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EFFECT OF BULLS' BREED, AGE AND BODY CONDITION SCORE ON QUANTITATIVE AND QUALITATIVE TRAITS OF THEIR SEMEN

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Abstract

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The objectives of this study were to determine and evaluate effect of breed, age and body condition score (BCS) on qualitative and quantitative parameters of bull's ejaculate. In total, 16 Holstein (H) and 15 Czech Fleckvieh (F) bulls were collected in September 2009 and May 2010. Volume of semen samples, sperm concentration and percentage of motile spermatozoa were evaluated immediately after collecting. Sperm motility was also evaluated after diluting and freezing/thawing of AI doses and subsequently during the short-term test of sperm survival. Percentages of live and pathologic sperm before diluting also were evaluated. The data set was analyzed using a generalized linear model in SAS/STAT software. A statistically significant effect of the breed, age and body condition on qualitative and quantitative traits of bull's sperm were determined ($P < 0.05–0.01$). Sperm activity after collection, dilution and freezing/thawing had significantly decreasing character.

bull semen, Holstein, Czech Fleckvieh, sperm survival

Reproductive capacity of dairy cows has significantly decreased tendency in the last decades (Flint, 2006). This trend has several reasons: selection aimed at increasing milk production (Hanuš *et al.*, 2010) and improving the breeding environment of dairy cows (Vacek *et al.*, 2007). The decreasing trend of dairy cow's conception is distinct in all animal breeders in developed countries (Lucy, 2001). Similar results are also documented by results of milk recording in the Czech Republic. Problems of cows' fertility were continuously studied in recent years, but only from female point of view (Vacek *et al.*, 2007; Stádník *et al.*, 2008; Bezdíček and Louda, 2009). The results of cow's reproduction, however, among other factors are also influenced by the male component, which is represented by the fertility of bull's ejaculate.

Artificial insemination (AI), the first biotechnology to be widely implemented in practice for improving reproduction traits and ensuring genetic gains, is important for selection and breeding of cattle (Gravance *et al.*, 2009). In the early days of their regular usage, AI doses were diluted and stored for short or medium periods before their use for insemination (Verberckmoes *et al.*, 2005). However, negative changes in sperm membranes in relation to storage time and extender used were demonstrated (e.g. Frydrychová *et al.*, 2010). The implementation of deep freezing technology completely changed selection and breeding processes, and AI became a very important tool for intensifying reproduction in cattle herds. The higher mating intensity and pregnancy rates for cows and heifers brought an enduring increase in the number of calves born. AI played an irreplaceable role in this process (Louda *et al.*, 2008), and its worldwide application was the main impulse for subsequent expansion of other procedures, e.g. management of estrus (Stádník *et al.*, 2008), freezing of sperm (Saragusty *et al.*, 2009), sperm sexing (Andersson *et al.*, 2006), collecting, freezing, cultivation and transfer of embryos (Říha *et al.*, 1998), and cloning (Liu *et al.*, 2010).

Sperm quality is influenced by many factors, e.g. by such internal factors as breed, variation between individuals, and age of sire (Štolc *et al.*, 2009a; Štolc *et al.*, 2009b), and by such external factors as feeding ration composition, content of specialized feeding supplements (Čeřovský *et al.*, 2009), environmental conditions, frequency of collecting ejaculate (Wolf and Smital, 2009), and season of year (Hajirezaee *et al.*, 2010) or even calendar month, and especially in animal species with seasonal sexual activity (Alavi *et al.*, 2010).

Conventional procedure for evaluating ejaculate that is performed before and after cryopreservation includes determining volume, density, activity, and sperm morphology. These parameters mostly detect cases of significantly inadequate reproduction or infertility in bulls (Rodriguez-Martinez, 1998).

We are supposed that knowledge of these relationships will enable breeders of sires and producers of AI doses using these factors to modify the management of breeding to increase quality of AI doses produced.

The present study's objectives were to determine and evaluate effects of breed, age and BCS of bulls on quantitative and qualitative parameters of bulls ejaculates.

