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FREEZABILITY OF SEMEN COLLECTED FROM GANDERS OF 12 BREEDS COVERED BY THE POLISH GENETIC RESOURCES CONSERVATION PROGRAM*

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Abstract

A rapid decline in wild animal biodiversity, as well as in the number of local livestock breeds of lower economical values, makes species and breeds protection and creation of genetic reserves a necessity and a challenge of the 21st century. For birds, semen freezing is still the best method of gene preservation *ex situ in vitro*. The aim of the presented study was to assess the susceptibility to freezing process and to establish the semen gene bank of twelve goose breeds covered by the Polish genetic resources conservation program. Sexually mature ganders were randomly selected from four northern local breeds: Kartuska, Pomorska, Rypinska, Suwalska, four southern local breeds: Garbonosa, Kielecka, Lubelska, Podkarpacka, and four foreign goose breeds: Kuban, Landes, Roman and Slovakian. Each breed was represented by 12 males, twelve repetitions were carried out. Semen samples were frozen in a computer-controlled cryogenic chamber using a method developed for White Koluda goose. The basic evaluation criterion was sperm morphology in fresh and freeze-thawed semen assessed in nigrosine-eosin stained histological smears. The percentage of live in total and live normal sperm in the fresh semen differed significantly ($P<0.05$) between breeds. Semen of Kartuska and Suwalska ganders (northern breeds), contained the highest percent of live sperm – 94.4 and 94.1 respectively, including 52.5% and 54.2% of live normal sperm, while the lowest number of these forms was observed for Slovakian goose – 86.4% and 29.2%, respectively. The freezing process resulted in a significant ($P<0.05$) reduction in the total number of live and live normal sperm and an increase in dead sperm in all groups. Cryopreservation process was the most effective in case of Suwalska goose semen (28.4% of live normal cells in thawed semen) and the less profitable for Landes (7.4% of live normal cells). The analyses showed significant differences between breeds in sperm morphology in fresh and thawed semen. Moreover, the high proportion of live spermatozoa in the fresh semen did not guarantee a high proportion of spermatozoa after thawing, indicating the differences in semen susceptibility to cryopreservation process depending on goose breed.

Key words: goose, semen, sperm morphology, cryopreservation, biodiversity

A rapid decline in the number of local domesticated livestock breeds of lower economical values can be observed all over the world (Blesbois and Brillard, 2007; Łukaszewicz et al., 2021). Preservation of native breeds and increasing their population should be a priority for every generation (Castillo et al., 2021). The genetic reserves *in vivo* (both *in situ* and *ex situ*) are insufficient gene protection due to the decline in biodiversity of animals kept in small isolated population and the risk of unexpected natural disasters. *In vitro* methods, such as cryopreservation of biological material (sperm, blastodermal cells, primordial germ cells, embryos, DNA), provide an excellent enhancement and protection of *in vivo* reserves (Gliozzi et al., 2011). Although in case of birds the conservation of genetic resources through semen cryopreservation will not be complete due to male karyotype (ZZ), nevertheless it will allow for the protection of genes located on the males' autosomal and sex chromosomes. According to many scientists (Blesbois, 2011; Tajima, 2013), it is the most advantageous form of avian gene preservation *in vitro*, so far. Cryopreserved material can be stored for long periods and be used to restore breeds in danger of extinction. From this point of view, long-term storage of semen in liquid nitrogen as a means of preserving the gene pool seems desirable. Problems related to maintaining the genetic diversity in small populations of endangered species can be solved by artificial insemination with frozen semen deposited in a gene bank (Blanco et al., 2009). So far, no standard freezing procedure has been developed for avian semen that would ensure fertilizing capacity at the desired high level (Gliozzi et al., 2011). The basis of success in obtaining such a number of offspring, which could ensure

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the preservation of a gene pool sufficient for a particular species, is the appropriate male selection and obtaining from them of the high-quality semen, especially in terms of the number of live normal sperm. The success in reproduction depends on these sperm because they possess the highest ability to reach and fertilize the ovum (Blesbois and Brillard, 2007). Experiments on cryopreservation of domestic bird semen, especially chickens, are very common and conducted in many research centers (Blesbois, 2011; Rakha et al., 2017; Mehdipour et al., 2018), however, experiments on evaluation and preservation of semen collected from birds covered by various conservation programs have been conducted relatively recently (Blesbois, 2011; Łukaszewicz, 2006; Svoradová et al., 2019).

