Relationship between *in vitro* semen parameters and bull fertility

[1]Marius Zăhan, [2]Emoke Pall, [3]Mihai Cenariu, [1]Ileana Miclea, [1]Alexandru S. Dascăl

[1] Department of Animal Reproduction, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania; [2] Institute of Life Sciences, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania; [3] Department of Reproduction and Obstetrics, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. Corresponding author: M. Zăhan, mzahan@usamvcluj.ro

Abstract. There are a lot of factors that influence bull semen quality used on commercial artificial insemination and makes fertility prediction difficult. The aim of the present study was to analyze *in vitro* quality of Flackvieh-Simmental bulls' parameters including different type of motility (computer assisted sperm analysis), viability, acrosome integrity and mitochondrial activity (flow cytometry), functional integrity (hypo-osmotic swelling test and thermoresistance test) and DNA integrity (DNA fragmentation index) in order to compare with fertility (non-return rate, NRR). After 1,235 artificial insemination with 10 bulls semen the percentage of NRR differs significantly ($P<0.0001$) between heifers and cows. The total motility ($r=0.8788$, $P<0.01$), velocity straight line ($r=0.6485$, $P<0.05$) and velocity average path ($r=0.6848$, $P<0.05$) were significantly correlated with NRR. No other correlations between bull fertility and computer assisted sperm analysis motility, viability, acrosome and sperm membrane integrity, DNA integrity and mitochondrial activity were observed. Concerning the results of the thermoresistance test, we observed a slow decrease trend of semen motility during the incubation period (from 82 to 69.5%) without significantly difference.

Key Words: bulls, artificial insemination, *in vitro* evaluation, fertility prediction.

Introduction. Bull management and quality control programs in artificial insemination (AI) are focused to ensure production and marketing of straws containing high fertile sperm in sufficient numbers to maximize the conception rate of each bull. Fertility is a characteristic with a substantial variability from one bull to another. In general, methods to predict fertility are better for identifying bulls with low fertility than for ranking bulls with high fertility (Kastelic 2013). Detecting high fertility bulls is the key to increasing livestock production and thus profitability. Fertility in the livestock enterprise is five to ten times more important economically than other production measures (Blezinger 2010).

Classical semen analysis consisting of sperm count, motility and morphology has been the main path of semen related bull infertility for many decades of time (Correa et al 1997). Yet there have been numerous situations where these parameters have good values but bulls show low fertility in terms of conception rate (Evenson 2016). The combination of competent physical/reproductive exams and semen evaluations can contribute greatly to the fertility and economics of individual herds as well as adding to understanding of those factors which affect cattle fertility (Chenoweth & McPherson 2016). Microscopic evaluation of motility and morphology has been the most important predictor of fertility in terms of utilization and the variation in fertility that these variables explain (Utt 2016). Sperm morphology was clearly associated with calving rate underlining the usefulness of morphological examination in the assessment of fertility (Attia et al 2016). Moreover both sperm and acrosome membrane integrity have been reported to be successfully associated with male *in vivo* fertility (Kordan et al 2013). Sperm DNA integrity can be used as an additional parameter to provide a more comprehensive description of semen quality (Takeda et al 2015). Single or double strand DNA brakes defined as sperm DNA fragmentation can lead to altering or losing genetic

information (Serafini et al 2015). On the other hand, mutations can also cause changes in sperm genetics with negative impact on male fertility. New knowledge on epigenetic modulation of sperm DNA, messenger RNA and protein is fundamental to refine and expand sperm function assays (Thundathil et al 2016).

Many factors play a role in a bull's fertility and breeding ability. Identifying the semen quality factors associated with fertility and development of techniques to accurately predict the fertility of bull semen is a goal of artificial insemination industry (Saacke 2006; Dalton 2011). Fertility of Flackvieh-Simmental bulls kept in Mures County was well characterized by different researchers (Onaciu & Jurco 2011; Gabor et al 2011; Cocan & Mureşan 2015). According to Rodriguez-Martinez & Barth (2007) due to the complex nature of male fertility any search for laboratory methods must include testing of most sperm attributes relevant for both fertilization and embryo development, not only in individual spermatozoa, but within a large, heterogeneous sperm population. In the present study, we have analyzed *in vitro* quality of a large number of Flackvieh-Simmental bulls parameters including different type of motility (computer assisted sperm analysis), viability, acrosome integrity and mitochondrial activity (flow cytometry), functional integrity (hypo-osmotic swelling test and thermoresistance test) and DNA integrity (DNA fragmentation index) in order to compare with fertility (non-return rate, NRR).