MATERIAL AND METHODS

Semen collecting and processing

The observations were made in a bull housing facility and laboratory at a single AI center, where 16 H and 15 F bulls were selected for monitoring. The bulls were fed an identical daily ration: hay (10 kg), straw (5 kg), soybean meal (0.5 kg) and mix of cereals: 1/3 oats, 1/3 wheat, 1/3 and barley (3 kg), plus the mineral mix Premin 22 Natural, VVS (0.1 kg). The selected bulls were from 1 to 7 years old and the frequency of semen collection for all was once weekly. One sample of ejaculate was obtained from each bull using an artificial vagina in September 2009 and in May 2010 repeatedly. The BCS of selected bulls was evaluated by five point scale with an accuracy of 0.25 points at the time of ejaculate collecting according to methodology of body condition scoring especially for H and F breeds, respectively. The optimum BCS on five point scale differed between evaluated breeds in relation to different requirements in accordance with production type – milk in H and dual purpose in F. The volume of semen samples (VOL) was measured using an electronic scale (Scout Pro, OHAUS®), sperm concentration (DEN) using a spectrophotometer (GENESYS 10vis, Thermo Scientific®), and percentage of motile spermatozoa (ACT) subjectively by phase contrast microscopy (LP 3000, Arsenal®) immediately after collecting. In addition, we evaluated the percentage of live sperm by staining before diluting and freezing in accordance with standard methodology (Ball and Peters, 2007): a drop of semen was mixed with eosin on a preheated microscope slide, spread, then examined under a phase contrast microscope at 1 000x magnification and with oil immersion. We classified a minimum of 100 spermatozoa as either dead (with red heads) or live (with white heads) and expressed this as a percentage rate. We also evaluated a percentage of pathologic sperm in accordance with Wells II. Method: a drop of semen was spread on microscope slide. After drying the smear was subsequently stained with Congo red, Bromothymol blue and Janus green. After that, the smear was examined under a phase contrast microscope at 1 000x magnification and with oil immersion. We classified a minimum of 200 spermatozoa as normal or pathologic.

Only fresh semen with required quality (minimum progressive motility 70% and sperm concentration 0.7×10^6 mm^{-3}) was used for the subsequent processing of samples for observation according to common standards used for producing AI doses.

The samples of semen were diluted with AndroMed® (Minitüb, Tiefenbach, Germany), a commercially produced extender containing soybean lecithin extract. Polyvinyl chloride (PVC) straws (0.25 cm^3; IMV) were filled, cooled to 4 °C and equilibrated for 90 min. Subsequently, they were frozen in a programmable freezing device (IMV-Digitcool, L'Aigle, France) then plunged into liquid nitrogen for storage.

Sperm survival test

We assessed sperm motility subjectively using phase contrast microscopy (LP 3000, Arsenal®) at 200x magnification immediately after collecting (ACT), after diluting (DIL) and after freezing/thawing (FRO). The motility values were detected at the beginning of the short-term heat test of sperm survival (ACT0, DIL0 and FRO0) and then after 30, 60 and 90 min (ACT30–90, DIL30–90) and FRO30–90, respectively) of the test duration in a dry heater (Thermo-block, FALC®) at a temperature of 38 ± 1 °C.

Straws were thawed in a water bath at 38 ± 1°C for 40 s., then input to preheated sterile tubes with sodium citrate physiological solution.

Statistical analysis

The data set was analyzed using a generalized linear model in the SAS statistical program (SAS/STAT® 9. 1., 2009). The following equation was used:

$$Y_{ijkl} = \mu + BREED_i + BCS_j + AGE_k + e_{ijkl},$$

where:

Y_{ijkl} ..... observed value of the dependent variable (volume of sperm in g, concentration of sperm in 10^6 mm^{-3}, percentage rate of live and pathologic sperm, motility of sperm after collecting, after diluting and after freezing/thawing in percent),

μ ..... average value of the dependent variable,

$BREED_i$ ... fixed effect of the i[th] breed (i = Holstein (H), n = 16; Czech Fleckvieh (F), n = 15),
BCS_j ..... fixed effect of the j[th] class of bull's BCS (j = to 2 points, n = 8; from 2.25 to 2.5 points, n = 15; up to 2.75 points, n = 8),
AGE_k ..... fixed effect of the k[th] class of bull's AGE (k = to 3 years of age, n = 14; from 4 years of age, n = 17),
e_{ijkl} ..... residual effects.

