Effect of semen extenders on viability of ISA Brown and Hubbard Flex roosters' sperm stored for 24 h

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ABSTRACT Artificial insemination is used in almost 95% of turkey reproductive flocks and is becoming more important in chickens, particularly broiler breeders, as well as in assisted reproduction of wild birds kept in breeding centers. Diluted semen is recommended for artificial insemination. Pooled semen samples collected twice a week by dorso-abdominal massage from 2 chicken lines: laying—ISA Brown (**ISA-B**) and meat type—Hubbard Flex (**H-F**) were divided into 5 parts: neat semen and diluted in 1:2 ratio with 4 extenders: basic EK; EK + 1 μg/mL organic selenium and 8 μg/mL vitamin E; EK + 10 mg/mL of royal jelly; and EK + 0.25 g/mL of lyophilized bovine colostrum. Diluted semen samples were evaluated after 15 min and then 24 h storage at 4°C. Sperm concentration, motility, motility parameters (with Sperm Class Analyzer), and morphology were evaluated in the neat semen, whereas in diluted and stored samples, the last 3 traits were determined. In case of both lines, dilution did not affect ($P > 0.05$) the number of live normal cells (78.0–81.1% in ISA Brown and 73.8–68.7% in Hubbard Flex) in relation to neat semen; however, bovine colostrum addition increased ($P < 0.05$) the percentage of bulb head sperm (5.7 vs. 10.0% and 12.1 vs. 17.6%, for ISA and Hubbard, respectively) and decreased sperm motility (67.4 vs. 92.9% and 67.3 vs. 98.5% for ISA and Hubbard). The 24 h storage of neat semen and semen diluted with colostrum caused ($P < 0.05$) the unfavorable changes in all evaluated traits and both chicken lines, whereas semen dilution with remaining extenders decreased the percentage of live normal cells (by 18.8–23.4% ISA and by 20.9–25.5% Hubbard) but did not affect sperm motility (81.5–87.6% for ISA and 81.1–96.6% for Hubbard). Sperm motility and motility parameters depended both on the extender and chicken line.

Key words: rooster semen, selenium, vitamin E, royal jelly, bovine colostrum

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INTRODUCTION

The constant increase in the world's population and the related growing demand for products of animal origin, including poultry (eggs, meat) necessitate the development of modern solutions to increase production efficiency at reducing its costs. In case of poultry reproduction, artificial insemination that allows to decrease the number of males in the flock, certainly contributes to this solution. The semen intended for insemination must be of very good quality, which depends, among others, on genetic origin (Siudzińska and Łukaszewicz, 2008a; Tabatabatei et al., 2009; Kowalczyk and Łukaszewicz, 2012; Ameen et al., 2014; Kotłowska et al., 2005), content of nutrients, especially antioxidants (Dimitrow et al., 2007; Jerysz and Łukaszewicz, 2013) that affect tissue activity during spermatogenesis (Surai et al., 1998; Barber et al., 2005), type of semen extenders selected for the species (Marzoni et al., 2003; Hudson et al., 2016; Kowalczyk et al., 2017), time elapsing from semen collection, and storage conditions before insemination (Siudzińska and Łukaszewicz, 2008b).

There are many different criteria for assessing semen quality. The most desired and valuable is semen containing as many live, viable sperm with proper morphological structure as possible, because only such cells have the highest potential fertilizing ability. However, it is well known that freshly collected, neat avian semen stored in vitro very quickly loses its viability and motility (Jafar et al., 2013; Das et al., 2015) and as the consequence, the fertilizing ability (Hudson et al., 2016). There is a growing interest in developing an efficient environment for storing semen in a liquid stage for prolonged time without significant decrease in its quality and fertilizing potency.

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A properly composed semen extender should provide optimal osmotic pressure, that is one which is close to the value of osmotic pressure of seminal plasma (Iaffaldano et al., 2005), as well as substances which support sperm viability and prevent from excessive lipid peroxidation. Extenders' osmotic pressure for poultry semen should vary between 250 and 460 mOsm/kg H_2O (Donoghue and Wishart, 2000; Iaffaldano et al., 2005). In the isotonic environment, sperm shows the highest metabolic activity, and any deviation from the norm can disrupt the integrity of their cell membranes and increase the population of dead sperm (Latif et al., 2005). Avian sperm are characterized by a high content of polyunsaturated fatty acids susceptible to peroxidation (Surai et al., 1998); therefore, one of the possibilities to improve the quality of semen designed for artificial insemination is the choice of appropriate components, especially those showing antioxidant properties. They can protect cell membranes and prevent the release of lipids and phospholipids from sperm into semen plasma during prolonged storage (Dimitrov et al., 2007). Certainly, selenium and vitamin E possess such properties (Barber et al., 2005; Jerysz and Łukaszewicz, 2013; Safa et al., 2016; Kowalczyk et al., 2017). The royal jelly (**RJ**)—a secretory product of cephalic glands of nurse bees that serves as the most important diet of honeybee larvae (Nagai et al., 2001; Pavel et al., 2011; Moradi et al., 2013) and bovine colostrum—a product of mammary gland secretion, which is of particular importance for the health of offspring, due to the content of both non-enzymatic antioxidants such as vitamin E, A, and C, but also microelements such as selenium, copper, and zinc (Przybylska et al., 2007), are also recognized as antioxidants. There is no information on the effect of the RJ and bovine colostrum addition to semen extender on quality of the avian sperm. Only the beneficial effect of colostrum as feed additive on quality of quail eggs was reported (Bayril et al., 2017) and expressed as the reduction of malondialdehyde content in egg yolk, which confirmed antioxidant properties of colostrum. Considering the properties of RJ and bovine colostrum, we have decided to test the effectiveness of both additives to semen extender for liquid storage of 2 chicken lines semen.