Due to the specific properties of gander semen and the way it is collected, the freezing process can greatly reduce the quality of the thawed semen and thus, the fertilizing ability of the sperm. Success in achieving high fertilization rate depends on many factors, including the initial quality of ejaculate, containing as many sperm as possible with a normal morphological structure (Jerysz and Łukaszewicz, 2013; Łukaszewicz et al., 2020 b; Svoradová et al., 2019). One of the crucial factors that have a significant impact on semen quality and its subsequent susceptibility to stress caused by freezing, is the method of semen collection, especially in case of ganders who have an intromittent copulatory organ (*phallus protrudens*) (King, 1981). The dorso-abdominal massage method may release from the cloaca a liquid substance containing glucose, which has a negative effect on sperm storage *in vitro* (Blesbois and Brillard, 2007), and may also cause some contamination with feces, blood or dirt from feathers (Almasi et al., 2002; Łukaszewicz, 2001). The specific structure of bird sperm is also a very important issue. They have a low content of cytoplasm and a large area of cell membrane, rich in fatty acids susceptible to lipid peroxidation (Surai et al., 1998; Partyka et al., 2012). This structure of the sperm makes it very susceptible to the unfavorable effects of the surrounding environment (Blesbois and Brillard, 2007; Long, 2006; Parks and Graham, 1992).

In the previous studies on goose semen cryopreservation, the impact of temperature decrease rate, type of diluents and cryoprotectants on sperm survival ability were analyzed (Chełmońska et al., 1984; Tai et al., 2001; Váradi et al., 2019). The experiments aiming to elaborate an effective and simple freezing protocol (semen diluent, cryoprotectant, equilibration time, temperature decrease rate, straws or pellets, nitrogen vapor or controlled freezing chamber), not only for avian species in general, but even for particular breed, are still ongoing. Based on the freezing method developed for the commercial goose line White Koluda® (Łukaszewicz, 2002 b), we decided to investigate semen susceptibility to freezing process and to establish a gene bank for twelve goose breeds covered by the Polish genetic resources conservation program.

The quality of fresh semen (volume, sperm concentration and morphology) collected individually from goose breeds covered by genetic resources conservation program has been discussed in details in our recent articles (Łukaszewicz et al., 2021, 2022). In the presented experiment, we focused on assessing the influence of goose breed on sperm susceptibility to the freezing process and the possibility of creating the gene bank in the form of frozen semen. It is well known that creating a genetic reserve *ex situ in vivo* is insufficient, as it involves the risk of losing valuable genes, therefore its support with *ex situ in vitro* method is highly recommended. This was also the aim of the research undertaken.

Since semen volume and sperm concentration have no effect on sperm viability after thawing, in the presented study we focused only on freezing process impact on the sperm morphology.

Material and methods

Birds and their maintenance

The experiment was carried out on sexually mature ganders (one-year-old, i.e. in the first reproductive season), randomly selected from twelve breeds (twelve males from each genetic group), kept as genetic reserve flocks in the Waterfowl Genetic Resources Station of the National Research Institute of Animal Production in Dworzyska, Poland. The following breeds were used: local northern breeds: Kartuska (Ka), Pomorska (Po), Rypinska (Ry), Suwalska (Su); local southern breeds: Garbonosa (Ga), Kielecka (Ki), Lubelska (Lu), Podkarpacka (Pd); and four foreign breeds: Kuban (Ku; from former USSR), Landes (LsD), Roman (Ro) and Slovakian (Sl). During the entire experimental period (form the beginning of January till the middle of April), the males were allocated individually in boxes (0.8 m × 1.0 m × 1.0 m) with a deep litter, and kept in unheated facilities, with natural day light and gravity ventilation. Free access to fresh, good quality water and 350–400 g of commercial food for breeding goose were provided daily per male.

Semen collection

One month prior to semen collection, the males were accustomed to this procedure (catching, massage, presence of operators). Dorso-abdominal massage (adopted from Chełmońska, 1967), performed twice a week for every male has been applied. Each day, the semen was collected simultaneously by two, three persons teams, for the first time at approximately 8:00–8:30 (from males of three breeds), and then after 1.5 hours (from another three breeds) on Mondays and Wednesdays, and on Tuesdays and Thursdays from other six breeds. Thanks to such organization, the total time of semen collection (from three breeds, *i.e.* 36 males) did not exceed 20–30 minutes. In order to maximize semen quality and quantity, the collection was performed always under the same conditions (environment, time, persons and massage procedure). The glass tubes used for semen collection have the shape and size adjusted to the size of gander copulatory organ. It was 3 cm in diameter at the mouth and 12 cm long. Clean, uncontaminated single ejaculates (evaluated macro-

scopically at collection for color, contamination: uric, fecal or blood) were transferred with an automatic pipette into the test tube to form uniform, pooled semen sample per every genetic group. Watery and contaminated ejaculates were poured out. Depending on goose breed and day of collection, the number of pooled ejaculates in evaluated semen sample varied between 8 and 12. Semen quality was evaluated (pooled volume, sperm concentration and morphology) immediately after collection, and then subjected to freezing.