The differences between the variables estimated were tested at the levels of significance $P < 0.05$ (*); $P < 0.01$ (**) and $P < 0.001$ (***). Spearman correlation coefficients were also determined.

RESULTS AND DISCUSSION

The effects of individual factors included in the statistical model are presented in Tab. I. Coefficient of the whole model repeatability ranged from $r^2 = 0.03$ to $r^2 = 0.36$ during the evaluation of observed traits. Effect of breed was significant in relation to the activity of sperm and percentage of pathologic sperm ($P < 0.05$). Effect of bull's BCS was significant in relation to the sperm concentration, activity of sperm ($P < 0.05$) and percentage of pathologic sperm ($P < 0.01$) as well, and effect of bull's age was significant only according to the sperm density and volume ($P < 0.05$).

The basic statistical characteristics of observed data are shown in Tab. II. The volume of ejaculate ranged from 2.8 to 20.0 g in Holstein bulls, respectively from 2.9 to 21.5 g in Fleckvieh bulls. The volumes of ejaculates correspond to the findings of other authors, e.g. Louda *et al.* (2007) reported the range 3–12 g in sires kept in AI centre. Ball and Peters (2007) mentioned the range from 5 to 6 g. The minimum volume of bull ejaculate suitable for long-term preservation is 3 g in adult bulls and 2 g in young bulls (Hafez and Hafez, 2000). Our results documented that the samples of semen obtained from selected bulls were suitable for processing and producing of semen doses.

The sperm density ranged from 0.4 to 1.6×10^6 mm^{-3} in the both groups of breed. Our results agreed with those of Louda *et al.* (2007), who reported sperm density in bulls' ejaculates from 0.8 to 2.0×10^6 mm^{-3}. However, Ball and Peters (2007) observed a range from 0 in azoospermic bulls to $3\,000 \times 10^6$ mm^{-3} in excellent sires. The minimum requirement for sperm density in relation to subsequent processing is 700.000 mm^{-3} to maintain fertility of sperm after freezing/thawing process (Hafez and Hafez, 2000). Our findings documented that the samples of semen obtained from selected bulls had a suitable density for processing and producing of semen doses.

Percentage of live sperm belongs to the main biological treatment of sperm quality. We take a reflection about viability and fertility of ejaculate. The rate of live sperm ranged from 30.0 to 94.0% in Holstein bulls and from 0.0 to 90.0 in Czech Fleckvieh.

The rate of pathologic sperm belongs to the morphological methods of ejaculate examination. Knowledge of sperm morphology may contribute for determination the causes of sires' infertility. The rate of pathologic sperm ranged from 3 to 33% in Holstein bulls, respectively from 4 to 30% in Czech Fleckvieh. Louda *et al.* (2007) reported percentage rate of pathologic sperm 20% maximal. So our results were slightly higher than the abovementioned limit, however selected bulls have been used regularly for AI doses production in AI center observed.

Indicators which are mentioned above belong to the fundamental characteristics of collected semen and determine the initial quality of ejaculate subsequently used for AI doses manufacturing. The last findings confirm the initial quality of ejaculate determines final quality of AI dose (Beran *et al.*, 2011).

Results of sperm survival in accordance with breed effect are shown in Tab. II and Fig. 1. The highest level of activity was detected after sperm dilution in both breeds evaluated. This fact confirms the suitable technology of sperm dilution and AI doses preparation for ensuring the highest possible quality of ejaculate for subsequent freezing. Freezing of AI doses, storage for long period and thawing is a demanding process for sperm membranes and its viability (Frydrychová *et al.*, 2010). Our results documented these relationships according to significant decline of activity after thawing. Evaluating interbreed differences, sperm activity after collection and dilution were higher in Holstein but activity of sperm after freezing/thawing in Czech Fleckvieh bulls. In Holstein sires were most significant decreases in ACT90 compared to Czech Fleckvieh. The H activity of sperm after freezing/thawing (FRO0 and FRO30) were higher

I: *Effects of individual factors in statistical model on basic quality traits of ejaculate*