MATERIALS AND METHODS

Birds and Semen Collection

Adults reproductive roosters (40-week-old at the onset of the experiment) of 2 lines: laying type–ISA Brown (**ISA-B**) and meat type–Hubbard Flex (**H-F**) were used as the semen donors. Each line was represented by 5 males previously selected from a larger population based on their response to massage and macroscopic evaluation of ejaculated semen. Semen donors were kept individually in large (60 × 50 × 75 cm) cages with straw-lined floor, under controlled environmental conditions (roofed room with a temperature of 18°C to 20°C and mechanical ventilation), and water was provided *ad libitum*, while feed according to the guidelines for particular line. Roosters were provided with appropriate environmental conditions to ensure a full welfare. For this type of experiments (semen collection), permission from the Local Ethics Commission for Experiments Carried on Animals is not required.

Semen was collected twice a week by dorso-abdominal massage (Burrows and Quinn, 1937). To maximize semen quality and to avoid roosters' stress, the collections were always performed by the same 2 persons and under the same conditions (time, massage procedure). Individual ejaculates of good quality evaluated visually were pooled within the line by transferring with automatic pipette to create one uniform semen sample. Semen collection was completed within 5 to 10 min.

Extenders and Semen Dilution

Collected semen samples were divided into 5 parts: neat semen and semen diluted threefold (1 part semen: 2 parts extender) with 4 extenders: basic EK (Łukaszewicz, 2002) (samples marked as **EK**); EK + 1 μg/mL organic selenium (selenomethionine; Sigma Aldrich) and 8 μg/mL vitamin E (TROLOX, Sigma–Aldrich) (marked as **EK + Se + E**) (Zawadzka and Łukaszewicz 2012); EK + 10 mg/mL of RJ (BARTPOL, Poland) (**EK + RJ**); and EK + 0.25 g/mL of lyophilized whey of bovine colostrum (**EK + COL**). The composition of EK extender was as follows: 0.14 g potassium citrate, 0.21 g sodium dihydrogen phosphate, 0.98 g disodium hydrogen phosphate, 0.7 g glucose, 1.4 g sodium glutamate, 0.2 g D-fructose, 0.7 g inositol, 0.1 g polyvinylpyrrolidone, and 0.02 g protamine sulfate per 100 mL of bi-distillate water. All components had a pure analytical quality and were supplied by POCH (Gliwice Poland); Fluka (Buchs, Germany), or Sigma (St. Louis, MO, USA). Colostrum was collected to the sterile 50 mL polypropylene container from a healthy cow, from the first milking after calving (within 2 h after calving) and then subjected to a freeze-drying process in Alpha 1-4 LSC freeze dryer (CHRIST, Germany). The osmotic pressure of the tested extenders was as follows: EK—385 mOsmol kg^{-1}; EK + Se + E—370; EK + RJ—430 and EK + COL—480 mOsmol kg^{-1} (Semimicro osmometer Type ML, Knauer, Berlin, Germany).

Diluted samples, placed in 1 mL sterile glass tubes, were evaluated within 15 min at room temperature (20°C–22°C), then covered with a cotton plug, transferred to the refrigerator and left at 4°C for 24 h. After this period, the samples were gently mixed with an automatic pipette and subjected to further evaluation. For every procedure, 12 repetitions were made.

Evaluation of Semen Samples

In the freshly collected pooled, neat semen the following parameters were evaluated: volume (with automatic pipette); sperm concentration (with hemocytometer using 3% eosin-NaCl solution (v/v) and Thoma-Zeiss chamber); motility and motility parameters; morphology and

osmotic pressure (Semi-Micro Osmometer, Knauer, Germany), whereas in the diluted semen (after 15 min) and samples stored for 24 h, the last 4 traits were determined.

