,5 %). Als Basisverdünner wurde der Trehalose haltige Verdünner nach Hanzawa et al. (2006) eingesetzt. Das Sperma wurde in 0,25 ml Straws abgefüllt, je Straw 300 Millionen Spermien. Das langsame Einfrieren erfolgte in einem Einfrierautomaten in zwei Schritten. Von 0 °C bis -35 °C fiel die Temperatur um -3 °C/min und von -35 °C bis -130 °C um -50 °C/min. Beim schnellen Einfrieren wurden die Proben in einer Styroporkiste im Stickstoffdampf 4 bis 4,5 cm über dem flüssigen Stickstoff eingefroren. Die Einfrierrate betrug -40 °C/min. Die Proben wurden im Wasserbad bei 4 °C aufgetaut. An jeden Einfrierversuch schloß sich ein Besamungsversuch an. Die Hennen wurden intravaginal mit dem Inhalt von einem bzw. von zwei Straws besamt. Folgende Ergebnisse wurden erzielt: 1 A) Das langsam eingefrorene Sperma befruchtete signifikant besser als das schnell eingefrorene (Befruchtungsrate von 49,3 zu 11,5 %); 1 B. Die Besamung mit zwei Straws erhöhte im Vergleich mit einem Straw die Befruchtungsrate nicht signifikant (52,1 zu 43,6 %); 2) Den höchsten Anteil befruchteter Eier erzielte Mix 2 (81,1 %), gefolgt von Mix 1 (77,3 %) und MA (39,8 %). Somit erwies sich das Mix 2-Medium in Verbindung mit langsamem Einfrieren als gut geeignet, eine Spermabank für genetische Ressourcen aufzubauen.

Schlüsselworte: Kryokonservierung, Hahnensperma, Genbank, Ex situ Erhaltung

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Introduction

Ex situ preservation of endangered breeds is a crucial part of measures to preserve genetic diversity in chickens as in other farm animal species. Up to now, cryopreservation of semen represents the only efficient method for the ex-situ management of avian genetic resources. So far, three national avian germplasm cryobank programs exist operating in North America, The Netherlands and France (Blesbois, 2006; Blesbois et al., 2008). All of them store mainly semen and blood samples. Compared to livestock species, chicken semen in general seems to react differently in the freezing process. This is obviously due to some unique physiological characteristics (Long, 2006). A good post-thaw survival rate of rooster semen is obtained with glycerol as cryoprotectant. However, glycerol acts as a contraceptive and should not exceed 0.7 % (v/v; 0.1 M) prior to insemination (Long and Kulkarni, 2004). Other successful techniques to freeze chicken semen use dimethylformamide (DMF), dimethylacetamide (DMA) or N-methylacetamide (MA) as cryoprotectants in connection with both fast and slow freezing rates (Tselutin et al., 1999; Woelders et al., 2006; Hanzawa et al., 2006; Schramm, 2008; Sasaki et al., 2010).

The aim of the present study was to establish an efficient semen cryopreservation system as a methodological prerequisite to create a semen cryobank for conservation of chicken resource populations in Germany.

Material and methods

Housing and Environment

The experimental design is shown in Table 1. Three chicken lines of White Leghorn (R11, M11, and R22) background were used for semen collection and females of line R22 for the insemination trials. The lines have been maintained at the Institute of Farm Animal Genetics Neustadt-Mariensee of FLI as genetic resources for many years. Hatching eggs of these lines were brought to the former Institute of Small Animal Breeding in Celle in 1965. More details about the origin of these lines have been reported elsewhere (Hartmann, 1997). For more than 20 generations, populations have been managed as closed breeding populations composed of 10 sire families, each made up by 1 sire and 10 dams. In every generation, male offspring have systematically been rotated across families via artificial insemination with no selection, and a generation interval of one year. The animals were kept in individual cages, and provided with a commercial layer feed (Agravis Mischfutter Ostwestfalen-Lippe GmbH) and water ad libitum. During the period of the study, the animals received 14 h of light per day starting at three o'clock in the morning. Roosters and hens were 28 to 36 weeks old at the beginning of the experiments.