TRAIT	MODEL		BREED		BCS		AGE	
	r^2	P	F-test	P	F-test	P	F-test	P
VOL	0.15	0.2109	0.05	0.8184	0.24	0.6249	4.56	< 0.05
DEN	0.29	0.0225	2.54	0.1229	6.13	< 0.05	5.22	< 0.05
ACT	0.16	0.1771	4.13	< 0.05	4.05	< 0.05	0.31	0.5829
PAT	0.36	< 0.01	6.31	< 0.05	14.95	< 0.001	0.24	0.6255
LIVE	0.03	0.8532	0.00	0.9786	0.34	0.5621	0.26	0.6114

VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm; PAT = percentage of pathological sperm; LIVE = percentage of live sperm

II: *Basic statistical characteristics of observed data of Holstein (n = 16) and Czech Fleckvieh (n = 15) bulls*

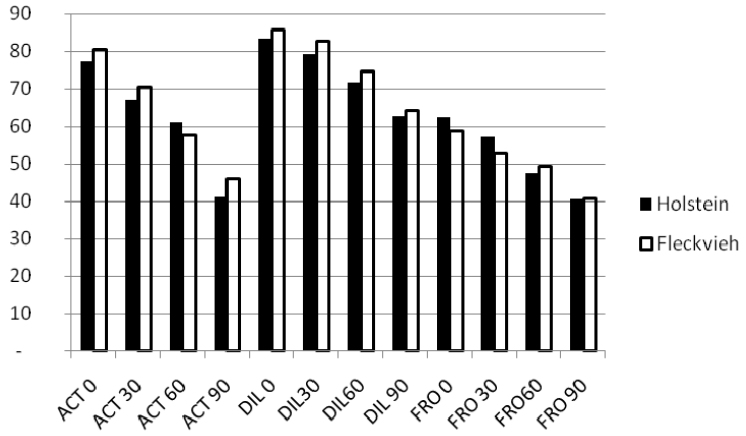
Variable	Unit	Breed	Min	Max	Mean	Variance	SD	SE
VOL	[g]	H	2.8	20	10.96	23.352	4.8323	1.2081
		F	2.9	21.5	10.98	28.985	5.3837	1.3901
DEN	[10^6. mm^{-3}]	H	0.4	1,6	0.98	0.126	0.3544	0.0886
		F	0.4	1.6	0.98	0.163	0.4039	0.1043
PAT	[%]	H	3	33	15.22	63.499	7.9686	1.9922
		F	4	30	14.90	65.364	8.0848	2.0875
LIVE	[%]	H	30	94	76.66	338.891	18.409	4.6022
		F	0	90	73.50	546.250	23.3720	6.0346
ACT0	[%]	H	50	95	77.19	183.229	13.5362	3.3841
		F	50	95	80.33	198.095	14.0746	3.6341
ACT30	[%]	H	0	90	66.88	552.917	23.5142	5.8785
		F	0	90	70.33	540.952	23.2584	6.0053
ACT60	[%]	H	0	80	61.25	515	22.6936	5.6734
		F	0	85	57.67	674.524	25.9716	6.7058
ACT90	[%]	H	0	80	41.25	651.667	25.5278	6.3819
		F	0	80	46.00	582.857	24.1424	6.2335
DIL0	[%]	H	60	90	83.44	69.063	8.3104	2.0776
		F	70	90	85.67	38.810	6.2297	1.6085
DIL30	[%]	H	60	90	79.06	104.063	10.2011	2.5503
		F	70	90	82.67	53.095	7.2866	1.8814
DIL60	[%]	H	30	90	71.56	305.729	17.4851	4.3713
		F	50	90	74.67	108.810	10.4312	2.6933
DIL90	[%]	H	0	90	62.81	693.229	26.3292	6.5823
		F	0	90	64.00	425.714	20.6328	5.3274
FRO0	[%]	H	30	80	62.50	193.333	13.9044	3.4761
		F	30	80	58.67	269.524	16.4172	4.2389
FRO30	[%]	H	10	80	57.19	279.896	16.7301	4.1825
		F	20	80	52.67	306.667	17.5119	4.5216
FRO60	[%]	H	0	70	47.50	286.667	16.9312	4.2328
		F	20	80	49.33	292.381	17.0992	4.4150
FRO90	[%]	H	0	70	40.63	339.583	18.4278	4.6069
		F	10	80	40.67	320.952	17.9151	4.6257

