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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

The effect of breed on freezability of semen of fancy fowl

Anna Siudzińska, Ewa Łukaszewicz*

Department of Poultry Breeding,
University of Environmental and Life Sciences of Wrocław,
Chełmońskiego 38c, 51-630 Wroclaw, Poland

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A simple cryopreservation method described in 1995 by Tselutin *et al*. was used for freezing the semen of four fancy fowl breeds: White Crested Black Polish (WCBP), Greenleg Partridge (GP), Italian Partridge (IP) and Black Minorca (BP). The differences in quality (ejaculate volume, osmotic pressure, sperm concentration and morphology) of fresh semen between evaluated breeds were observed, as well as the differences in semen freezability. The freezing-thawing process caused significant (P≤0.01) decrease in percentage of live, normal spermatozoa, with coincident increase in percentage of dead spermatozoa and spermatozoa with acrosome defect. In relation to the fresh semen, the number of live, normal spermatozoa that survived cryopreservation procedure constituted 18.1% in WCBP, 25.1% in GP, 26.2% in IP and 33.6% in BM semen.

KEY WORDS: cryopreservation / fancy fowl / freezability / semen / sperm

Cryopreservation of fancy fowl semen along with artificial insemination could make an important contribution to the amateur poultry breeding, particularly in gene exchange. However, there is still a necessity for developing optimum cryopreservation method(s), which would allow storing semen in LN_2 (-196°C) for long periods of time with minimum loss in cell viability and fertilizing potential.

*Corresponding author: ewa.lukaszewicz@up.wroc.pl

Compared to mammals, avian spermatozoa possess unique physiological features which make their survival under freezing-thawing conditions difficult. The uptake of cryoprotectant by the cell may be less effective than in mammals because of elongated head with little cytoplasm content [Donoghue and Wishart 2000]. Structural damage to spermatozoa concerns first of all the plasma membrane, mitochondria and acrosome region [Harris *et al*. 1973, Maeda *et al*. 1990]. Xia *et al*. [1988] reported that in the domestic fowl freezing-thawing process caused significant damages to mitochondria, midpiece, and perforatorium, but not to sperm nucleus.

Although many attempts at freezing semen of domestic bird species have been undertaken the cryopreservation is still in experimental stage, mostly due to relatively small improvements made and appreciable losses of spermatozoa viability after freezing-thawing process. The critical steps in successful cryopreservation of fowl semen are: selection of suitable semen extender, proper cryoprotectant, as well as freezing-thawing method. Despite the variety of methods, appreciable losses of spermatozoa viability after freezing-thawing process are still observed. Tselutin *et al.* [1995] developed simple and inexpensive method for poultry semen cryopreservation which can be easily adapted to farm conditions.

Amateur and fancy fowl breeds have become more and more popular among breeders and pet animals keepers. Polish Greenleg Partridge chicken (also known as Green-legged Partridge chicken) is kept as gene pool reserve flock, in small households or agroturist farms in Poland. It also yields tasty meat of high quality and eggs with relatively low cholesterol content [Stępińska *et al*. 1993]. Italian Partridge and Black Minorca are breeds kept by amateurs for egg lying simultaneously being decorative birds, while White Crested Black Polish is very famous as typical fancy breed. The exchange of genetic material is often limited by long distance between farms resulting in decrease of genetic diversity. Instead of animal transport the cryopreserved semen can be sent making breeder's work much easier and effective.

In this study the possibility was tested of using a simple cryopreservation method developed by Tselutin *et al.* [1995] to freeze the semen collected from roosters of four fancy breeds and evaluation performed of interbreed differences in semen quality and freezability.

Materials and methods

Semen was collected from roosters of four breeds of domestic fowl (*Gallus domesticus*): White Crested Black Polish (WCBP), Greenleg Partridge (GP), Italian Partridge (IP) and Black Minorca (BM). Each breed was represented by seven sexually matured, six-months-old males. Birds were housed in large individual cages at 15-18ºC under 14h/10h L programme and offered commercial feed assigned for reproductive chicken, and water *ad libitum*.