Sperm morphology was examined on the basis of histological smears vital stained with nigrosine-eosin, at 1250× magnification (Nikon Eclipse E100 light microscope). In every smear, the dead sperm, live in total, live normal (morphologically undamaged and unchanged), bulb head, bent neck, and other deformities (not included to any former class) were distinguished. All pink-stained sperm were counted as dead. The results were expressed as the percentage of particular categories of sperm (300 cells evaluated in every slide = 100%; Łukaszewicz et al., 2008). Motility and motility parameters were examined using Sperm Class Analyzer (SCA system, Version 5.1, Microptic, Barcelona, Spain), light microscope (Nikon Eclipse E200), with × 10 negative phase objective, Basler camera (scA 780-54fc, Ahrensburg, Germany), warm stage, and computer to analyze and store data. Prior analysis, semen was diluted 1:60 with warm (25°C) physiological saline. Four µL of prepared sample was placed in a Leja 8 analysis chamber (Leja Products B.V., Holland) of thickness 20.0 µm. Following motility parameters were analyzed: percentage of motile sperm, curvilinear velocity (**VCL**), straight-line velocity (**VSL**), path velocity (**VAP**), linearity (**LIN**), and amplitude of lateral head displacement (**ALH**).

Statistical Analysis

Data of the fresh semen characteristics were analyzed by one-way ANOVA. The impact of main effects (chicken line, extenders, time of storage) and interactions between effects were analyzed by multiple analysis of variance. The significance of differences between the examined groups was determined using the Duncan test (Statistica, version 12.5 StatSoft, Inc., Kraków, Poland, sp. z o.o.).

RESULTS

Chicken Line Effect–Fresh Semen Quality

The comparative analysis of assessed rooster lines fresh semen is presented in Table 1. The genetic line of roosters affected the osmotic pressure, sperm concentration, and motility parameters. Higher, however statistically not significant ($P > 0.05$) value of osmotic pressure and sperm concentration was observed for semen of H-F meat line compared with the ISA-Brown (ISA-B) laying line. In the ISA-B semen, a higher number ($P < 0.05$) of live normal sperm and higher ($P > 0.05$) volume of pooled ejaculates was recorded. Moreover, a significant ($P < 0.05$) line effect on the percentage of damaged and abnormal forms was measured. The bulb head sperm were the most frequent (Table 1).

In motility parameters, significant differences ($P < 0.05$) were found only in straight-line velocity (VSL), considered more favorable in ISA-B sperm. Other motility values did not differ significantly ($P > 0.05$), although sperm motility was higher in H-F sperm and the remaining parameters in ISA-B sperm (Table 2).

Extender and Storage Effect on Laying Line ISA Brown Sperm Quality

The effect of extender composition and storage time on semen characteristics of ISA-B roosters is presented in Tables 3 and 4. Semen dilution with tested extenders had significant ($P < 0.05$) effect on osmotic pressure, the total number of live sperm and bulb head sperm, as well as on percentage of motile sperm and motility parameters. The osmotic pressure was the highest in sample with bovine colostrum (EK-COL)—530.0 ± 14.1 mOsmol kg^{-1} vs. 303.6 ± 3.8 mOsmol kg^{-1} in the neat semen (Table 3). Only the addition of EK + RJ (sample evaluated 15 min after dilution) caused the decrease in total number of live sperm, whereas the presence of colostrum in the basic EK extender (EK + COL) caused an increase ($P < 0.05$) in bulb head sperm. However, there was no extender effect ($P > 0.05$) on the percentage of live normal, bent neck sperm, and sperm with other deformities (Table 3). ISA-B semen dilution itself, generally reduced sperm motility, but only in the EK + RJ and EK + COL, the differences were significant ($P < 0.05$). Also almost all motility parameters (VCL, VSL, VAP, LIN, and ALH) were higher in the neat samples compared with those diluted with any of tested extenders.

Semen storage for 24 h at 4°C caused different changes in ISA-B semen quality. Negative and significant ($P < 0.05$) changes in sperm morphology, regardless the extender's additives were observed, whereas osmotic pressure remained unchanged (Table 3). In the neat semen, the number of live normal cells decreased, compared with unstored sample, by 59.0% (22.6 vs. 81.6%), whereas in the diluted semen, it varied from 20.9 (for EK + Se + E, most effective) to 32.6 (for EK + COL, less effective). The EK + COL extender was also the least effective because of high proportion of damaged sperm (Table 3), as well as

Table 1. Comparison of ISA Brown and Hubbard Flex roosters' fresh semen quality (n = 12; means; SEM).

Chicken line	Pooled ejaculate volume [mL]	Sperm concentration [n x$10^9$$mL^{-1}$]	Osmotic pressure [mOsmol kg^{-1}]	Morphological forms [%]				
				Live in total	Live normal	Bulb head	Bent neck	Other deform.
ISA Brown	2.23	401.7[b]	303.6[b]	93.0	81.4[a]	5.7[b]	2.1	3.6
Hubbard Flex	2.10	464.1[a]	310.8[a]	92.1	74.0[b]	12.1[a]	2.8	3.0
SEM	0.147	12.78	9.852	0.671	1.558	1.174	0.470	0.340
Effect[1]	0.678	0.011	0.048	0.516	0.013	0.003	0.437	0.341

[a,b]means in columns followed by different superscripts differ significantly ($P < 0.05$).
[1]Interactions between the main effect (chicken line) were not significant ($P > 0.05$).