Table 1:
Experimental design

	Experiment 1		Experiment 2
	A	B	
Cryoprotective agents (CPA)	Mix 2	Mix 2	MA, Mix 1, Mix 2
Freezing method	fast or slow	slow	slow
Insemination dose	1 straw	1 or 2 straws	1 straw
Roosters			
hatching date		28-10-2009	
genetic lines		R22, R 11, M11	
number		11	
Semen collection period	June 2010	August 2010	February 2011
Hens			
hatching date	28-10-2009		15-12-2011
genetic line		R22	
number	49	44	34
Insemination period	July 2010	September 2010	July 2011

Experiment 1 A. Comparison of fast and slow freezing

Ejaculates of 11 White Leghorn roosters of three lines (R11, M11, and R22) were pooled and diluted 1:2 at room temperature with HS1 extender. The extender was prepared as described by Hanzawa et al. (2006): Sodium glutamate (H_2O) 1.2 g, potassium acetate (anhydrous) 0.3 g, trehalose 3.8 g, glucose (anhydrous) 0.2 g, N,N-Bis(2-hydroxyethyl)-2-aminoethansulfonic acid (BES) 0.5 g, Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-tris) 0.5 g, gentamicin sulfate 0.001 g per 100 ml distilled water, osmolarity 360 mOsm/kg, pH 6.8. After 60 minutes at 5 °C, ejaculates were further diluted by mixing one part of prediluted semen with one part of freezing extender Mix 2. The composition of the cryodiluents is shown in Table 2. The diluted sperm contained 6.5 % cryoprotectant in the final concentration. The semen was diluted to a standardized number of 1200 million spermatozoa per ml before freezing. Consequently, in each 0.25 ml straw 300 million sperm were included. For slow freezing procedure, a programmable freezer (Ice Cube 14S, Minitube, Tiefenbach, Germany) was used. The start temperature was 0 °C, followed by a 2-step freezing method with a cooling rate of -3 °C/min until -35 °C in the first step, and cooling rate of -50 °C/min until -130 °C. Fast freezing was carried out in a box by exposing the semen samples to liquid nitrogen vapour at 4 to 4.5 cm above the surface of liquid nitrogen. In this one-step freezing procedure the

temperature declined with -40 °C/min. Afterwards semen samples frozen with both procedures were placed in liquid nitrogen for long-term storage.

Table 2:
Composition of rooster sperm cryodiluents (ml)

	MA-diluent	DMF-diluent
Base diluent HS-1	87	87
Methyl acetamide (MA)	13	
Dimethyl formamide (DMF)		13

Mix 1- diluent: mixing one part MA-diluent with one part DMF-diluent
Mix 2- diluent: mixing two parts MA-diluent with one part DMF-diluent

Experiment 1 B. Comparison of one and two straws for insemination

The same roosters as in experiment 1 A were used (11 ejaculates per pool). Collection of semen, processing and examination were as described in experiment 1 A, and only the slow freezing method was used because freezing fast had achieved a low fertilization result. The same females as in experiment 1 A were inseminated with either one or two straws.

Experiment 2. Comparison of different cryoprotectants

The same roosters were used as in experiment 1 A. Semen collection and processing as well as slow freezing procedure were carried out as described in experiment 1 A. Three different freezing extenders, Mix 1, Mix 2 and MA, were used (composition see Table 2). The final concentration of all cryoprotectants was in each case 6.5 %.

Semen evaluation

Sperm cell concentration was determined by the NucleoCounter SP-100 system (ChemoMetec, Denmark). The fresh and frozen-thawed semen was evaluated for motility and morphological alterations. The straws were thawed in 4 °C water bath for 1 min. The motility characteristics of spermatozoa were evaluated by means of CASA (computer-assisted sperm analysis) with a Hamilton Thorne Biosciences-IVOS (Beverly, USA) and 4- Leja analysis chambers of a thickness of 20 µm (Minitube, Tiefenbach, Germany). The instrument's setting was adapted from Klimowicz et al. (2008); only the temperature of analysis was increased from 24 °C to 37 °C. Fresh and thawed semen were diluted 1:200 and 1:50, respectively, in HS1 medium before measurement. The parameters measured were: percentage of total motile sperm (MOT%), percentage of progressive motile sperm (ProgMot%), average path velocity (VAP, µm/s), progressive velocity (VSL µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness of track (STR %, ratio of VSL/VAP) and linearity of track (LIN %, ratio of VSL/VCL). For the morphological examination, sperm cells were fixed in formol citrate solution and microscopically evaluated using a 100x oil immersion objective (Alkan et al., 2002). In each preparation, 100 spermatozoa were counted, and the percentage of defect types was calculated.