Material and Method

Semen. Commercial straws of semen from ten bulls of Flackvieh-Simmental breed (2-4 years old) frozen in Triladyl egg-yolk extender (Minitübe, Germany) were available from the SC Semtest-BVN Târgu-Mureș, Romania. Each straw contained approximately 25 million spermatozoa. For *in vitro* analysis the straws were thawed in a water bath at 37°C for 20 s.

Computer assisted sperm analysis system (CASA System). Sperm motility was assessed using Sperm Class Analyzer® CASA System. An aliquot (5 µL) of semen was placed on a warmed microscope slide at 37°C and covered with a coverslip (18 × 18 mm). Images captured by camera microscope at a magnification of x 200 were analyzed by software. The mean values were calculated for each of the following parameters based on approximately 1000 spermatozoa: total motility (TM %), progressive motility (PM %), velocity curved line (VCL µm/s), velocity straight line (VSL µm/s), velocity average path (VAP µm/s), linearity index (LIN %), straightness index (STR %), oscillation index or wobble (WOB %), amplitude of lateral head displacement (ALH µm) and beat cross frequency (BCF Hz).

Flow-cytometry sperm analysis. Sperm viability, acrosome integrity and mitochondrial membrane status were assessed using the BD FACS Canto II flow cytometer. Fluorescence detection was set at 530 nm (green fluorescence), 285 (orange fluorescence) and 670 nm (red fluorescence).

Sperm viability was assessed using Live-Dead Sperm Viability Kit based on SYBR-14 and propidium iodide (PI) labeled. A sample of 60 µL of semen was mixed with 6 µL SYBR-14 and 2.5 µL PI, agitated well and incubated for 20 min at 37°C. Fluorescence was detected using 530 nm filter for SYBR-14 and 670 nm filter for PI. Ten thousand sperm per sample were evaluated and three sperm populations were detected: live sperm (green fluorescence), death sperm (red fluorescence) and dying sperm (double stained).

Sperm acrosome status was assessed using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and PI labeling. A sample of 100 µL of semen was mixed with 900 µL phosphate buffer solution (PBS) and 2 µL FITC-PNA. After 10 min at 37°C incubation the FITC-PNA fluorescence was detected at 530 nm, while PI was detected at 670 nm. Ten thousand sperm events per sample were analyzed and the sperm populations were classified in four categories: live sperm with acrosome reacted, dead

sperm with acrosome damage, live sperm with non-reacted acrosome (LNRA) and dead sperm with non-reacted acrosome.

Sperm mitochondrial membrane status was measured based on the respiratory activity using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanineiodide (JC-1). A sample of 100 µL of semen was mixed with 400 µL PBS and 1 µL JC-1. After 40 min at 37°C incubation the JC-1 fluorescence was measured at 530 nm and 585 nm. Ten thousand sperm per sample were analyzed and the sperm populations were classified in two categories: high mitochondrial activity expressed orange fluorescence (HMA) and low mitochondrial activity expressed green fluorescence.

Assessment of functional integrity

Hypo-osmotic swelling test (HOST) was accomplished by mixing 30 µL semen with 300 µL of HOS (0.0375 mg/mL Natriumcitrate x $2H_2O$ and 13.5 mg/mL fructose, 100 mOsm/kg). After the incubation in a water bath at 37°C for one hour, a drop of the sperm suspension was placed on a slide and covered with a glass coverslip. The slides were evaluated at phase contrast microscope using 400 x magnification. A total number of 200 sperm cells were counted, those with any degree of a coiled tail (HOST positive sperm) as well as those with a straight tail (negative HOST sperm).