VOL = volume of ejaculate; DEN = density of sperm; PAT = percentage of pathologic sperm; LIVE = percentage of live sperm; ACT0 = sperm motility after collection; DIL0 = sperm motility after diluting; FRO0 = sperm motility after freezing/thawing; ACT30–90, DIL30–90 and FRO30–90 = sperm motility after 30, 60 and 90 minutes of short-term heat test of sperm survival; H = Holstein breed; F = Czech Fleckvieh breed

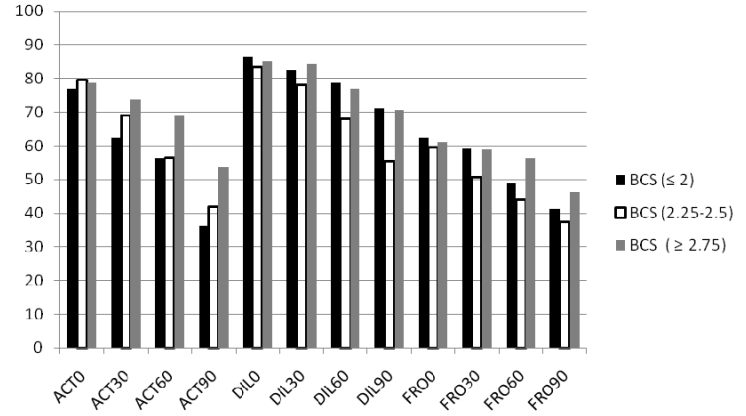
compare to F. These findings indicate possible different requirements for management of sires breeding and AI doses manufacturing according to breed, respectively different production type of sires.

The sperm survival in relation to the BCS of bulls is shown in Fig. 2. Results described higher level of sperm activity immediately after collection in bulls with higher BCS (> 2.75), especially during the entire short-term test ($P < 0.05$). The same trend of sperm activity decline was detected after dilution and freezing/thawing as well, and sires with BCS higher 2.75 demonstrated lower level of sperm activity decline simultaneously. These findings recommended the sires BCS on the level 2.75 and higher as the most suitable. However, subsequent research focused to determination of the upper line of suitable BCS is required. Traits of sires' sperm quality were not yet evaluated in relation to its BCS and breeders of sires can used these results for the changes in management of sires breeding.

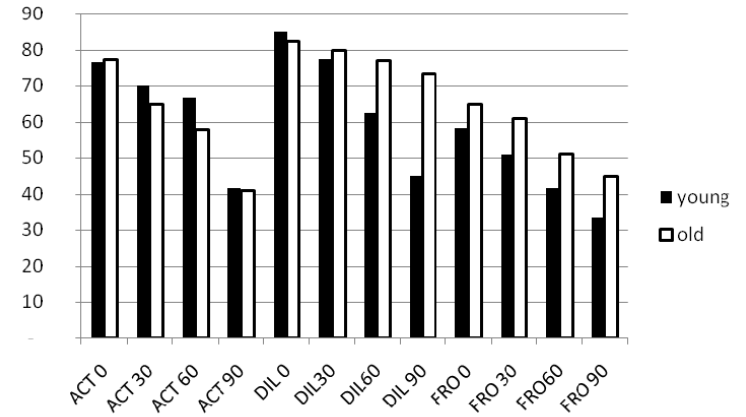
The sperm survival in relation to the age of bulls is shown in Fig. 3 and 4. In Holstein, the activity of the sperm after collection was almost the same in both categories of age during the whole test. Holstein sires demonstrated significantly ($P < 0.05$) higher decline in sperm activity after 90 minutes of the test comparing with Czech Fleckvieh sires. This fact could relate to higher intensity of metabolism in Holstein breed and evoke requirement the different