Fertilization trials

The fertilization ability of spermatozoa was tested by intravaginal artificial insemination (AI). Hens of the line R22 were used. Each hen was inseminated three times (day 1, 4 and 8) with the content of one (Exp. 1 A/2) or either one or two straws (Exp. 1 B). The semen was thawed in a water bath at 4 °C and was immediately used for insemination. Hens inseminated with fresh spermatozoa served as controls. Insemination started at 11 o'clock in the morning. Eggs were collected daily, beginning at the second day after the first AI, and continued for 10 d. Eggs were stored at 14 °C up to the end of the collection period, and subsequently placed together into an incubator. Fertility was checked at 7th day of incubation by candling. Fertility rate was calculated as ratio of eggs fertilized and eggs laid during the 10 d period of collection.

Statistical analyses

Statistical analyses were performed using SigmaStat 2.0 (Jandel Scientific, Erkrath, Germany). Semen quality traits were analyzed by One-way ANOVA. In the case of a statistically significant difference, all pair wise comparisons were carried out using Tukey procedure. If distribution of data significantly deviated from normal distribution, the Kruskal-Wallis ANOVA on Ranks was carried out. In this case, differences of pair wise comparisons were tested using the Dunn's method. Chi-square tests were applied to test the influence of freezing rate (slow or fast), insemination dose (1 or 2 straws) and the kind of the cryoprotectant (Mix 1, Mix 2, MA) on fertility.

Results

The fertility data are summarized in Table 3. In the first experiment (part A) a significantly higher fertility with slowly frozen (49.3 %) than with fast frozen (11.5 %) semen was found. The semen had been taken only two times to check quality after thawing. It was very similar between the slowly frozen and the fast frozen samples (Table 4). Merely at the second semen collection, the qual-

ity of the semen was lower after fast freezing. In particular, the portion of motile sperm and their speed were lower than in slowly frozen and thawed samples.

Table 3:
Fertilization rates with fresh and frozen-thawed semen

Exp. No.	No. of hens	No. of eggs			Straws/ AI	Semen processing	Freeze rate
		incu-bated	fertile	% fertile			
1 A	10	79	74	93.7[a]	-	Fresh, diluted	-
	20	148	73	49.3[b]	1	Frozen with Mix 2	slow
	19	148	17	11.5[c]	1	Frozen with Mix 2	fast
1 B	5	38	34	89.5[a]	-	Fresh, diluted	-
	19	133	58	43.6[b]	1	Frozen with Mix 2	slow
	20	146	76	52.1[b]	2	Frozen with Mix 2	slow
2	34	374	365	97.6[a]	-	Fresh, diluted	-
	12	97	75	77.3[b]	1	Frozen with Mix 1	slow
	11	90	73	81.1[b]	1	Frozen with Mix 2	slow
	11	93	37	39.8[c]	1	Frozen with MA	slow

Within experiment, different superscripts indicate significant differences ($P < 0.05$)

In experiment 1 B, we tested whether the fertility rate can be improved by increasing the insemination dose. Although the percentage of fertile eggs was slightly higher after the insemination with two straws (52.1 %) instead of one (43.6 %), the differences were statistically not significant (Table 3). The semen quality before and after freezing is shown in Table 5. The frozen-thawed sperm showed a significantly lower portion of total and progressively motile spermatozoa, lower velocity parameters as well as a higher percentage of sperm with morphological modifications in comparison with the fresh sperm. Only parameters amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) were hardly changed after freezing.

Table 4:
Quality parameters of fresh and frozen-thawed rooster semen in experiment 1 A

Sperm Parameters	Fresh semen		Frozen-thawed semen			
	21-06-2010	23-06-2010	21-06-2010		23-06-2010	
			slow	fast	slow	fast
% motile	74	75.3	49.3	49	46	35.5
% ProgMot	47	40.7	27.7	27	21	14
VAP (µm/s)	81.1	72.6	73.5	69	68.4	60.8
VSL (µm/s)	67.7	57.3	58.6	55.1	52.7	46.6
VCL (µm/s)	120.7	115.5	115.8	111.4	112.6	104.2
ALH (µm)	4.8	4.9	5.4	5.4	5.6	5.2
BCF (Hz)	30.8	33.5	31.4	31.1	33.7	34.2
STR (%)	80	76.3	76.7	77	74	74.3
LIN (%)	53	48.3	49.7	49	45	43.5
% abnormal	21	18	44	45	24	40