Evaluation of sperm morphology

Sperm integrity and morphology (n = 300 cells per slide) was evaluated in nigrosine–eosin stained histological smears, under light microscope, at 1250x magnification (Jenaval, Carl Zeiss, Jena, Germany). Sperm were assigned to seven categories: six of them, named "live in total", were as follows: morphologically normal (typical spindle-shaped head and well-marked acrosome); bulb-head; bent neck; midpiece deformed (swelling, ragged or lack of midpiece); spermatids (immature forms); spermatozoa with other deformations (not included in any of the previous category), and dead sperm (pink stained by eosin) (Łukaszewicz et al., 2011). The results of morphological evaluation were expressed as the percentage of particular sperm categories (300 cells = 100%). To better illustrate the effect of freezing process on sperm morphology, the changes between fresh and thawed semen for individual sperm forms (changes in percentage points, pp) were presented in Table 3 and for live normal cells on Figure 1, as the relative changes in relation to the fresh semen treated as 100%.

Freezing process

Semen diluted at room temperature (about 20–22°C) in 2:1 ratio (two parts of semen: one part of extender) with EK extender (Łukaszewicz, 2001), was placed in the refrigerator for 15 minutes at +4°C, then mixed with 6% (v/v) of cool dimethyl-formamide (DMF, SERVA, Heidelberg, Germany) in a final concentration, respired into 0.25 ml plastic straws and immediately placed in a Minidigitcool 1400 freezer previously chilled to 4°C (IMV, L'Aigle, France, ref. ZB280). After 5 minutes of further equilibration, the semen samples were pre-frozen to –140°C, with linear temperature decrease rate of 60°C/min, then plunged into liquid nitrogen (–196°C) container. Prior to semen evaluation, the straws were thawed for 2–3 sec in 60°C water-bath (Łukaszewicz, 2002 a, b).

Statistical analysis

Shapiro-Wilk test showed no significant deviations from normality, therefore the one-way ANOVA was applied to evaluate the effect of breed and cryopreservation on semen characteristics. The interactions between effects were analyzed by multiple analysis of variance. The significance of differences between the examined gander semen characteristics was determined using Duncan's multiple range test (Statistica, version 12.5, StatSoft, Inc., Kraków, Poland, sp. z o.o.).

Results

Characteristics of the fresh semen

The morphological analysis of fresh semen is presented in Table 1. The percentage of live sperm in total in the pooled fresh semen of all studied goose breeds was high and ranged from 77.7% to 97.7%, the highest average amount was found in semen of Kartuska (94.4%) and Suwalska (94.1%) breeds from the northern varieties, while the lowest in semen of Slovakian (86.4%), Roman (88.2%) and Landes (89.3%) goose belonging to the foreign breeds (Table 1). The recorded differences were statistically significant ($P<0.05$). The share of live normal sperm was significantly lower (from 14.7% in Slovakian to 66.1% in Suwalska goose semen). The percentage of normal sperm in semen of Suwalska (54.2%), Kartuska (52.5%) and Podkarpacka (50.7%) breeds was significantly higher ($P<0.05$) compared to all foreign breeds, Pomorska and some of the southern breeds (Garbonosa, Kielecka, Lubelska). Bulb-head sperm were predominant among the distinguished abnormal forms. In the particular pooled semen samples, their number ranged from 11.0% in Kartuska to 52.7% in Slovakian goose. The highest average share (35.0%) of bulb-head sperm was found in semen of Garbonosa goose and this value was significantly higher ($P<0.05$) than in the majority of other breeds (except Slovakian goose – 29.8%). Noteworthy is the high percentage of spermatids in semen of foreign breeds – Kuban (mean – 13.0%; from 1.7% to 24.7%) and Slovakian (mean – 11.5%; from 5.0% to 19.3%), which was significantly ($P<0.05$) higher than in Suwalska, Lubelska and Rypinska breeds. The average number of bent neck sperm was also rather high, the highest amount was stated in Lubelska gander semen (mean – 13.3%; from 8.3% to 19.7%), that differed significantly ($P<0.05$) comparing to semen of the other breeds. The smallest and similar (but not significant, $P>0.05$) numbers of bent neck sperm were observed in the semen of Podkarpacka, Suwalska and Kielecka ganders (Table 1). We want to pay particular attention to high percentage of the deformed sperm in total (from 24.7% in Kuban to 68.7% in Lubelska goose). The average share of all deformed sperm was the highest in Garbonosa gander semen (53.4%) and it differed significantly ($P<0.05$) from the semen of the other breeds (Table 1).