Table 2. Comparison of ISA Brown and Hubbard Flex roosters' fresh semen motility and motility parameters (n = 12; means; SEM).

		Motility parameters				
Chicken line	Sperm motility [%]	VCL [Mms^{-1}]	VSL [μms^{-1}]	VAP [μms^{-1}]	LIN [%]	ALH [μm]
ISA Brown	92.9	91.2	39.8^{a}	60.7	42.9	3.8
Hubbard Flex	98.5	80.4	32.4^{b}	51.6	40.6	3.8
SEM	1.615	3.148	3.562	3.199	2.970	4.627
Effect[1]	0.097	0.094	0.043	0.068	0.251	0.804

$^{a\ b}$means in columns followed by different superscripts differ significantly ($P < 0.05$).

Abbreviations: ALH, amplitude of lateral head displacement; LIN, linearity; VAP, path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

[1]Interactions between the main effect (line) were not significant ($P > 0.05$).

poor motility and motility parameters (Table 4). Only 9.3% of the sperm was motile after 24 h storage, whereas in EK + Se + E, there was still 92.0%, and VCL was 9.0 and 52.7, respectively. As in case of sperm morphology, the EK extender enriched with Se + E had also the most beneficial effect on sperm motility after 24 h storage. All motility parameters were the lowest in semen with EK + COL addition, followed by those in the neat semen. After 24 h storage in EK, EK + Se + E, and EK + RJ sperm motility was similar ($P > 0.05$), and it remained at similar level (decrease in value from 0.5 to 4.9%) in diluted but unstored semen, whereas in the neat semen and diluted with EK + COL, motility was drastically ($P < 0.05$) reduced (by 58%). Also, some motility parameters were affected by 24 h semen storage, despite the extender used (Table 4).

Extender and Storage Effect on Meat Type Hubbard Flex Sperm Quality

The impact of analyzed extenders on meat line H-F rooster semen (Table 5 and Table 6) was very similar to those of laying line ISA-B (Table 3 and 4). Significant changes were found in the osmotic pressure, total number of live sperm, bulb head sperm, as well as in sperm motility and motility parameters (except LIN parameter). The content of live sperm in total was the lowest in the basic EK extender but differed significantly ($P < 0.05$) only when compared with semen with EK + COL. The effect of extender composition on bulb head sperm was different than in ISA-B semen. The highest ($P < 0.05$) amount of these deformities was found in the semen with EK + COL and EK alone. No significant ($P > 0.05$) effect of extenders on the content of live normal, bent neck, and sperm with other deformities was observed (Table 5).

Despite extender used, the dilution of semen generally reduced the sperm motility and motility parameters, with the exception of LIN, which was similar ($P > 0.05$) in all samples and ranged between 40.6% (neat semen evaluated 15 min after collection) and 44.1% (EK + RJ). The highest percentage of motile sperm ($P < 0.05$) was observed in the fresh neat sperm and the lowest in sperm with the addition of bovine colostrum (EK + COL). In EK, EK + Se + E, and EK + RJ, the percentage of motile sperm was similar

Table 3. Extender and storage effect on ISA Brown roosters' semen osmotic pressure and sperm morphology (n = 12; means; SEM).

		Morphological forms [%]				
Semen sample	Osmotic pressure [mOsmol kg^{-1}]	Live in total	Live normal	Bulb head	Bent neck	Other deform
Neat						
Fresh	303.6^{d}	93.0^{a,b,1}	81.6^{1}	5.7^{b}	2.1^{1}	3.6^{1}
Stored	300.8^{c}	71.1^{c,2}	22.6^{c,2}	7.1^{b}	32.5^{a,2}	8.2^{2}
EK						
Fresh	400.7^{b}	92.1^{a,b,1}	81.1^{1}	5.3^{b,1}	2.0^{1}	3.4^{1}
Stored	392.5^{b}	79.2^{b,2}	57.7^{a,2}	9.0^{b,2}	6.9^{b,2}	5.4^{2}
EK + Se + E						
Fresh	349.3^{c}	93.1^{a,b,1}	81.4^{1}	6.2^{b}	2.4	2.9^{1}
Stored	367.5^{b}	79.0^{b,2}	60.7^{a,2}	7.4^{b}	6.2^{b}	4.5^{2}
Fresh	377.7^{b,c}	88.7^{b,1}	78.0^{1}	6.2^{b}	1.9	2.4^{1}
EK + RJ						
Stored	388.3^{b}	76.1^{b,c,1}*	59.2^{a,2}	6.9^{b}	4.9^{b}	4.9^{2}
EK + COL						
Fresh	530.0^{a}	93.3^{a,1}	76.6^{1}	10.0^{a,1}	2.5	4.6^{1}
Stored	546.0^{a}	85.5^{a,2}	49.0^{b,2}	16.9^{a,2}	5.3^{b}	14.1^{2}
SEM	9.852	0.934	1.891	0.456	0.903	0.472
Effects[3]						
Extender	0.001	0.001	0.001	0.001	0.001	0.001
Storage	0.419	0.001	0.001	0.001	0.001	0.001
Extender × Storage	0.404	0.010	0.001	0.027	0.001	0.006

$^{a\text{-}d}$Mean values in columns within time of storage (fresh, unstored, and stored) signed with different letters differ significantly ($P < 0.05$).