Thermoresistance test was used in order to establish semen ability to retain motility during incubation time. Sperm rectilinear motility was analyzed at 15, 60, 120, 180 and 240 min of post-thaw incubation at 37°C.

DNA integrity (DNA fragmentation index). For DNA fragmentation index (DFI) semen was processed according to the instruction of the Bovine-Halomax® kit (ChromaCell SL, Madrid, Spain). After 5 min in a 90–100°C water bath, vials with agarose were left into a thermostatic water bath at 37°C to equilibrate for 5 min. When the agarose reached 37°C, 25 µL of semen were added to the vial and mixed with a pipette. Then, a drop of the cell suspension was placed on a previously treated and pre-cooled (5°C) slide and it was covered with a glass coverslip at 4°C for 5 min. The coverslip was smoothly removed and the slide was introduced into 10 mL of the lyses solution and maintained for 5 min at room temperature. The slide was then washed in MilliQ water for 5 min, dehydrated in sequential 70 and 100% ethanol baths for 2 min each and air dried. Just before analyzing under bright-field microscopy, the slides were immersed for 6 min in staining solution A and 7 min in staining solution B. According to the kit instructions, using 1000 x magnification at the microscope bright field 200 sperm were counted. Sperm showing a halo of dispersion equal or wider than the core minor diameter were considered positive for high DFI.

Fertility (non-return rate, NRR). In order to establish bull fertility, a number of 1235 artificial inseminations (AI) were carried out by well-trained technicians. Semen from each bull was used for AI for 20 to 200 cows. NRR is a general criteria used widely for determining pregnancy and was also used as measure of fertility. NRR represents percentage of cattle with no estrous cycle detected 60^{th} days after AI.

Statistical analysis. The software GraphPad Prism v. 6.0 (Graph Pad Software Inc., San Diego, CA, USA) was used for data analysis. The results were statistically analyzed using one way analysis of variance (ANOVA) with Tukey's Multiple Comparisons Test. The relationship between *in vitro* determined sperm quality (TM, PM, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, viability, LRNA, HMA, HOST and DFI) and fertility (the 60-day non-return rate) were analyzed using Spearman rank correlation. In all cases, $P<0.05$ was considered to be significant.

Results and Discussion. The average, standard deviation and standard error of mean for *in vitro* sperm quality parameters are presented in Table 1. Sperm motility analyzed by CASA indicated an average value of total motility higher than 80% with a progressive motility for half of them. In order to establish some correlation between fertility and *in vitro* sperm quality, ten CASA sperm parameters were analyzed.

Table 1

In vitro semen quality parameters and fertility in ten Flackvieh-Simmental bulls

Parameters	*X*	*SD*	*SEM*	*Min*	*Max*
TM (%)	84.92	6.29	1.99	73.39	91.27
PM (%)	42.20	5.92	1.88	35.99	51.23
VCL (µm/s)	47.05	3.82	1.21	41.14	53.41
VSL (µm/s)	19.05	2.85	0.90	17.13	25.57
VAP (µm/s)	28.52	2.73	0.86	25.34	33.10
LIN (%)	38.48	4.33	1.37	31.97	43.17
STR (%)	61.21	3.23	1.02	56.60	66.55
WOB (%)	59.50	4.15	1.31	53.36	64.45
ALH (µm)	2.22	0.17	0.05	1.99	2.50
BCF (Hz)	6.17	0.73	0.23	5.30	7.53
VIAB (%)	47.19	16.20	5.12	16.50	77.70
LNRA (%)	76.44	6.03	1.91	65.90	83.10
HMA (%)	70.93	17.19	5.44	33.10	92.40
HOST (%)	46.10	11.45	3.62	24.00	60.50
DFI (%)	3.90	1.22	0.39	2.00	6.00
NRR (%)	55.74	4.25	1.34	49.40	61.15

TM - total motility, PM - progressive motility, VCL - velocity curved line, VSL - velocity straight line, VAP - velocity average path, LIN - linearity index, STR - straightness index, WOB - oscillation index or wobble, ALH - amplitude of lateral head displacement, BCF - beat cross frequency, VIAB - viability, LNRA – live non-reacted acrosome, HMA - high mitochondrial activity, HOST - hypo-osmotic test, DFI - DNA fragmentation index and NRR - non-return rate.