Characteristics of the frozen/thawed semen

The results of sperm morphology analysis of frozen/thawed semen are presented in Table 2. The highest number of live sperm in total was noted in Garbonosa gander semen (mean – 53.7%; from 29.3% to 64.7%). This value was significantly ($P<0.05$) higher than in the semen of the other breeds. The lowest total number of live sperm was found in Landes gander semen (mean – 22.6%; from 0.0% to 51.3%). The share of live, normal sperm in thawed semen ranged from 0.0% to 41.0%, and its average amount was the highest in semen of Suwalska goose, and then slightly lower in Garbonosa and Kuban goose. In relation to the other breeds, these values were much higher and differed significantly ($P<0.05$) (Table 2).

Table 1. Fresh semen morphology of ganders covered by genetic resources conservation program (means ± SD)

Goose breed		Sperm morphological forms (%)							
		Live in total	Live normal	Bulb head	Spermatids	Bent neck	Other deformed	Dead sperm	Deformed together**
North goose breeds	Kartuska	94.4±2.1 f	52.5±6.7 a	20.7±4.3 c	4.9±2.1 cde	8.8±2.4 de	7.4±2.7 cd	5.6±2.1 f	37.0±5.2 e
	Pomorska	90.8±3.4 bcd	38.5±8.6 de	26.5±8.9 bc	4.4±1.9 cde	12.6±3.7 ab	8.7±2.9 bc	9.2±3.4 bcd	47.9±7.6 abc
	Rypinska	91.9±1.6 cdef	47.9±4.5 abc	24.4±4.1 bc	3.8±1.8 de	6.9±1.6 ef	8.9±2.2 bc	8.1±1.6 cdef	40.3±4.2 de
	Suwalska	94.1±2.6 ef	54.2±7.3 a	22.4±6.4 c0	3.2±1.1 e	5.6±1.9 f	8.7±1.9 bc	5.9±2.6 ef	36.8±7.5 e
South goose breeds	Garbonosa	90.7±3.5 bcd	31.1±10.9 f	35.0±7.1 a	6.3±1.7 bcd	5.9±2.9 f	12.5±3.3 a	9.3±3.5 bcd	53.4±8.0 a
	Kielecka	93.6±2.2 def	44.7±7.4 bcd	25.3±6.5 bc	7.2±3.5 bc	6.5±1.9 ef	9.9±2.2 b	6.4±2.2 def	41.6±7.0 cde
	Lubelska	91.1±5.4 cde	38.4±12.0 de	25.2±6.6 bc	3.5±0.8 de	13.3±3.9 a	10.8±3.4 ab	8.9±5.4 cde	49.2±9. 8ab
	Podkarpacka	93.4±2.2 def	50.7±7.3 ab	21.7±5.3 c	5.8±2.2 bcde	5.4±2.1 f	9.6±2.0 b	6.6±2.2 def	36.8±6.5 e
Foreign goose breeds	Kuban	92.5±3.1 def	41.6±5.7 bcd	23.0±5.8 c	13.0±7.4 a	8.8±2.2 de	6.1±1.9 d	7.5±3.1 def	37.9±6.5 e
	Landes	89.3±3.6 bc	40.1±9.2 de	24.0±5.1 bc	4.1±2.4 de	11.8±4.1 abc	9.3±3.7 b	10.7±3.6 bc	45.0±7.0 bcd
	Roman	88.2±3.7 ab	33.4±9.7 ef	26.9±7.3 bc	8.2±2.8 b	10.3±2.3 bcd	9.3±2.9 b	11.8±3.7 ab	46.5±8.2 bcd
	Slovakian	86.4±4.4 a	29.2±8.3 f	29.8±10.3 ab	11.5±4.3 a	9.4±3.3 bcd	6.5±1.2 d	13.6±4.4 a	45.7±11.0 bcd

a–f – mean values in columns followed by different letters differ significantly (P<0.05).
**Deformed together means the sum of: bulb head + bent neck + other deformations.

