CRYOPRESERVATION OF LANDESE GANDER SEMEN

F. Dubos [1*], F. Seigneurin [2], M. M. Mialon-Richard[3], I. Grasseau [4], G. Guy [1], and E. Blesbois[4]

[1] INRA, Unité Expérimentale des Palmipèdes à Foie Gras, 40280 Benquet France
[2] SYSAAF, Station de Recherches Avicoles, 37380 Nouzilly France
[3] INRA, Station d'Amélioration Génétique des Animaux, 31326 Castanet-Tolosan France
[4] INRA, Station de Recherches Avicoles, 37380 Nouzilly France
* Corresponding author e-mail. fdubos@bordeaux.inra.fr

ABSTRACT

The aim of this project was to set a freezing technique of grey Landese gander semen in order to constitute a gene stock stored in the French National Cryobank of Domestic Animals. Several adaptations of semen freezing methods originating from two procedures established with other geese strains were performed. The efficiency of the methods was analysed by controlling sperm motility, the ratio of normal alive spermatozoa (eosin-nigrosin smears) and the fertility obtained by intra vaginal artificial insemination of frozen-thawed semen. Average quality of fresh semen was low and initial percentage of alive normal spermatozoa did not exceed 40%. Fertility rates obtained with frozen-thawed semen were very low during the first season and reached a mean of 25% during the second. These last results are sufficient to preserve gene stock, but it is careful to store the semen of a large number of ganders to obtain a satisfying *ex-situ* conservation of the line.

KEY WORDS: Cryopreservation, Fertility, Gander, Gene stock, Spermatozoa.

INTRODUCTION

Geese raised for "foie gras" production, remains at a low level of production and do not account for more than 3.5 % of French market in 2003. Genetic resources are by the way seriously threatened. This threat is emphasized by the risks of exposure to the avian influenza contamination. The success of freezing semen should partly allow storing gene stock in the form of male gametes in the French National Cryobank of Domestic Animals. Freezing gander semen is currently used for geese of *anser cygnoides* type (Tai *et al*., 2001) and in course of development for south and east European strains (Lukascewicz, 2001 and 2004). To our knowledge no investigation of this field has been carried out for Landese geese. Our goal during two years of trial was to set a method of preservation of ~~the~~ Landese gander semen by adapting the methods described by Tai *et al*. (2001), and Lukascewicz (2001). These methods use the same internal cryoprotectant, the dimethyl acetamide (DMA) and the same sperm packaging (straws), but they differ by the temperature kinetics and the diluent composition. These two methods were compared in our experimental conditions and then several variants were studied in order to purchase the maximum efficiency for spermatozoa survival.

MATERIALS AND METHODS

Animals: the same flock of 46 males and 56 females of the INRA 07 experimental strain hatched in 2003 was used in spring 2004 and 2005. From birth to thirty weeks of age they

were raised in natural lighting, fed commercial diets and were given access to an open yard. Onwards males were placed in individual cages in a dark windowless building and submitted to 9 hours light/day. Females were placed in five outside collective pens, and consequently set natural and seasonal lays. The feeding program was based on laying performances. Birds were fed a "lay" diet (9 MJ/Kg and 17% crude protein (CP)) up to the laying rate of 25 % and then a second diet (11 MJ/Kg and 19.2% CP). Females were fed ad libitum while males were restricted daily (2.5 MJ/day).

***Semen collection**:* during the two short reproductive periods (Ferbruary to April), ganders were solicited twice a week by dorso-abdominal massage.

Freezing methods: According to the low quality of production of INRA 07 ganders (mean volume, 0.24 ml and concentration, $651*10^6$ spz/ml; Mialon-Richard, 2004), sperm originating from ten ejaculates were pooled before freezing. Based on the methods of Tai *et al*. (2001) and Lukaszewicz (2001) we modified several parameters to test a total of ten variants during the two years of trials. These adaptations are described on Table 1.

Table 1. Different freezing methods tested on INRA 07 grey Landese gander.