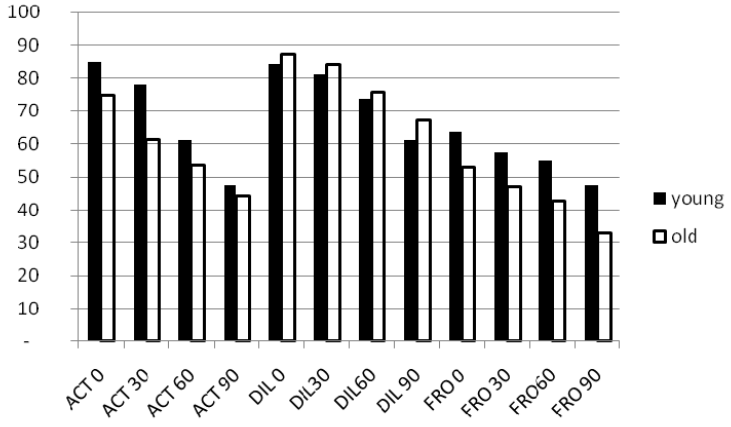
1: *Results of sperm survival depending on the bull's breed*
ACT0 = sperm motility after collection; DIL0 = sperm motility after diluting; FRO0 = sperm motility after freezing/thawing; ACT30–90, DIL30–90 and FRO30–90 = sperm motility after 30, 60 and 90 minutes of short-term heat test of sperm survival



2: *Results of sperm survival depending on the bull's BCS*
ACT0 = sperm motility after collection; DIL0 = sperm motility after diluting; FRO0 = sperm motility after freezing/thawing; ACT30–90, DIL30–90 and FRO30–90 = sperm motility after 30, 60 and 90 minutes of short-term heat test of sperm survival



3: *Results of sperm survival depending on the Holstein bull's age*
ACT0 = sperm motility after collection; DIL0 = sperm motility after diluting; FRO0 = sperm motility after freezing/thawing; ACT30–90, DIL30–90 and FRO30–90 = sperm motility after 30, 60 and 90 minutes of short-term heat test of sperm survival



4: *Results of sperm survival depending on the Czech Fleckvieh bull's age*
ACT0 = sperm motility after collection; DIL0 = sperm motility after diluting; FRO0 = sperm motility after freezing/thawing; ACT30–90, DIL30–90 and FRO30–90 = sperm motility after 30, 60 and 90 minutes of short-term heat test of sperm survival

III: *Spearman correlation coefficients r and related statistical significance P among basic traits of ejaculate*

	BCS	ACT [%]	DEN [10^6 . mm^{-3}]	VOL [g]	PAT [%]	LIVE [%]
AGE	–0.14534	0.14898	0.23233	**0.52098**	–0.05853	–0.13971
	0.4353	0.4238	0.2085	**0.0027**	0.7545	0.4535
BCS	1	–0.7374	–0.32864	0.13842	0.24655	–0.11146
		0.6934	0.0711	0.4577	0.1812	0.5505
ACT [%]		1	**0.3586**	0.14448	**–0.49875**	**0.36013**
			0.0476	0.4381	**0.0043**	**0.0466**
DEN [10^6 . mm^{-3}]			1	–0.05556	**–0.52421**	0.28316
				0.7666	**0.0025**	0.1227
VOL [g]				1	–0.01623	–0.11017
					0.931	0.5552
PAT [%]					1	–0.08887
						0.6345

ACT = activity of sperm; DEN = density of sperm; VOL = volume of ejaculate; PAT = percentage of pathologic sperm; LIVE = percentage of live sperm

composition of diluters for Holstein sires. Sperm activity after dilution was the same at the beginning and after 30 minutes of the test, but after 60 and 90 minutes was significantly higher in old Holstein bulls. Sperm activity after freezing/thawing was higher in old Holstein bulls during the whole test. These findings correspond with those of Štolc *et al.* (2009a) and indicate the significant effect of age as well as that processing of ejaculate – dilution and freezing/thawing significantly decline level of sperm activity in total and especially after 60 and 90 minutes. Producers of AI doses could use results of young bulls for ensuring the higher quality of their AI doses by low level of dilution of their ejaculate. However, this possibility contradicts economy of AI doses manufacturing. The similar tendencies were detected in sperm activity of Czech Fleckvieh sires after collection and dilution. Nevertheless, sperm activity after freezing/thawing was higher in young bulls during the whole test of sperm survival. This fact is opposing to results detected in Holstein bulls and indicates need the differences in AI doses manufacturing according to breed, respectively production type, of bulls, although the technology of AI doses production is not different in present practice. Again, our findings agreed with those of Štolc *et al.* (2009a) or Štolc *et al.* (2009b) as well.