Table 2. Morphology of frozen/thawed semen of ganders covered by genetic resources conservation program (means ± SD)

Goose breed		Sperm morphological forms (%)							
		Live in total	Live normal	Bulb head	Spermatids	Bent neck	Other deformed	Dead sperm	Deformed together**
North goose breeds	Kartuska	44.7±17.4 bc	19.6±8.0 bcd	10.1±3.7 cd	1.6±1.4 b	7.3±3.6 ab	6.1±3.7 de	55.3±17.4 bc	23.5±9.7 abc
	Pomorska	45.3±13.0 bc	13.1±5.4 defg	8.6±2.9 de	2.5±1.6 b	9.6±5.1 a	11.4±4.4 a	54.7±13.0 bc	29.6±10.5 a
	Rypinska	41.2±8.8 bc	13.6±4.3 defg	15.6±3.4 a	1.8±1.1 b	3.8±2.1 cd	6.4±3.2 cde	58.8±8.8 bc	25.8±8.2 ab
	Suwalska	49.6±11.0 bc	28.4±7.2 a	9.7±2.8 cd	1.2±1.0 b	5.3±2.9 bcd	4.9±1.7 e	50.4±11.0 bc	19.9±5.8 bcd
South goose breeds	Garbonosa	53.7±11.0 c	22.6±4.9 abc	14.0±4.2 ab	2.8±1.6 b	4.2±1.5 cd	10.1±3.4 ab	46.3±11.0 c	28.3±7.0 a
	Kielecka	36.2±15.5 b	11.7±6.9 efg	13.8±5.8 ab	1.9±1.1 b	3.4±1.5 cd	5.4±3.2 e	63.8±15.5 b	22.6±9.3 abc
	Lubelska	46.0±10.6 bc	18.5±5.5 bcd	12.3±3.3 abc	1.7±1.1 b	7.8±3.4 ab	5.8±1.0 e	54.0±10.6 bc	25.8±6.1 ab
	Podkarpacka	36.2±15.9 b	17.5±9.7 bcde	9.4±4.2 cd	1.8±1.1 b	2.7±1.7 d	4.7±2.6 e	63.8±15.9 b	16.8±7.7 cd
Foreign goose breeds	Kuban	43.8±17.0 bc	24.0±10.8 ab	5.5±2.3 ef	5.9±4.7 a	4.5±1.8 cd	3.8±2.1 e	56.2±17.0 bc	13.9±4.9 d
	Landes	22.6±17.0 a	7.4±7.4 g	4.4±3.3 f	1.1±1.0 b	5.3±4.7 bcd	4.4±3.5 e	77.4±17.0 a	14.1±9.8 d
	Roman	36.4±7.2 b	10.7±4.1f g	8.3±4.0 de	3.0±1.7 b	5.8±2.6 bc	8.5±1.8 bcd	63.6±7.2 b	22.7±4.7 abc
	Slovakian	46.3±14.9 bc	16.8±7.7 cdef	11.5±3.7 bcd	4.6±3.2 ab	4.5±1.8 cd	8.9±3.2 abc	53.7±14.9 bc	24.9±7.5 ab

a–g – mean values in columns followed by different letters differ significantly (P<0.05).
**Deformed together means the sum of: bulb head + bent neck + other deformations.

Table 3. The decline (in pp) of particular sperm forms in thawed gander semen compared to the fresh semen indicating the effect of cryopreservation process[1]

Goose breed		Sperm morphological forms (%)							
		Live in total	Live normal	Bulb head	Spermatids	Bent neck	Other deform.	Dead sperm	Deformed together**
North goose breeds	Kartuska	49.7#	32.9#	10.6#	3.3#	1.5	1.3	49.7#	13.5#
	Pomorska	45.5#	25.4#	17.9#	1.9	3.0#	2.6	45.5#	18.3#
	Rypinska	50.7#	34.3#	8.8#	1.0	3.1	2.6#	50.7#	14.5#
	Suwalska	44.5#	25.8#	12.7#	2.0	0.3#	3.8#	44.5#	16.9#
South goose breeds	Garbonosa	37.0#	8.5#	21.0#	3.5#	1.7	2.4	37.0#	17.4#
	Kielecka	57.4#	33.0#	11.5#	5.3#	3.1#	4.5#	57.4#	19.0#
	Lubelska	45.1#	19.9#	12.9#	1.8	5.5#	5.0#	45.1#	23.4#
	Podkarpacka	57.2#	33.2#	12.3#	4.0#	2.7	4.9#	57.2#	20.0#
Foreign goose breeds	Kuban	48.7#	17.6#	17.5#	7.1#	4.3#	2.3	48.7#	24.0#
	Landes	66.7#	32.7	19.6#	3.0#	6.5#	4.9#	66.7#	30.9#
	Roman	51.8#	22.7#	18.7#	5.2#	4.5#	0.8	51.8#	23.8#
	Slovakian	40.1#	12.4#	18.3#	6.9#	4.9#	2.4	40.1#	20.8#

[1]Presented values constitute the differences between fresh and frozen/thawed semen.
#Indicates significant differences (P<0.05) between fresh and frozen/thawed semen in participation of particular sperm forms (amount in %).
**Deformed together means the sum of: bulb head + bent neck + other deformations.