Freezing method	LUKA V 1	TAÏ	TAÏ V 1	TAÏ V 2	TAÏ V 3	TAÏ V 4	TAÏ V 5	TAÏ V 6	Glycerol 2	Glycérol 3
Diluent	EK	TAÏ							IGGK + Inositol	
dilution	1/2	1/3				1/2		2/3	1/2	
Diluent supply + sperm + cryopr.	100 0	100 0	50 50						100 0	50 50
Cryopr.	DMA	DMA							Glycerol	
Level	6%	9%							11%	
Supply	Undiluted	Undiluted	+ diluent						Undiluted	+ diluent
Temp supply	5°C	5°C	Ambient						5°C	Ambient
Equilibr.	5 min	10 min							10 min	8 min
Freezing	LN2 vapour 5 cm	Carbonic Ice	LN2 vap 7 cm	Programmable freezer					Programmable freezer	
Time / speed	15 min	15 min	15 min	-55°C / min		-7°C / min		55°C / min	-55°C / min	-7°C / min

Cryopr = cryoprotectant; Level = % of cryoprotectant in diluted sperm. Temp = temperature; Temp supply = temperature supply for cryoprotectant; equilibr. = time of cryoprotectant equilibration before freezing; LN2 Vap x cm = freezing semen in nitrogen vapour x centimetres up to liquid nitrogen. Time/speed = contact duration to nitrogen vapour or carbonic ice or freezing speed with programmable freezer.

In vitro quality evaluation of semen: - Morphological integrity and viability of spermatozoa (% alive, % normal and viable, % abnormal, % dead spermatozoa) were evaluated under optical microscope after eosine/nicrosine staining (Blom, 1950) and were summarized as % alive-normal spermatozoa (% AN). The ratio % (AN of fresh spermatozoa/ AN of frozen-thawed spermatozoa) was called survival rate.

Massal motility of semen was observed under a light microscope (X 200) and subjectively noted on a scale ranging from 0 to 6.

Fertility: intra vaginal insemination of 10-20 females/semen pool was realized each 3-4 days for 2 to 3 successive weeks followed by a two weeks period without insemination before a new period of test. The mean dose of insemination was 20 million spermatozoa/ female/insemination during the first reproductive period and 40 million during the second. Fertility rates (% fertile/incubated eggs) were evaluated by candling at day 7 of incubation.
Statistical analysis : Massal motility, % AN and survival rates were analysed by Analysis of Variance. Fertility rate was appreciated using the Pearson Khi2 test (SAS software).

RESULTS AND DISCUSSION

Year 2004 (Table 2):

Motility and % AN of fresh sperm were low as fertility (57 % fertility rate). Sixty percent of normal alive cells were additionally lost after semen cryopreservation. Motility also decreased with freezing. The survival rates (34.5% to 43.9%) did not differ significantly between treatments indicating the same efficiency for each method. These survival rates were close to these measured by Lukaszewicz (2001-2004) that could show a good use of the freezing method. However, the quality of fresh semen was so low that after cryopreservation there were very few normal and viable spermatozoa (2.8 to 2.7 million of alive and normal spermatozoa per insemination dose) and much probably fewer spermatozoa able to fertilize. As a consequence, results of fertility with frozen-thawed semen were very low (3.7 to 8.8%). These fertility results were far lower than these obtained by Tai (12.4 to 94.7%) and Lukaszewicz (66.1%) with other strains. This result could probably partly be explained by the low total number of inseminated spermatozoa (20 millions) chosen to show differences between treatments if they did exist, but also by the characteristic of the line.

Table 2. In vitro and fertility results in year 2004

Freezing procedure	TAÏ Control	TAÏ Variant 1	TAÏ Variant 2	Lukaszewicz Variant 1
Number trials	8	8	11	8
Motility Fresh sperm	3,3 ± 0.7	3,4 ± 1,4	3,5 ± 0,9	3,3 ± 0,5
Motility frozen-thawed sperm	0,7 ± 0,3	0,9 ± 0,4	0,8 ± 0,3	1,1 ± 0,5
% AN fresh	39,1 ± 6,6	44,7 ± 8,5	43,1 ± 8,8	40,5 ± 4,3
% AN frozen	16,8 ± 3,7	17,4 ± 6,1	18,7 ± 6,5	14,3 ± 3,3
Survival rate (%)	43,9 ± 11,7	42,4 ± 20,1	43,2 ± 10,8	34,5 ± 7,3
Fertility	3,7 (108 eggs)[1]	5,5 (75)[1]	8,8 (113)[1]	8,5 (117)[1]

[1]*number eggs (between brackets).*

Year 2005 (Table 3):