[1]Mean values in columns within extender signed with*.

[2]Show significant differences between freshly diluted and stored semen ($P < 0.05$).

[3]Interactions between the main effects were not significant ($P > 0.05$).

Table 4. Extender and storage effect on ISA Brown rooster sperm motility and motility parameters (n = 12; means; SEM).

Semen sample	Motility [%]	Motility parameters				
		VCL [μms^{-1}]	VSL [μms^{-1}]	VAP [μms^{-1}]	LIN [%]	ALH [μm]
Neat						
Fresh	92.9^{a,1}	91.2^{a,1}	39.8^{a,1}	60.7^{a,1}	42.9^{a,b,1}	3.8^{a,1}
Stored	34.9^{b,2}	26.0^{b,2}	7.9^{b,2}	13.6^{b,2}	31.2^{b,2}	2.5^{b,2}
EK						
Fresh	87.6^{a,b}	72.9^{b,1}	28.8^{b,1}	45.3^{b,1}	37.6^{b,c}	3.7^{a,b}
Stored	88.1^{a}	47.3^{a,2}	18.1^{a,2}	28.7^{a,2}	38.3^{a}	3.2^{a}
EK + Se + E						
Fresh	87.5^{a,b}	68.3^{b,1}	30.5^{b,1}	47.4^{b,1}	45.4^{a,1}	3.4^{b,c}
Stored	92.0^{a}	52.7^{a,2}	19.5^{a,2}	31.8^{a,2}	37.3^{a,2}	3.3^{a}
EK + RJ						
Fresh	81.5^{b}	67.5^{b,1}	31.5^{b,1}	45.9^{b,1}	46.0^{a}	3.2^{c}
Stored	86.4^{a}	44.6^{a,2}	18.4^{a,2}	28.0^{a,2}	40.8^{a}	3.0^{a}
EK + COL						
Fresh	67.4^{c,x}	48.8^{c,1}	17.7^{c,1}	28.0^{c,1}	35.5^{c,1}	3.3^{b,c,1}
Stored	9.3^{c,y}	9.0^{c,2}	2.9^{b,2}	5.3^{c,2}	9.5^{c,2}	0.4^{c,2}
SEM	2.840	2.500	1.237	1.795	1.177	0.105
Effects[3]						
Extender	0.001	0.001	0.001	0.001	0.001	0.001
Storage	0.001	0.001	0.001	0.001	0.001	0.001
Extender × Storage	0.001	0.001	0.001	0.001	0.001	0.001

[a-d]Mean values in columns within storage time (fresh, unstored, and stored) with different letters differ significantly ($P < 0.05$).
Abbreviations: ALH, amplitude of lateral head displacement; LIN, linearity; VAP, path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.
[1]Mean values in columns within extender signed with*-.
[2]Show significant differences between freshly diluted and stored semen ($P < 0.05$).
[3]Interactions between the main effects were not significant ($P > 0.05$).

(Table 6). All motility parameters (VCL, VSL, VAP, LIN, and ALH) were the highest (and significantly $P < 0.05$ higher compared to most extenders) in the fresh neat semen, and only in EK + Se + E extender, the motility parameters were similar.

Semen storage at 4°C had no significant effect ($P > 0.05$) on the osmotic pressure only, whose relationship between groups was the same as in unstored semen. The highest pressure was observed in EK + COL and the lowest in the neat semen. There was a significant ($P < 0.05$) decrease in the total live sperm and live normal sperm, with a simultaneous increase in the percentage of all abnormal forms. The number of live sperm in total was the lowest in neat semen and decreased by 15.9% compared with unstored semen, by 7.4% in EK + COL, and by 11.8% in EK + RJ. As it could be

Table 5. Extender and storage effect on Hubbard Flex rooster semen osmotic pressure and sperm morphology (n = 12; means; SEM).