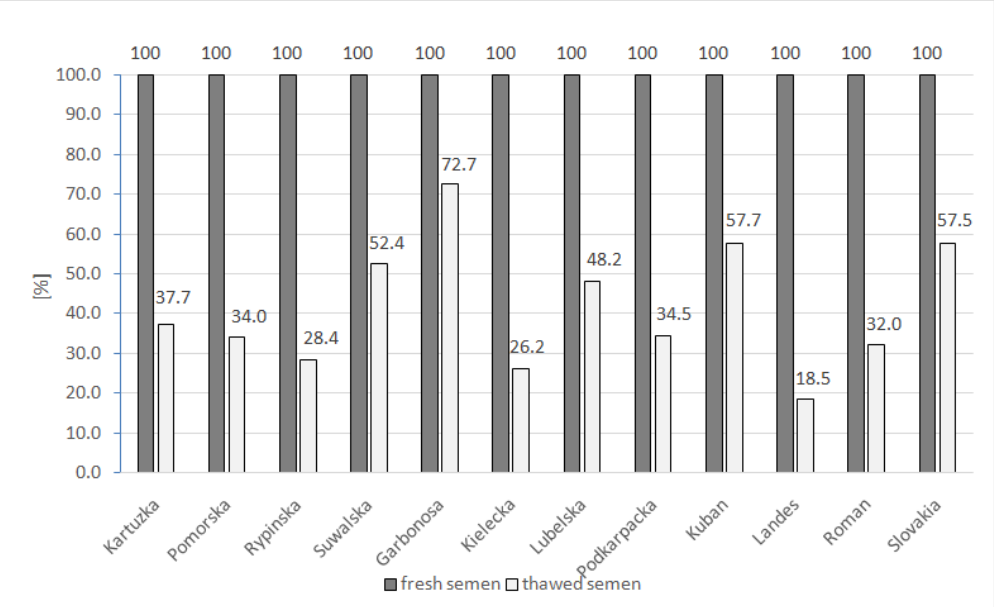


Figure 1. Changes in percentage of live sperm in total in the frozen-thawed semen in relation to their content in the fresh semen (%). With the exception of Landes goose, all declines were statistically significant (P<0.05)

Similarly, as in the fresh semen, the bulb head sperm were the most frequent among the deformed forms (from 0.0% to 23.7%) in the thawed semen, and the highest percentage of them was found in the semen of Rypinska ganders, while the smallest amounts in the semen of foreign breeds – Landes and Kuban (Table 2).

The content of bent neck sperm was the highest in thawed Pomorska goose semen (mean – 9.6%; from 3.3% to 22.0%). In the semen of other goose breeds, there were much less sperm with this deformation, and the lowest share was found in the semen of Podkarpacka ganders (mean – 2.7%; from 0.0% to 5.7%).

The total number of sperm with various deformations was similar to that in the fresh semen, ranging from 0.0% to 52.3%. The highest number of deformed sperm was observed in Pomorska gander semen (29.6% on average). This value was significantly higher (P<0.05) in comparison to semen of Suwalska, Podkarpacka, Kuban and Landes ganders.

The effect of semen freezing on goose sperm morphology

The statistical analysis of frozen/thawed semen showed that, comparing to the fresh semen, the smallest reduction in the amount of live sperm in total was in Garbonosa goose semen – by 37.0 pp (percentage points; Table 3; decrease from 90.7% in the fresh to 53.7% in the thawed semen), and the highest in semen of Landes goose – by 66.7 pp (Table 3) (from 89.3% to 22.6%; Table 1 and Table 2). Also, in terms of the live normal sperm, the lowest decrease occurred in Garbonosa gander semen – by 8.5 pp (decrease from 31.1% to 22.6%), while the highest – by 34.3 pp (from 47.9% to 13.6%) in semen of Rypinska breed. Comparing to the fresh semen, the amount of bulb head sperm in the semen after thawing decreased by 8.8 pp in Rypinska gander semen (from 24.4% to 15.6%) to 21.0 pp in Garbonosa goose (from 35.0% to 14.0%). The total amount of deformed sperm decreased by 13.5 pp in Kartuska (from 37.0% to 23.5%) to 30.9 pp in Landes ganders (from 48.5% to 25.8%). The increase in the amount of eosin-stained sperm (dead one) in thawed semen resulted from both the death of live normal sperm, as well as from previously damaged cells, that did not survive the freezing process.