The freezing method Tai variant 2 was retained as "control" method for the first series of insemination of the second period of reproduction, and new adaptations were then tested. Additionally, the cryoprotectant glycerol that is very efficient in chicken (Seigneurin and Blesbois, 1995; Tselutin *et al*, 1999) was tested.
The quality of fresh spermatozoa was low and was then decreased by cryopreservation. The survival rates were close to these obtained during the first season or higher (but there were no significant difference). The glycerol showed a positive effect on motility of frozen-thawed spermatozoa. The final number of alive and normal frozen-thawed spermatozoa inseminated was higher than during the first season and equivalent for each treatment (5.84 to 7.12

millions over a constant total of 40 million spermatozoa inseminated). Although the fertility results obtained with frozen semen were lower than those obtained with fresh semen, they were improved in 2005 up to a mean of 25 %. We can assume that the insemination dose (40 million), new material (programmable freezer) and operator technical have led to this results.

Table 3. In vitro and fertility results in year 2005

Freezing method	TAÏ Variant 2	TAÏ Variant 3	TAÏ Variant 4	TAÏ Variant 5	TAÏ Variant 6	Glycérol 1	Glycérol 2
Number trials	7	7	15	6	8	11	7
Mot. fresh sp.	3,4 ± 1,4	3,1 ± 0,9	4,0 ± 1,3	3,5 ± 1,2	4,1 ± 1,3	3,1 ± 0,5	4,0 ± 1,2
Mot. frozen. sp	0,6 ± 0,2	0,7 ± 0,3	0,8 ± 0,4	1,2 ± 0,3	1,2 ± 0,5	2,5 ± 0,7	2,3 ± 0,6
% AN/fresh	41,0 ± 5,9	38,1 ± 5,3	36,1 ± 7,3	29,7 ± 2,4	42,6 ± 5,4	30,1 ± 5,2	28,5 ± 3,5
% AN/frozen	17,2 ± 2,5	16,3 ± 1,4	15,0 ± 2,2	16,2 ± 2,6	17,8 ± 2,6	14,8 ± 4,2	14,6 ± 1,8
Survival r (%)	43,0 ± 5,0	43,0 ± 7,7	43,1 ± 6,7	54,6 ± 8,7	41,9 ± 5,4	49,2 ±13,8	51,2 ± 5,0
Fertility	26,4 (72) [1]	23,2 (99)	25,0 (240)	21,6 (74)	24,8 (109)	7,8 (77)	33,3 (15)

Mot=Motility; sp=spermatozoa; Survival r=survival rate; [1]number eggs (between brackets).

CONCLUSION

All together these results show clearly that the initial quality of grey Landese gander semen is not sufficient to reach quickly a convenient fertility rate with frozen semen. Furthermore, semen production is seasonal and allows only to 2 or 3 months of work per year which is detrimental to a rapid improvement. However mean fertility rates of 25 % have been obtained with frozen semen. These results are sufficient to preserve a strain but necessitate collecting a high number of ganders. The comparison between the two years suggests that ~~some~~ improvements are still possible. New studies, particularly with the use of glycerol as cryoprotectant will be interesting.

ACKNOWLEDGMENTS

The authors wish to thanks the genetic resources office for his financial support, and all those who provide help in the management of waterfowls : Josette Barrieu, M. Laguë, JB. Laverze, JM. Mallet and P. Morganx

REFERENCE

Blom E. 1950. A one minute live-dead sperm stain by means of eosine nicrosine.Fertil. Steril. 1 : 176-177

Lukaszewicz E. 2001. Effects of semen filtration and dilution rate on morphology and fertility of frozen gaznder spermatozoa. Theriogenol. 55 : 1819-1829.

Lukaszewicz E. 2004. Attemps on freezing the Greylag (Anser anser L.) gander semen Anim. Repro. Sc. 80 : 163-173

Mialon-Richard M.M. 2004. Facteurs de variation de la production spermatique de jars Landais sous programme lumineux. 6èmes Journées de Recherche sur les Palmipèdes à Foie Gras, Arcachon, France

Seigneurin, F., Blesbois, E. 1995. Effects of freezing rate on viability and fertility of frozen-thawed fowl spermatozoa. Theriogenology, 43:1351-1358

Tai J. J. L., Chen J. C., Wu K. C., Wang S. D., Tai C. 2001. Cryopreservation of gander semen Br. Poult. Sci. 42 : 384-388.

Tselutin, K., Seigneurin, F., Blesbois, E. 1999. Comparison of cryoprotectants methods of cryopreservation of fowl spermatozoa. Poultry Science, 78:586-590.