% motile, percentage of motile sperm; % ProgMot, percentage of progressive motile sperm; VAP, velocity average path; VSL, velocity straight line; VCL, velocity curve line; ALH, average lateral head displacement; BCF, beat-cross frequency; STR, straightness ((VSL/VAP)x100); LIN, linearity ((VSL/VCL)x100); % abnormal, percentage of morphologically abnormal sperm

Table 5:
Quality parameters of fresh and frozen-thawed rooster semen (mean ± SD) in experiment 1 B

Sperm Parameters	Experiment 1 B		Test
	Fresh semen	Mix 2 frozen	
% motile	81.3 ± 2.3[a]	49.8 ± 4.5[b]	t- Test
% ProgMot	53.3 ± 3.9[a]	29.3 ± 3.5[b]	t- Test
VAP (µm/s)	89.8 ± 5.1[a]	74.0 ± 4.0[b]	t- Test
VSL (µm/s)	74.2 ± 4.8[a]	61.1 ± 4.0[b]	t- Test
VCL (µm/s)	131.4 ± 3.9[a]	112.1 ± 3.3[b]	t- Test
ALH (µm)	5.3 ± 0.2[a]	5.0 ± 0.1[a]	MW*
BCF (Hz)	31.4 ± 0.9[a]	31.0 ± 1.1[a]	t- Test
STR (%)	79.2 ± 1.4[a]	79.1 ± 0.9[a]	t- Test
LIN (%)	54.8 ± 2.7[a]	52.9 ± 1.9[a]	t- Test
% abnormal	13.0 ± 1.0[a]	39.2 ± 13.0[b]	t- Test

Different superscripts indicate significant differences ($P < 0.05$)
*Mann-Whitney-Rank Sum Test
% motile, percentage of motile sperm; % ProgMot, percentage of progressive motile sperm; VAP, velocity average path; VSL, velocity straight line; VCL, velocity curve line; ALH, average lateral head displacement; BCF, beat-cross frequency; STR, straightness ((VSL/VAP)x100); LIN, linearity ((VSL/VCL)x100);
% abnormal, percentage of morphologically abnormal sperm

In the second experiment, very good fertilization results (Table 3) were achieved with frozen sperm in both Mix 1 (77.3 %) and Mix 2 (81.1 %). Hens inseminated with fresh sperm diluted in HS1 produced 97.6 % fertility. The fertilization result at application using MA as cryodiluent

was worst (39.8 %). The post-thaw quality of MA frozen semen was also notably lower than with the other two cryoprotectants (Table 6). Particularly, the portions of total and progressively motile sperm as well as the speed of the sperm were significantly less in MA frozen samples. Figure 1 shows the portion of daily-fertilized eggs after insemination with Mix 2 frozen-thawed semen. Collecting hatching eggs started one day after first insemination. On this day, the portion of fertile eggs was still low between 15 and 30 % and increased gradually thereafter. The course of fertility rates over time emphasised the outstanding result of the experiment 2. The fertilization rate of this experiment remained on a very high level till the end of the testing period. In the other experiments, the fertilization rate decreased between the inseminations and increased after that again.

Table 6:
Quality parameters of fresh and frozen-thawed rooster semen (mean ± SD) in experiment 2

Sperm Parameters	Experiment 2				Test
	Fresh semen	MA frozen	Mix 1 frozen	Mix 2 frozen	
% motile	81.3 ± 6.4^{a}	32.7 ± 3.9^{b}	47.7 ± 6.2^{c}	44.3 ± 5.6^{c}	Tukey
% ProgMot	58.4 ± 7.8^{a}	15.8 ± 1.8^{b}	26.4 ± 4.4^{a,b}	24.8 ± 2.1^{a,b}	Dunn's Method
VAP (µm/s)	97.3 ± 9.7^{a}	60.8 ± 3.7^{b}	75.6 ± 3.5^{c}	73.6 ± 2.7^{c}	Tukey
VSL (µm/s)	84.0 ± 11.2^{a}	50.5 ± 3.0^{b}	61.8 ± 2.6^{a,b}	59.9 ± 2.2^{a,b}	Dunn's Method
VCL (µm/s)	132.4 ± 6.3^{a}	97.0 ± 4.1^{b}	113.9 ± 3.9^{c}	112.7 ± 2.4^{c}	Tukey
ALH (µm)	5.0 ± 0.3^{a}	4.7 ± 0.5^{a}	5.1 ± 0.3^{a}	5.0 ± 0.3a	F- Test
BCF (Hz)	30.9 ± 0.9^{a}	31.8 ± 0.7^{a}	31.8 ± 0.7^{a}	32.0 ± 0.9^{a}	F- Test
STR (%)	82.5 ± 3.0^{a}	79.6 ± 1.2^{a,b}	78.1 ± 1.0^{b}	77.7 ± 1.2^{b}	Tukey
LIN (%)	61.0 ± 5.1^{a}	50.9 ± 2.1^{b}	51.8 ± 1.2^{b}	51.4 ± 1.2^{b}	Tukey
% abnormal	21.0 ± 6.0^{a}	45.0 ± 5.0^{b}	35.4 ± 10.0^{b,c}	31.5 ± 8.6^{a,c}	Tukey