Tab. III contains Spearman correlation coefficients among the basic traits of ejaculate. Significant coefficients were detected between age of bulls and volume of sperm ($P < 0.05$), sperm density and pathologic sperm ratio ($P < 0.01$) and between activity of sperm immediately after collection and sperm density and percentage of live and pathologic sperm ($P < 0.05$).

Tab. IV shows Spearman correlation coefficients among sperm activity after collection, diluting, freezing/thawing and during the short-term test

IV: *Spearman correlation coefficients r and related statistical significance P among sperm activity after collection, diluting, freezing/thawing and during the test of sperm survival*

[%]	DIL0	FRO0	ACT30	DIL30	FRO30	ACT60	DIL60	FRO60	ACT90	DIL90	FRO90
ACT0	0.16802	0.11775	**0.59952**	0.24726	0.05748	**0.39723**	0.16955	0.078	0.05879	0.12384	0.06495
	0.3663	0.5281	**0.0004**	0.1799	0.7587	**0.0269**	0.3619	0.6766	0.7534	0.5069	0.7285
DIL0	1	0.10855	0.03508	**0.81732**	0.16039	–0.06759	**0.43345**	0.14247	–0.13797	0.24792	0.15481
		0.5611	0.8514	**<.0001**	0.3887	0.7179	**0.0149**	0.4445	0.4592	0.1787	0.4057
FRO0		1	0.09407	0.11977	**0.91358**	–0.05453	0.28382	**0.82445**	–0.21386	0.15486	**0.70503**
			0.6147	0.521	**<.0001**	0.7708	0.1218	**<.0001**	0.248	0.4055	**<.0001**
ACT30			1	0.24395	0.09149	**0.78028**	0.14222	0.15333	**0.42114**	0.09141	0.09936
				0.186	0.6245	**<.0001**	0.4453	0.4102	**0.0183**	0.6248	0.5949
DIL30				1	0.13703	0.08723	**0.58952**	0.19786	–0.01344	**0.48856**	0.21532
					0.4623	0.6408	**0.0005**	0.286	0.9428	**0.0053**	0.2447
FRO30					1	–0.01432	**0.3931**	**0.92572**	–0.17977	0.25422	**0.84555**
						0.9391	**0.0287**	**<.0001**	0.3332	0.1676	**<.0001**
ACT60						1	–0.05845	0.00214	**0.58264**	–0.06835	0.00075
							0.7548	0.9909	**0.0006**	0.7148	0.9968
DIL60							1	**0.51323**	–0.14053	**0.90728**	**0.48557**
								0.0032	0.4508	**<.0001**	**0.0056**
FRO60								1	–0.12331	**0.39348**	**0.9163**
									0.5087	**0.0285**	**<.0001**
AC90									1	–0.04778	–0.08887
										0.7986	0.6345
DIL90										1	**0.42565**
											0.017

ACT0 = sperm motility after collection; DIL0 = sperm motility after diluting; FRO0 = sperm motility after freezing/thawing; ACT30–90, DIL30–90 and FRO30–90 = sperm motility after 30, 60 and 90 minutes of short-term heat test of sperm survival

of sperm survival. The significant correlation coefficients ranged from 0.3931 to 0.9163 ($P < 0.05–0.0001$).

CONCLUSION

A statistically significant effect of the breed, age and body condition on qualitative and quantitative traits of bull's sperm was determined ($P < 0.05–0.01$). Sperm activity after collection, dilution and freezing/thawing had significantly decreasing character during the entire short-term heat test of sperm survival. The highest values of sperm activity were detected in diluted semen and the lowest values had frozen/thawed samples of bull semen in both bull's breed and age. Selected relationships between observed traits were confirmed by Spearman correlation coefficients ($P < 0.05–0.0001$).

We can conclude that freezing process reduces the activity of sperm so the initial semen quality is critical for final quality of AI dose after thawing. It is therefore important to use all information ensuring the highest quality of semen immediately after collecting and diluting before the processing of AI dose of sires.

Acknowledgments

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