Semen was collected two times a week by dorso-abdominal massage [Burrows and Quinn 1937]. Clean, uncontaminated ejaculates obtained from males of the same

breed were pooled and further evaluated as one sample. From each rooster semen was collected ten times. Special care was taken to avoid contamination of semen which reduces its quality.

In freshly collected semen the volume of pooled ejaculates, osmolarity, concentration and sperm morphology were assessed. On the basis of total volume of pooled ejaculates the mean volume of a single ejaculate was derived, calculated within one collection and breed. The spermatozoa concentration was determined haemocytometrically. Osmolarity was measured in fresh, undiluted semen, in equilibrated and frozen-thawed semen samples, with the use of Semi-Micro Osmometer (KNAUER, Germany). Sperm morphology as a good indicator of spermatozoa condition and fertilizing ability was investigated in nigrosin-eosin smears and evaluated under 1250x with a light microscope (JENAVAL, CARL ZEISS, Jena, Germany). In every slide 300 cells were counted and considered to be viable when they were not stained, or dead when they were totally or partly pink-stained with eosin. Within the fraction of live, unstained cells, spermatozoa were categorized into six classes:

1. Morphologically normal (spindle-shaped head with well-marked acrosome and visible tail).
2. Swollen head.
3. Acrosome defect.
4. Bent neck.
5. Defective midpiece.
6. Spermatozoa with other defects (coiled tails, lack of tail, spermatids, etc.).

The results of morphological evaluation were expressed as the percentage of particular categories of spermatozoa (300 cells = 100%). Moreover, for better comparison of the fresh semen quality of evaluated breeds, the Semen Quality Factor – SQF – was calculated as follows:

$$SQF = \frac{\text{spermatozoa concentration per ml} \times \text{ejaculate volume (ml)} \times \text{normal spermatozoa (\%)}}{100\%}$$

Within 20 min following collection the semen samples were subjected to cryopreservation procedure adopted from Tselutin *et al*. [1995]. Prior to freezing, aliquots of the semen from examined breeds were diluted at room temperature with EK diluent [Łukaszewicz 2002b] and stored for 15 min at -8ºC. Dimethylacetamide (DMA) was added to diluted samples to a final concentration of 6%. After further five minutes of equilibration the solution was pipetted and plunged drop-by-drop directly into liquid nitrogen. Frozen pellets were transferred into LN_2 container and stored for several days before being thawed.

To determine the influence of cryopreservation process upon spermatozoa examined, their morphology and osmotic pressure were assessed after the equilibration with cryoprotectant and after thawing.

The interbreed differences within parametres measured were verified with ANOVA and Duncan's multiple range test (STATISTICA, version 7.1 StatSoft, Inc. Data Analysis Software System).

Results and discussion

The results obtained revealed the significant differences among evaluated fancy fowl breeds, both in relation to the fresh semen quality and freezability. Moreover, it was shown that quality of semen collected from roosters of fancy breeds and its resistance to cryoinjury stress are both lower than in commercial meat or laying type chicken lines.

The ejaculates of largest volume were obtained from GP and BM males and the smallest from WCBP, with significant differences ($P \leq 0.01$) between the latter and the other breeds (Tab. 1). Concentration of spermatozoa (Tab. 1) was the lowest in GP and the highest in WCBP males ($P \leq 0.01$).