We would like to draw particular attention to how important the way of analyzing and interpreting the results obtained is. Comparing the data presented in Table 1 and Table 2, it is difficult to clearly determine whether the quality of fresh semen is the only factor influencing the quality of thawed semen. It is a bit easier to draw conclusions by analyzing the differences (decrease in percentage points) of individual sperm forms between fresh and thawed semen (Table 3). They show that in semen of Kartuska, Kielecka, Podkarpacka and Landes goose, the decrease in the amount of live normal sperm was similar and fluctuated around ±30%. However, it does not clearly indicate the role of fresh semen quality and its susceptibility to freezing process. Calculating the percentage of particular sperm in thawed semen in relation to the fresh semen treated as 100% shows the real differences in sperm susceptibility to freezing. Among the breeds mentioned above, the highest percentage of live sperm after thawing (37.3% in relation to the fresh semen) was found in the semen of the Kartuska goose, and the lowest (18.5%) in the Landes (Figure 1). In case of Garbonosa goose, where live normal sperm in the fresh semen constituted only 31.1%, 72.7% of them survived the freezing process, while in semen of Suwalska goose (54.2% of live normal cells) only 52.4% (Figure 1). Such comparison confirms the evident goose breed effect on semen susceptibility to freezing.

Discussion

Our first studies on semen evaluation of goose covered by the genetic conservation program were carried out in the 1980s. They mainly concerned the analysis of fresh and frozen semen of Kuban goose (Chełmońska et al., 1984). After a long break, the semen of Biłgorajska (Opałka et al., 2008) and Zatorska (Gumułka and Rozenboim, 2015) goose was assessed. Regardless of our research, similar experiments were conducted in France on the freezing of Landes goose semen (Dubos et al., 2006; Váradi et al., 2019), and in the Slovak Republic – on local Slovak goose (Svoradová et al., 2019).

In the discussed studies, where the susceptibility to freezing regime of semen of 12 goose breeds from gene conservation flocks has been assessed for the first time, we found significant differences in the quality of both the fresh and thawed semen. In general, the quality of semen obtained from northern goose breeds was better than that of southern and foreign breeds. Their semen had more live in total and live morphologically normal sperm, and fewer deformed and dead sperm. At the onset of semen collection, despite being of the same age, the northern ganders were better "matured", because their semen contained less spermatids (immature forms of sperm), especially in comparison to the foreign breeds. The breed effect on semen quality has also been found in studies conducted on other bird species (Siudzińska and Łukaszewicz, 2008; Zawadzka et al., 2015). Observed differences may indicate a different course of spermatogenesis in individual males, as well as different stages of sexual maturation.

Chełmońska et al. (1984) observed a lower content of live sperm in the fresh semen of Kuban goose, both compared to the results obtained by us in a similar genetic group as well as in relation to all northern and southern goose breeds. Values similar to those obtained in our experiment for the local goose breeds were described by Gumułka and Rozenboim (2015) for Zatorska goose, and Opałka et al. (2008) for Biłgorajska goose, which are also covered by the genetic resources conservation program. Váradi et al. (2019) found a lower content of live sperm in semen of Landes ganders, not only compared to those examined in our experiment, but also compared to all ganders of the local northern and southern Polish breeds. Almasi et al. (2002) and Varga et al. (2003) who analyzed the fresh semen of Hungarian ganders also pointed to a low semen quality of local goose breeds kept as gene pool flock. The percentage of live sperm was lower compared to our results obtained in the northern and southern varieties, as also observed by Iolchiev et al. (2018) in *Anser anser* L. goose semen and Tai et al. (2001) in Chinese brown goose, which also has been observed in Biłgorajska (Opałka et al., 2008) and Kuban goose (Chełmońska et al., 1984).

Information of Dubos et al. (2006) indicated that they obtained a similar amount of live normal sperm for Landes ganders as in our study, whereas Liu et al. (2008, 2014), who analyzed semen of Yangzhou, Zi and Rhin goose breeds, recorded a similar amount of normal sperm to that obtained by us in the southern and foreign breeds, and Al-Daraji et al. (2011) – in semen of local Iraqi ganders.