Different superscripts indicate significant differences (P<0.05)
% motile, percentage of motile sperm; % ProgMot, percentage of progressive motile sperm; VAP, velocity average path; VSL, velocity straight line; VCL, velocity curve line; ALH, average lateral head displacement; BCF, beat-cross frequency; STR, straightness ((VSL/VAP)x100); LIN, linearity ((VSL/VCL)x100);
% abnormal, percentage of morphologically abnormal sperm

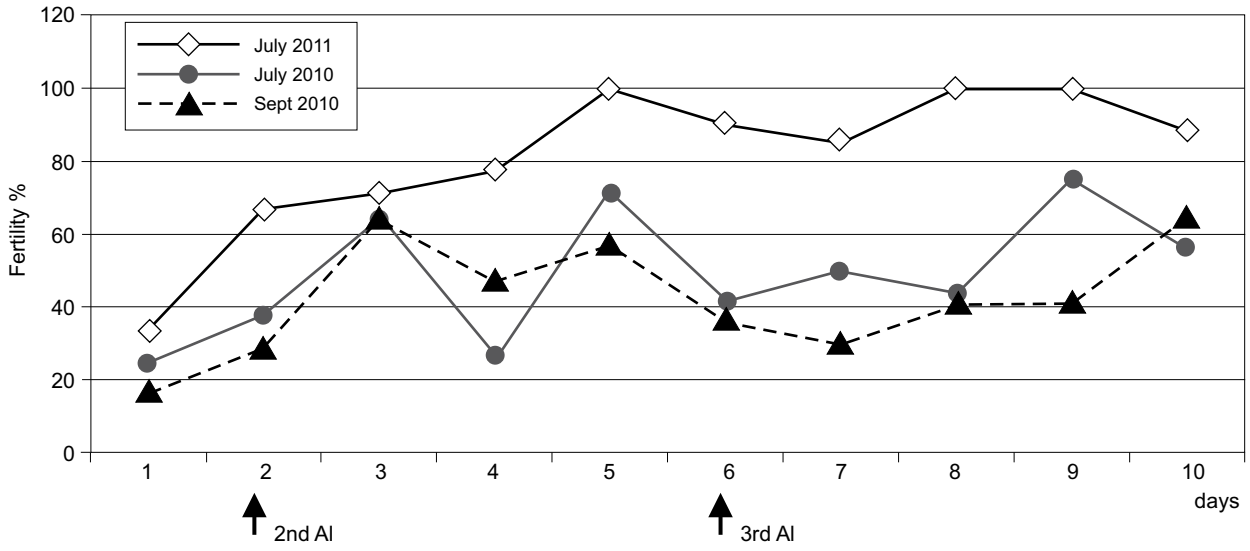

Figure 1:
Daily fertility after inseminations with frozen-thawed semen in Mix 2 (Day 1 = second day after first insemination)