Semen sample	Osmotic pressure [mOsmol kg^{-1}]	Morphological forms [%]				
		Live in total	Live normal	Bulb head	Bent neck	Other deform.
Neat						
Fresh	310.8^{d}	92.1^{a,b,1}	74.0^{1}	12.1^{a,b}	2.8^{1}	3.0^{1}
Stored	309.2^{d}	76.2^{b,2}	22.6^{b,2}	10.6^{b}	27.9^{a,2}	15.0^{a,2}
EK						
Fresh	381.7^{b}	86.6^{b,1}	68.7^{1}	12.3^{b}	2.3^{1}	3.1^{1}
Stored	395.0^{b}	76.2^{b,2}	47.8^{a,2}	12.6^{b}	9.0^{b,2}	6.6^{b,2}
EK + Se + E						
Fresh	362.5^{c}	91.2^{a,b,1}	73.8^{1}	11.9^{b}	2.2^{1}	3.0^{1}
Stored	375.0^{c}	81.8^{a,b,2}	48.2^{a,2}	13.8^{b}	11.7^{b,2}	7.8^{b,2}
EK + RJ						
Fresh	385.0^{b}	89.9^{a,b,1}	73.8^{1}	10.8^{b}	2.7^{1}	2.4^{1}
Stored	398.3^{b}	78.1^{b,2}	51.0^{a,2}	11.7^{b}	8.0^{b,c,2}	7.3^{b,2}
EK + COL						
Fresh	543.3^{a}	93.8^{a,1}	69.3^{1}	17.6^{a,1}	2.9	3.7^{1}
Stored	557.0^{a}	86.4^{a,2}	45.7^{a,2}	23.9^{a,2}	4.7^{c}	11.9^{a,2}
SEM	10.364	0.863	1.843	0.618	0.883	0.527
Effects[3]						
Extender	0.010	0.001	0.001	0.001	0.001	0.001
Storage	0.060	0.001	0.001	0.123	0.001	0.001
Extender × Storage	0.536	0.260	0.001	0.156	0.001	0.002

[a-d]Mean values in columns within time of storage (fresh, unstored, and stored) signed with different letters differ significantly ($P < 0.05$).
[1]Mean values in columns within extender signed with*.
[2]Show significant differences between freshly diluted and stored semen ($P < 0.05$).
[3]Interactions between the main effects were not significant ($P > 0.05$).

Table 6. Semen extender and storage effect on Hubbard Flex rooster sperm motility and motility parameters (n = 12; means; SEM).

Semen sample	Motility [%]	Motility parameters				
		VCL [μms^{-1}]	VSL [μms^{-1}]	VAP [μms^{-1}]	LIN [%]	ALH [μm]
Neat						
Fresh	98.5^{a,1}	80.4^{a,1}	32.4^{a,1}	51.6^{a,1}	40.6	3.8^{a,1}
Stored	54.3^{b,2}	31.1^{b,2}	10.9^{b,2}	16.4^{b,2}	34.3^{a,b}	3.2^{a,2}
EK						
Fresh	81.1^{b}	63.8^{b,1}	26.1^{b,c,1}	40.9^{c,1}	40.6	3.4^{a,b,c}
Stored	90.2^{a}	52.2^{a,2}	20.2^{a,2}	32.1^{a,2}	39.7^{a}	3.4^{a}
EK + Se + E						
Fresh	87.5^{b}	72.6^{a,1}	31.4^{a,1}	48.2^{a,b,1}	43.5	3.6^{a,b}
Stored	89.6^{a}	55.9^{a,2}	21.8^{a,2}	33.0^{a,2}	37.4^{a}	3.3^{a}
EK + RJ						
Fresh	86.2^{b}	64.5^{b,1}	28.2^{a,b,1}	43.7^{b,c,1}	44.1	3.2^{b,c}
Stored	90.4^{a}	47.8^{a,2}	20.1^{a,2}	30.4^{a,2}	40.7^{a}	3.2^{a}
EK + COL						
Fresh	67.3^{c,1}	50.7^{c,1}	23.1^{c,1}	32.5^{d,1}	41.0^{1}	3.0^{c,1}
Stored	29.1^{c,2}	16.4^{2}	7.6^{b,2}	11.4^{b,2}	27.4^{b,2}	2.1^{b,2}
SEM	2.283	2.001	0.912	1.382	0.908	0.067
Effects[3]						
Extender	0.001	0.001	0.001	0.001	0.022	0.001
Storage	0.001	0.001	0.001	0.001	0.041	0.001
Extender × Storage	0.001	0.001	0.001	0.001	0.154	0.043

[a-d] Mean values in columns within storage time (fresh, unstored, and stored) with different letters differ significantly ($P < 0.05$).
Abbreviations: ALH, amplitude of lateral head displacement; LIN, linearity; VAP, path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.
[1]Mean values in columns within extender signed with*-.
[2]Show significant differences between freshly diluted and stored semen ($P < 0.05$).
[3]Interactions between the main effects were not significant ($P > 0.05$).

expected, the lowest number of live normal cells was observed in the neat semen, and compared with freshly collected semen, it decreased by 51.0%, whereas in diluted samples, by 20.9% (in EK) and 25.6% in EK + Se + E. Storage of neat semen increased by 25.1% the number of bent neck sperm, whereas in the EK + COL extender, the bulb head sperm (by 6.3%).