In the present study, we found that in the fresh semen, bulb head and bent neck sperm were predominating among all deformed sperm. These observations are consistent with those obtained by Gumułka and Rozenboim (2015) and Opałka et al. (2008). This relationship was also found in our previous studies conducted on other bird species (Kowalczyk et al., 2012; Łukaszewicz et al., 2020 a; Zawadzka et al., 2015).

For many years, the authors of the presented paper have been conducting intensive experiments on assessing the semen quality of various goose species and breeds, as well as the other bird species. Obtained results indicate that the sperm is highly diversified, especially in terms of the morphological structure (Kowalczyk and Łukaszewicz, 2012; Łukaszewicz, 2001, 2002 a, 2006; Łukaszewicz and Kruszyński, 2003; Łukaszewicz et al., 2003, 2004).

The data discussed in the presented studies shows that despite breed, the total amount of live sperm in the fresh gander semen is at a similar and rather high level, while significant differences occur in the content of live normal sperm, which are the most valuable and desired from fertilizing ability viewpoint. Therefore, when analyzing the quality of gander semen, especially in terms of its further use, e.g. for artificial insemination, short- or long-term storage *in vitro*, it is not enough to consider the number of live sperm in total, because only normal sperm with intact morphological structure have a potentially full fertilizing ability.

The freezing process significantly reduced the content of live in total and live normal sperm, with a simultaneous increase in dead sperm in the semen of all assessed gander groups. The highest number of live sperm in total was found in the semen of southern variety – Garbonosa, while live normal cells in the semen of northern breed – Suwalska. The lowest amount of these two forms was observed in the semen of Landes ganders, belonging to the foreign breeds. Similarly as in the fresh semen, the bulb head sperm constituted the highest percentage among the deformed forms also in the thawed semen.

Similarly, in our previous experiments, a decrease in the number of live sperm in total and live normal sperm caused by freezing process was found, regardless of species and breed – in Graylag gander semen (Łukaszewicz et al., 2004; Chełmońska et al., 2004), Canada goose (Zaik, 2013; Kowalczyk and Łukaszewicz, 2012), White Italian ganders (Łukaszewicz, 2001), White Koluda goose (Łukaszewicz, 2006; Chełmońska et al., 2004) and in Kuban ganders (Chełmońska et al., 1984). Our results were also confirmed by reports of the other authors. Iolchiev et al. (2018) reported a decrease in the number of live sperm in semen of *Anser anser* goose and Tai et al. (2001) – in the Chinese brown goose, while Dubos et al. (2006) and Váradi et al. (2019) – in semen of Landes ganders.

Artificial insemination, routinely used in chicken, duck and turkey reproductive farms still causes many problems in goose reproduction, therefore it must be constantly improved. One of the main problems is a poor quality of the fresh semen, as well as a low survival rate of sperm caused by damaged integrity of cell membrane occurring during freeze-thawing processes (Olexikova et al., 2019; Parks and Graham, 1992). Long (2006) suggests that essential sperm functions being disrupted by cryogenic protocols involve compromises to the plasma membrane. Also, Blesbois et al. (2005) and Gliozzi et al. (2011) reported that cryopreservation causes a significant decrease in the viability and integrity of the sperm cell membrane, contributing to a significant increase in the percentage of dead sperm. As reported by Blesbois et al. (2005), the fluidity of the cell membrane is an indicator of the ability of sperm to freezing.

Conclusion

The obtained results confirmed the breed effect on fresh semen quality of ganders and its susceptibility to freezing procedure. When analyzing the quality of goose semen, due to great differences in amount of live sperm in total and live normal cells, both forms should be considered not only the number of live sperm in total. The freezing process caused a significant ($P<0.05$) reduction in the number of live in total and live normal sperm, and increase in dead sperm, in all groups of goose; however, the impact of goose breed on freezing efficiency has been observed. When evaluating semen susceptibility to freezing procedure on the basis of sperm morphology, the relative changes in relation to the fresh semen should be considered.

Ethics approval

The study was carried out in strict accordance with the recommendations of the National Ethics Commission (Warsaw, Poland), and according to Polish law and the EU directive (no 2010/63/EU), the experiment conducted within this study did not require the formal approval of the Ethics Commission. Semen collection is a routine procedure for poultry reproductive flock. All members of the research staff were trained in animal care and handling by PolLASA (Polish Laboratory Animal Science Association).

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