Discussion

In previous studies, we applied a fast freezing method to cryopreserve rooster sperm. As cryoprotectants DMA and DMF were used in final concentrations of 6.5 %. Fertilization success, however, was very unsatisfying with a rate of fertilized eggs of 10 %. Fast freezing with a mixture of DMF and MA (Mix 2) improved fertilization results, but were still modest (29.9 %, Ehling, unpublished data). Therefore, first the effects of a fast and slow freezing rate on the fertility results by using of Mix 2 were examined in the present study (experiment 1A). According to our former study, the fast freezing protocol led to a rather low fertility success, although in vitro semen quality after thawing did not differ between slow and fast freezing procedure (CASA values and morphology). Other functional tests of sperm and examination of the acrosome integrity would give deeper insight into differences like in the examinations of Santiago-Moreno et al. (2011). A low fertility was also observed in other studies, using DMA as cryoprotectant and a fast freezing protocol (Blesbois et al., 2007; Baguio et al., 2008; Santiago-Moreno et al., 2011). Santiago-Moreno et al. (2011) examined three different freezing rates (slow-one step, medium-2 step and rapid) which led to different semen qualities, but had no significant effect on fertility rate. The medium-rate 2-step method (-7 °C/min from 5 °C to -35 °C, -60 °C/min from -35 °C to -140 °C) achieved the best fertility of 40.7 % and preserved best the motility and the acrosome and membrane integrity. Similar good fertilization results were found with a slow freezing method and the use of DMA or DMF (Lake and Ravie, 1984; Schramm, 1991; Chalah et al., 1999; Schramm, 2008). There are numerous studies reporting very good fertility rates with cryoprotectants of the amide group, straws and fast freezing (Woelders et al., 2006; Hanzawa et al., 2006; Purdy et al., 2009; Sasaki et al., 2010). However, the best fertility rates were obtained with semen frozen rapidly as pellets with DMA (Tselutin et al., 1999; Chalah et al., 1999; Woelders et al., 2006). Different procedures are not directly comparable as they are affected by various factors and may also be dependent on genotypes of chickens. In our hands, however, the slow freezing protocol worked well, and better than a fast freezing procedure.

Experiment 1 B, based on two straws per insemination compared to one, resulted only in a slightly higher fertilization result. Bielefeld (1985) also reported that doubling of insemination dose to 360 million sperm did not lead to a significant increase in the fertilization rate. Brillard and McDaniel (1986) concluded that the changes in semen doses modify duration rather than maximum levels of fertilization. The FAO guidelines for the Management of Small Populations at Risk (FAO, 1998) recommend a total dose of 600 million rooster sperm per insemination. The insemination volume should be approximately 60 to 100 µl. In our experiments the insemination dose was 200 µl, what corresponds to the content of one straw. The higher insemination volume (2 straws) in experiment 1 B possibly led to a partial sperm loss by reflow.

In the second experiment, MA was tested as a cryoprotectant. Hanzawa et al. (2006) and Sasaki et al. (2010) obtained higher fertility with MA (60.8 %) than with DMF (47.6 %), DMA (32.9 %) or DMSO (41.3 %). However, in the present experiment the fertilization rate was only moderate (39.8 %) when using MA as single cryoprotectant. In contrast, in our experiment the combination of MA and DMF led to very high fertilization results of approximately 80 %. The altered mixing ratio of DMF and MA in Mix 1 did not produce a higher fertility in the fertilization test. In all experiments, the same group of roosters was used. Their semen was inseminated in experiment 1 A and B into the identical group of hens, for experiment 2 another set females was used. These new hens had a high laying performance at the time of inseminations with cryopreserved semen. This might have contributed to the high fertilization success in this experiment.

In the experiment 2, the sperm had the same quality than in the other tests. The age of the animals was possibly of influence. The roosters were older (already 16 months) than in the experiment 1 A (8 months) and B (10 months). Long et al. (2010) accounted for an influence of rooster's age on the hydrolyzing ability of frozen-thawed sperm among elite layer lines. Sperm from 12 month old males hydrolyzed more holes in the inner perivitelline membrane than sperm from six month old males. Whether the age of the genetic pool roosters really influences the fertility results has to be verified in other experiments.

The movement of spermatozoa is one of the important parameters of semen quality. Motility assessment depends on the structural and functional integrity of spermatozoa and allows prediction of the fertilizing potential of semen (Froman, 2007; Blesbois et al., 2008). In the present study sperm quality was assessed by motility parameters, objectively measured by means of CASA. Most of CASA parameters were affected by cryopreservation. Primarily, the percentage of motile and progressively motile spermatozoa was reduced in approximately 50 % of the cryopreserved samples, when compared with fresh samples. Additionally the post-thaw sperm showed a significant decrease of their velocity. Blesbois et al. (2008), Sontakke et al. (2004), Fungfuang et al. (2009), Lemoine et al. (2011) and Santiago-Moreno et al. (2011) also reported such changes following freezing and thawing of chicken sperm.

Cryopreservation significantly affected the percentage of morphologically normal spermatozoa. In the present examination, percentage of abnormal sperm is, however,

fundamentally less than in studies by Schramm (1991; 2008) and Fungfuang et al. (2009).

In conclusion, best fertility results of 80 % were achieved using a mixture of DMF and MA in combination with a 2-phase slow freezing procedure. These results suggest that this protocol can be used to establish a cryobank for conserving chicken genetic resource populations for an unlimited period of time.

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