Semen viability was analyzed by flow cytometry, taking into account only the green fluorescence sperm because the double stained sperm (green and red) did not have intact sperm membrane (dying spermatozoa). For acrosome integrity analysis we focused only on live non-reacted acrosome sperm population. The mean value of sperm with intense mitochondrial activity justifies the mean values of sperm motility and velocity. Regarding sperm membrane and DNA integrity, the mean values of hypo-osmotic test and DNA fragmentation index revealed good sperm quality.

The results of thermoresistance test have shown a slow decrease trend in the first four hours of incubation (Figure 1). However, the differences between motility in different time periods were no significant from statistic point of view.

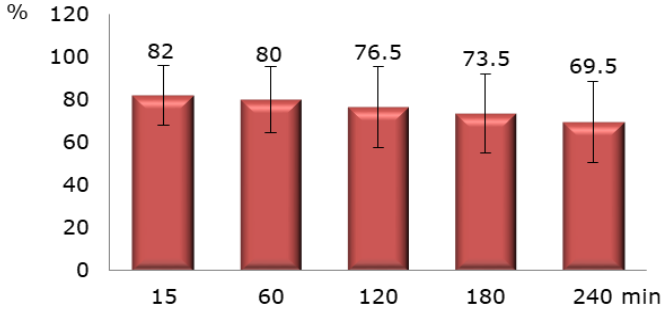

Figure 1. Dynamics of motility during the thermoresistance test.

According to Vianna et al (2009), rapid (46°C/30 min) and slow (38°C/5 h) thermoresistance tests are not reliable to predicting *in vivo* behavior of bull frozen semen

and are not effective to estimate fertility. In an interesting study no significant differences were observed in progressive motility and thermoresistance between semen of two Angus bull groups with high and low fertility *in vivo*, but vitality, HOST and acrosome integrity showed significant differences (Morado et al 2015).

For *in vivo* testing of bull semen fertility we used both heifers and cows. The percentage of NRR was 67.34±1.59 for the primiparous and 52.72±1.39 for the multiparous, with significant statistical differences (P<0.0001). However, for a better estimation of potential fertility, the percentage of NRR was calculated including both categories. This value was used to analyze the correlation between NRR and *in vitro* semen parameters (Table 2).

Table 2

Spearman correlation coefficients between *in vitro* semen quality parameters and fertility in ten Flackvieh-Simmental bulls

Parameters	*r*	*P-value*	*Significance*
TM	0.8788	0.0016	**
PM	0.4303	0.2182	Ns
VCL	0.3697	0.2957	Ns
VSL	0.6485	0.0490	*
VAP	0.6848	0.0347	*
LIN	0.3818	0.2788	Ns
STR	0.4377	0.2070	Ns
WOB	0.2970	0.4069	Ns
ALH	0.1152	0.7589	Ns
BCF	0.4667	0.1786	Ns
VIAB	0.1879	0.1786	Ns
LNRA	-0.2727	0.4483	Ns
HMA	-0.4788	0.1663	Ns
HOST	-0.2848	0.4271	Ns
DFI	0.5293	0.1189	Ns

TM - total motility, PM - progressive motility, VCL - velocity curved line, VSL - velocity straight line, VAP - velocity average path, LIN - linearity index, STR - straightness index, WOB - oscillation index or wobble, ALH - amplitude of lateral head displacement, BCF - beat cross frequency, VIAB - viability, LNRA - live non-reacted acrosome, HMA - high mitochondrial activity, HOST - hypo-osmotic test and DFI - DNA fragmentation index

As we can see, we found a few correlations between *in vivo* and *in vitro* parameters (Figure 2). Only in the case of motility (TM) and velocity (VSL and VAP) the correlation with NRR was significant (P<0.05).