Table 1. Means and their standard deviations (SD) for traits of fresh semen collected from roosters of four fancy breeds

Trait		Breed			
		White Crested Black Polish	Greenleg Partridge	Italian Partridge	Black Minorca
Ejaculate volume/male (ml)	mean	0.23^{A}	0.46^{B}	0.42^{B}	0.46^{B}
	SD	0.03	0.09	0.06	0.09
Osmotic pressure (mOsm/kg)	mean	318.00^{A}	319.50^{AB}	325.00^{B}	324.00^{AB}
	SD	5.37	5.50	2.36	5.16
Sperm concentration ($n \times 10^9$/ml)	mean	4.69^{B}	3.18^{A}	3.68^{AB}	4.19^{AB}
	SD	1.05	0.69	0.67	1.28
Sperm morphology (%)					
normal	mean	66.39^{B}	66.91^{B}	56.47^{A}	64.18^{B}
	SD	7.56	6.99	8.30	20.29
swollen head	mean	2.78	3.58	2.17	3.28
	SD	2.98	3.55	1.64	2.28
acrosome defect	mean	0.44	0.76	1.03	0.69
	SD	0.84	1.42	0.83	0.81
bent neck	mean	2.33^{B}	3.00^{B}	5.64^{A}	1.95^{B}
	SD	1.27	1.63	2.12	0.98
defective midpiece	mean	4.33	3.09	1.25	2.67
	SD	5.19	2.42	0.92	2.64
other defects	mean	6.25	5.85	7.17	6.28
	SD	3.16	3.51	2.74	3.82
dead	mean	17.47^{A}	16.82^{A}	26.28^{B}	14.38^{A}
	SD	9.26	7.33	8.56	4.75
SQF/male*	mean	75.94^{B}	97.27^{B}	84.34^{B}	132.24^{A}
	SD	24.17	26.46	14.95	51.06

^{AB}Within lines means bearing different superscripts differ significantly at $P \leq 0.01$.
*SQF – Semen Quality Factor = semen concentration ($n \times 10^9$/ml) × ejaculate volume/male (ml) × morphologically normal spermatozoa (%)/100%.

Although in this study the volume of ejaculates, osmolarity and concentration of spermatozoa were found similar to those already reported by Polish authors [Podgórny *et al*. [1976], Partyka *et al*. [2007], the semen quality expressed by the number of live, morphologically normal spermatozoa occurred lower compared to commercial fowl lines. Tselutin *et al*. [1999] reported from 92 to 94% of live morphologically normal spermatozoa in semen of chicken males from commercial lines, which were comparable to values given by Ansah *et al*. [1985] and Blesbois *et al*. [2005] – 89.1 and 87.0%, respectively. Higher percentage of morphologically normal spermatozoa (84%) in the fresh semen of GP males was reported also by Partyka *et al*. [2007].

While relating to all classes of spermatozoa, no significant differences in fresh semen were identified among GP, BM and WCBP males. Significantly lower ($P \leq 0.01$) percentage of live normal spermatozoa, higher percentage of spermatozoa with bent neck, and dead spermatozoa was observed in IP semen, compared to semen of the remaining three breeds (Tab. 1). Among evaluated breeds significant differences were also identified in SQF. It was the highest in BM and the lowest in WCBP (means of 132.2 and 75.94, respectively) – Table 1.

The osmotic pressure of the fresh semen varied from 318 to 325 mOsm/kg, being the lowest in WCBP and the highest ($P \leq 0.01$) in IP males (Tab. 1).

With no reference to breed, the addition of cryoprotectant and semen equilibration in presence of 6% DMA resulted in increase ($P \leq 0.01$) of osmolarity compared with the the fresh semen, which was caused by high osmolarity of DMA. It varied from 1158.0 mOsm/kg in BM to 1199.0 mOsm/kg in IP semen. Equilibration process had also the negative effect on sperm morphology. Blanco *et al*. [2000] found that viability of sperm of different bird species decreased with increasing DMA concentration. Chełmońska *et al.* [2006] reported the detrimental effect of rising DMA level on quail sperm morphology, while Tselutin *et al.* [1999] observed the drop in percentage of morphologically normal spermatozoa from 92% in fresh fowl semen to 62-68% in semen incubated in diluent with different levels of DMA. Chalah *et al.* [1999], comparing the viability of fowl spermatozoa prepared for cryopreservation in different cryoprotectants found 74% of viable spermatozoa when DMA was used. Simultaneously, the per cent of live sperms in fresh semen reached 83%.