After 24 h storage, the motility and motility parameters were the lowest in neat semen and semen diluted with extender enriched with colostrum (decrease by 44.2 and 38.2% in relation to unstored sperm). In the remaining extenders, the motility was similar and did not differ significantly from values in unstored semen (Table 6). Three motility parameters (VCL, VSL, and VAP) were affected by 24 h storage at 4°C. The largest decrease in these parameters occurred in neat semen (by 49.3, 21.5, and 35.2%, respectively) and in semen with EK + COL (by 34.3, 15.5, and 21.1%). In the remaining samples, the VCL decrease by 11.6% (in EK) and by 16.7% (EK + Se + E and EK + RJ), the straight-line velocity (VSL) by 9.6% (in EK + Se + E) and 5.9% (EK), whereas path velocity (VAP) by 8.8% (EK) and 15.2% (EK + Se + E).

DISCUSSION

Chicken Line Effect

The presented studies reveal the impact of roosters' origin on both, the quantitative and qualitative characteristics of sperm and its susceptibility to various extenders and short-time storage in a liquid stage. This confirms the results of our previous research (Siudzińska and Łukaszewicz, 2008a; 2008b) on 4 fancy fowl breeds and those of Ameen et al. (2014), who showed significant differences between Hubbard and ISA White lines in ejaculate volume, active motile and sluggish motile sperm, and ejaculate concentration. In addition, Ameen et al. (2014) described a higher sperm concentration and better motility in Hubbard semen than in ISA. Tabatabatei et al. (2009) found that semen of indigenous breeds had a higher sperm concentration, higher content of morphologically defected sperm, lower sperm motility, and sperm viability when compared with Ross-308 broiler breeder roosters. Hermiz et al. (2016) when comparing semen from roosters of ISA-B commercial line, different local breeds, and their hybrids with ISA-B observed significant differences among genetic groups in ejaculate volume, sperm concentration, presence of abnormal sperm, mass motility, and individual sperm motility. Local breeds and their hybrids with ISA-B had lower ejaculate volume (0.523 mL vs. 0.288–0.515 mL) and sperm concentration (5.55×10^9 mL^{-1} vs. $3.65–4.67 \times 10^9$ mL^{-1}), but similar number of live sperm (91.60% and 90.80–93.08%), comparing with ISA-B line.

Extender and Storage Effect

The extenders tested when added to semen of both rooster lines caused significant changes in osmotic pressure, number of bulb head sperm, and their motility, whereas they did not elicit changes in the percentage of live normal cells, bent neck, or other abnormal forms. Semen storage for 24 h did not affect the osmotic pressure, whereas changes in other semen characteristics

were observed. The size of changes depended on extender composition and analyzed sperm traits.

Semen dilution with basic EK extender, with an osmotic pressure between 380 and 400 mOsmol kg^{-1}, increased the pressure to the same value as EK extender. The addition of Se + E, as well as of RJ, caused a slight reduction in the osmotic pressure, but bovine COL significantly increased this value to 530.0 mOsmol kg^{-1} in ISA-B and 543.3 mOsmol kg^{-1} in H-F semen. According to Donoghue and Wishart (2000), the best environmental pressure for poultry semen should be between 250 and 460 mOsmol kg^{-1}. In our experiment, these parameters were met in the basic EK, EK + Se + E, and EK + RJ extenders, while colostrum addition significantly exceeded values recommended by Donoghue and Wishart (2000) and Latif et al. (2005). At varying level of osmotic pressure between sperm and surrounding environment (seminal plasma or extender), a movement of fluid from the environment to the cells and vice versa, which results in pressure equalization can be observed. In a hyperosmotic environment, sperm cells become shrunk because of the loss of water, whereas in a hypoosmotic solutions, sperm cells swell because of water penetration (Donghue and Wishart, 2000; Latif et al., 2005). In the present study, the highest number of bulb head sperm was observed for samples diluted with extender of the highest osmotic pressure, that is with bovine colostrum, regardless chicken line and storage time; nevertheless, it should be stressed that also for this extender, the highest number of live sperm in total, both in fresh semen and semen after 24 h storage was observed. Colostrum contains about 250 natural chemicals including immunoglobulins, resistance regulators, amino acids, minerals, and probably some of them, not higher osmotic pressure, caused sperm head swelling. Łukaszewicz and Fujihara (2000) and Łukaszewicz (2001) when examining the effect of 6% dimethylformamide addition to EK extender used for goose insemination did not observe significant differences in sperm morphology of diluted semen whose osmotic pressure was around 1100 mOsmol kg^{-1}, comparing to the neat gander semen whose osmotic pressure was 250 to 260 mOsmol kg^{-1}. Moreover, the osmotic pressure of ISA-B semen was similar to H-F line semen, whereas the percentage of bulb head sperm in H-F has been twice as high as ISA-B, what allows to assume that probably this deformation was not caused by the osmotic pressure.