After freezing-thawing procedure a slight decrease in osmolarity was observed (by 96 in WCBP to 180 mOsm/kg in GP semen). However, no significant differences were identified among breeds with regard to osmolarity, both of equilibrated and frozen-thawed semen samples.

Cryopreservation procedure applied in this investigation caused significant and unfavourable changes in semen quality, manifested mostly by the proportion of morphologically normal spermatozoa, dead spermatozoa and spermatozoa with acrosome defect. With no reference to breed, freezing-thawing process resulted in further increase ($P \leq 0.01$) in the number of dead spermatozoa and spermatozoa with acrosome defect, accompanied by the decreased content of live, morphologically normal spermatozoa (Fig. 1, 2, 3 and 4).

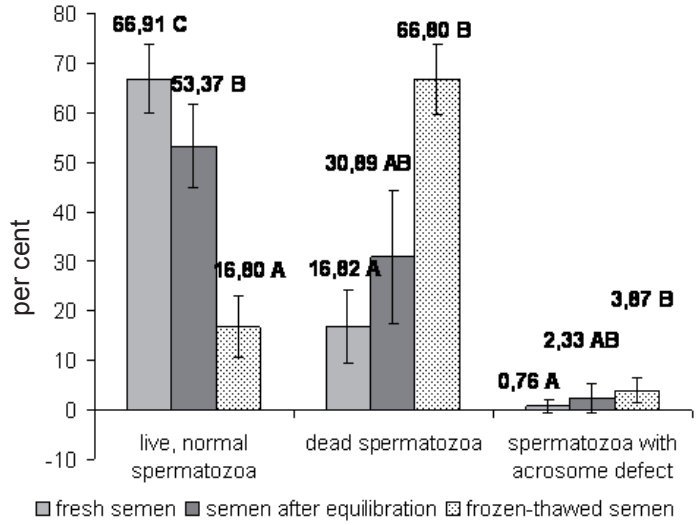

Fig. 1. The effect of subsequent steps of cryopreservation process on selected classes of spermatozoa in Greenleg Partridge males (means and their standard deviations, n=10).
[AB...]Within classes, means bearing different superscripts differ significantly at P≤0.01.

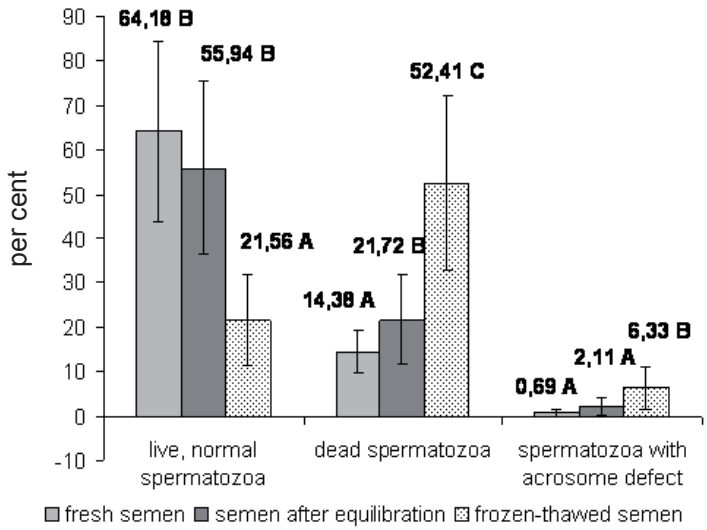

Fig. 2. The effect of subsequent steps of cryopreservation process on selected classes of spermatozoa in Black Minorca males (means and their standard deviations, n=10).
[AB...]Within classes, means bearing different superscripts differ significantly at P≤0.01.

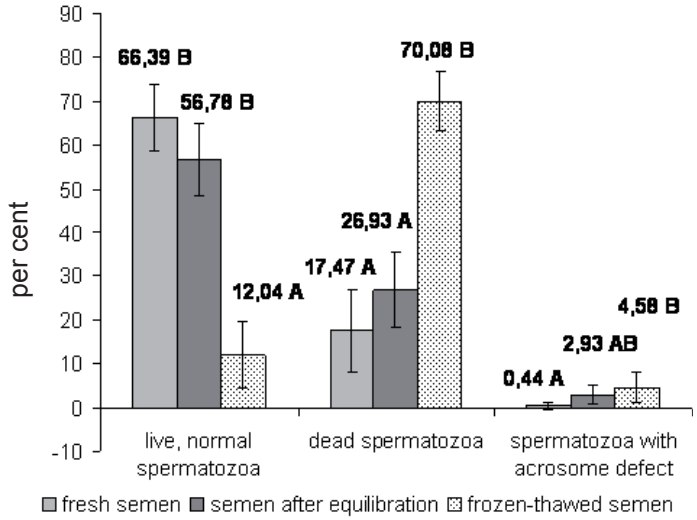

Fig. 3. The effect of subsequent steps of cryopreservation process on selected classes of spermatozoa in White Crested Black Polish males (means and their standard deviations, n=10).
[AB...]Within classes, means bearing different superscripts differ significantly at P≤0.01.

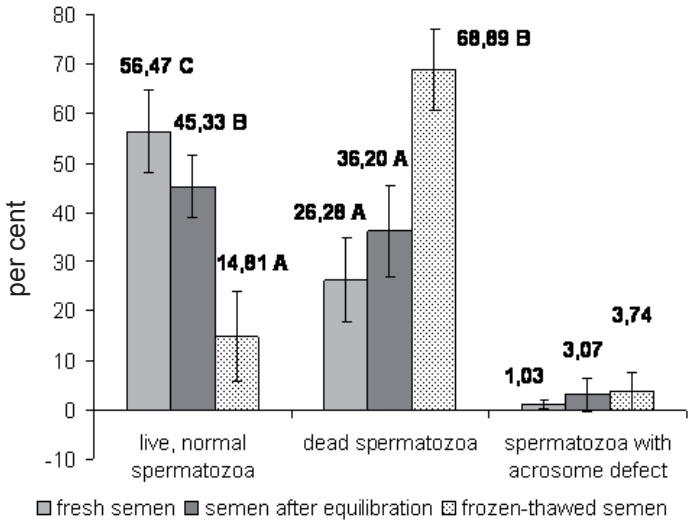

Fig. 4. The effect of subsequent steps of cryopreservation process on selected classes of spermatozoa in Italian Partridge males (means and their standard deviations, n=10).
[AB...]Within classes, means bearing different superscripts differ significantly at P≤0.01.

Similarly to the fresh semen, the lowest percentages of live, morphologically normal spermatozoa and the highest percentages of dead spermatozoa in equilibrated semen were found in IP males (Fig. 4). The highest proportion of morphologically normal spermatozoa was observed in WCBP (Fig. 3) and the lowest proportion of dead spermatozoa – in BM semen (Fig. 2). Significant differences ($P \leq 0.01$) were identified in the number of morphologically normal spermatozoa between IP and WCBP or IP and BM semen.

Observed changes in sperm morphology caused by cryoinjury stress were probably due to membrane damage as described in ultrastructure of frozen-thawed spermatozoa by Harris *et al*. [1973] and Maeda *et al*. [1990].