Owing to the specific structure of avian sperm and fatty acid-rich, elongated cell membrane, many studies are conducted on the role of antioxidants, occurring in both sperm plasma and extenders, in maintaining the fertilizing capacity of sperm. Our analysis of sperm morphology confirmed that during storage, selenium, vitamin E, RJ, and bovine colostrum addition to the basic EK extender had significant protective effect on the sperm cell membrane. In unstored semen, there were no significant differences in percentage of live normal sperm between neat and diluted semen, but after 24 h of storage, differences in the participation of individual morphological forms became significant. Although 24 h storage of chicken semen caused significant decrease in the number of live normal sperm, regardless of extender, it was significantly lower than in neat, undiluted semen samples, what may indicate a beneficial effect of extender additives tested in this experiment. Similar as in our experiment, many researchers also discussed that during poultry semen storage, the number of live properly formed sperm decreases and deformed or dead sperm increases, regardless of the type of diluent and the antioxidant added (Barber et al., 2005; Hudson et al., 2016). Although antioxidants do not prevent completely the adverse changes caused by storage, they significantly reduce their effects. For example, Kowalczyk et al. (2017), after 24 h storage of capercaillie semen in the EK extender found a significant decrease in number of live sperm (by 3.8%), but after addition of antioxidants (Se + E), the drop was insignificant (by 1.5%). Similarly, the positive effect of selenium and vitamin E on viability, membrane integrity, and reduction of abnormal sperm cells in stored rooster semen was observed by Safa et al. (2016). Das et al. (2015) using EK extender for rooster semen of various origin also stated the decrease in correctly formed sperm from 79.4–83.0% to 72.8–79.5%. Moradi et al. (2013) examining the protective properties of RJ during sperm storage for up to 120 h described that its addition caused an increase in sperm viability depending on RJ concentration and length of storage.

As in case of sperm morphology, the type of semen extender and storage time affect the kinetic values of sperm. Three diluents: basic EK, EK + Se + E, and EK + RJ did not cause significant differences in sperm motility, but regardless of extender, 24 h storage significantly reduced sperm motility and motility parameters. The poorest, and significantly lower, comparing to other extenders, sperm motility was measured in sample with bovine colostrum addition. It could be caused by differences in osmotic pressure. Latif et al. (2005), observing the diluted semen of Hubbard breeders stored for 4, 24, and 48 h, affirmed that osmotic pressure between 350 mOsmol kg^{-1} and 400 mOsmol kg^{-1} does not affect sperm motility only during the first 4 h of storage, but with time, the changes in sperm motility become significant. This information confirms that storage time, not the osmotic pressure of sperm environment, may have a greater impact on sperm motility, which is consistent with our observations. Both, in ISA-B and H-F semen, a decrease in sperm motility in all evaluated samples could be observed, regardless of osmotic pressure level (from 304 in the fresh, neat semen to 543 mOsmol kg^{-1} in EK + COL sample). Our observations are in accordance with the opinion of other authors (Long and Conn, 2012). Das et al. (2015) assessing sperm motility during 24 h storage found a decrease in sperm motility from 65.63 to 58.13% (i.e. by 7.5%). Studies by other authors also showed that the addition of antioxidants reduced the negative impact of storage time and thus loss in sperm quality (Dimitrov et al., 2007; Safa et al., 2016). In our previous studies (Kowalczyk et al.,

2017), we proved that in relation to the fresh semen, sperm motility was significantly reduced by 27.0% in samples stored for 24 h in EK extender and 14.3% in samples enriched with Se and vit. E. Also, percent of motile sperm in EK + Se + E was higher than in EK (71.6 vs. 58.9%, i.e. by 12.7%). Mentioned observations were confirmed in the presented studies in which sperm motility in diluted and stored ISA-Brown and H-F semen decreased by 0.5 and 9.1%p in EK; by 4.5 and 2.1%p in EK + Se + E; and by 4.9 and 4.2%p in EK + RJ extender, in relations to freshly diluted, unstored semen, whereas in the neat semen, the percentage of motile sperm decreased by 58% in ISA-B and by 44.2% in H-F rooster semen. As in our experiment, the effect of storage time on sperm motility was also described by Moradi et al. (2013) in research on the use of RJ. After 24 h storage, semen with RJ addition had higher sperm kinematics parameters (VAP, VCL, VSL, and LIN), which was probably caused by beneficial effect of RJ as the effective antioxidant.

As it is well known, the main role of antioxidants is to protect cells against excessive lipid peroxidation of cell membranes, especially unsaturated fatty acids, which because of the existence of 1 or more double bonds, show particular susceptibility to oxidation (Surai et al. 1998; Safa et al., 2016). Semen extender enrichment with antioxidants can bring benefits by reducing sperm susceptibility to lipid peroxidation; however, the diverse results of our experiment and those of other authors indicate the need of conducting further research on extender supplementation with various antioxidants, especially in the aspect of liquid semen storage for artificial insemination purposes. The RJ and bovine colostrum used in the presented experiment seem to be good a additives that decrease the adverse effects of rooster sperm storage in a liquid state, but perhaps more effective levels of their addition should be examined.

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