In this investigation, depending on fowl breed, the percentage of live, normal spermatozoa that survived cryopreservation constituted from about one-fourth to one-third of normal spermatozoa in the fresh semen (WCBP – 18.1%, GP – 25.1%, IP – 26.2%, BM – 33.6%). Chalah *et al*. [1999] reported that depending on technique used, one-third to one-half of the initial population of spermatozoa survive the cryopreservation procedure. When semen was frozen in pellets using DMA as a cryoprotectant, only 19% of viable spermatozoa were observed in thawed semen, what is in accordance with the present results. Seigneurin and Blesbois [1995] obtained about 34-48% normal, viable spermatozoa in fowl semen frozen with addition of glycerol, what constituted 36-51% of viable spermatozoa in the fresh semen. In frozen-thawed semen of White Koluda® and Greylag (*Anser anser* L.) ganders, the cryoinjury stress survived, in relation to the fresh semen content, respectively 28-53% and 60% of spermatozoa [Łukaszewicz 2002ab, Łukaszewicz *et al*. 2004].

The ability of spermatozoa to survive under cryopreservation is different in different bird species. The freezability was found to be higher in roosters and ganders than in the other domestic bird males [Massip *et al.* 2004]. Blesbois *et al.* [2005] obtained significantly higher percentage of viable and morphologically normal spermatozoa in cryopreserved fowl semen (32%) as compared to semen of turkey (25%) and guinea fowl (13%). On the basis of results presented here it can be stated that significant differences in semen freezability exist also among WCBP, GP, IP and BM breeds. Semen resistance to freezing-thawing process was the highest in BM (Fig. 2) and the lowest in WCBP males (Fig. 3).

Łukaszewicz [2002a] pointed to the significance of fresh semen quality, which has a crucial effect on final success of cryopreservation. Although the live, normal spermatozoa content of fresh semen in WCBP (66.4%) and GP (66.9%) males was higher than in the remaining two breeds, their content of the thawed semen decreased by 54.4 and 50.1 per cent points, respectively. The latter confirms our observations on differences between fowl breeds concerning the spermatozoa resistance to cryoinjury stress.

Perhaps the applied technique of cryopreservation (choice of cryoprotectant, and its concentration, time of equilibration, freezing and thawing method, etc.) should be adjusted not only to species, but also to a particular breed and quality of produced semen, in order to obtain more satisfying results.

Although the present report indicates the interbreed fowl differences in spermatozoa freezability, it can be concluded that simple, inexpensive, and easily adaptable to field conditions method of cryopreservation semen in pellets can be used to preserve spermatozoa of fancy fowl breeds and to facilitate gene exchange between breeders.

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Anna Siudzińska, Ewa Łukaszewicz

Podatność nasienia kogutów ras amatorskich na mrożenie

S t r e s z c z e n i e

Badano przydatność prostej metody mrożenia opracowanej i opisanej w 1995 r. przez Tselutina i współautorów do zamrożenia nasienia kogutów czterech amatorskich ras kur: czubatki polskiej (*White Crested Black Polish* – WCBP), zielononóżki kuropatwianej (*Greenleg Partridge* – GP), włoszki kuropatwianej (*Italian Partridge* – IP) i minorki czarnej (*Black Minorca* – BM). Stwierdzono różnice w jakości nasienia świeżego (objętość ejakulatów, ciśnienie osmotyczne, koncentracja i morfologia plemników) między kogutami ocenianych ras, a także różnice między rasami w podatności nasienia na mrożenie. Proces ekwilibracji nasienia w obecności 6% DMA oraz jego zamrożenie-rozmrożenie powodowały istotny ($P \leq 0,01$) spadek liczby plemników żywych, prawidłowo ukształtowanych, przy równoczesnym wzroście liczby plemników martwych lub z uszkodzonym akrosomem. Bez względu na rasę kogutów, mrożenie nasienia zastosowaną metodą powodowało spadek liczby plemników żywych prawidłowo ukształtowanych, których udział w stosunku do udziału w nasieniu świeżym stanowił w przypadku czubatki polskiej 18,1%, zielononóżki kuropatwianej 25,1%, włoszki kuropatwianej 26,2% oraz 33,6% w przypadku minorki czarnej.