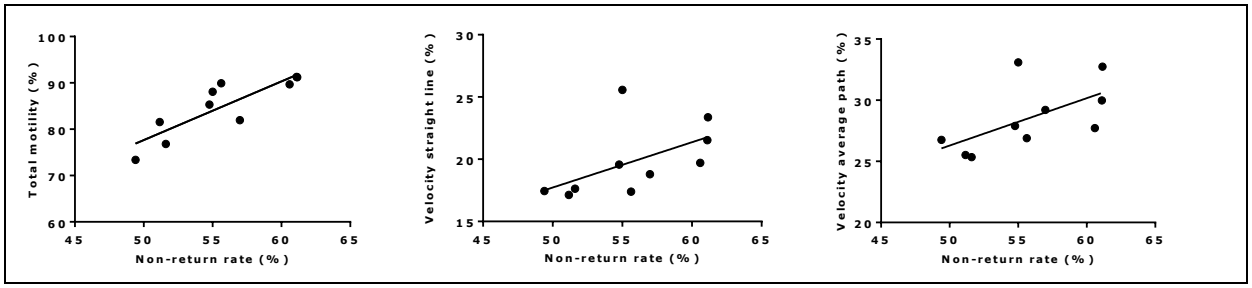

Figure 2. Relationship between non-return rate and some CASA parameters.

No other correlation was observed between NRR and CASA parameters. On the other hand, we found a weak negative relationship between NRR and LNRA, HMA and HOST without significant differences from statistic point of view. In order to establish which kinetic parameters are better correlated with bull fertility (NRR and pregnancy rate), Nagy et al (2015) found that velocity (curvilinear, straight-line and average path velocity) could be used to predict fertility potential of Holstein Friesian bulls. Using the same bull breed (Holstein Friesian), Cojkić et al (2017) observed a significantly correlations between fertility (expressed by pregnancy rate per cycle) and total sperm motility (r=0.67), progressive sperm motility (r=0.65) and rapid sperm movement (r=0.71). Other researchers show significant Spearman correlation coefficient (r=0.84) between nine sperm quality parameters and Holstein bull fertility (Gliozzi et al 2017). Thus, total motility, active cells, hyperactive cells, straightness, straight line velocity, linearity, viable sperm with high membrane lipid disorder, Alpha-T standard deviation and fragmented DNA sperm were proposed to use in order to predict bull fertility.

However, not all studies have shown strong correlation between bull fertility and semen quality parameters. The relationship between semen quality parameters and field fertility is due by many factors, animal species, extender or medium, specimen chamber, intensity of illumination, imaging hardware and software, instrument settings, technician, etc., all affect accuracy and precision of output values (Amann & Waberski 2014). Besides this, the most important factor is represented by breed (Rabidas et al 2012; Kumar et al 2015). Eight out of ten semen motility parameters (measured by CASA) differed significantly between dairy bull breeds (Holstein and Swedish Red) and beef bull breeds (Limousin, Charolais, Simmental, Hereford, Angus and Blonde D'Aquitaine), as well as DFI, acrosome integrity of high and low respiratory activity (Morrell et al 2018).

Conclusions. Significant correlations were seen between NRR and total motility, velocity straight line and velocity average path, while no correlations were found between other type of motility, viability, acrosome and sperm membrane integrity, DNA integrity and mitochondrial activity.

Acknowledgements. This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS/CCCDI–UEFISCDI, project number PN-III-P2-2.1-BG-2016-0131, within PNCDI III.

References

Amann R. P., Waberski D., 2014 Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology 81(1):5-17.

Attia S., Katila T., Andersson M., 2016 The effect of sperm morphology and sire fertility on calving rate of Finnish Ayrshire AI bulls. Reprod Dom Anim 51(1):54-58.

Blezinger S. B., 2010 Bull fertility critical to a successful breeding program. Cattle Today Newspaper.

Chenoweth P. J., McPherson F. J., 2016 Bull breeding soundness, semen evaluation and cattle productivity. Anim Reprod Sci 169:32-36.

Cocan B., Mureșan G., 2015 Researches regarding the influence of the bulls age on the raw and processed semen. Bulletin UASVM Animal Science and Biotechnologies 72(1):20-23.

Cojkić A., Dimitrijević V., Savić M., Jeremić I., Vuković D., Čobanović N., Obradović S., Petrujkić B. T., 2017 The correlation between selected computer assisted sperm analysis parameters and bull fertility. Veterinarski Arhiv 87(2):129-137.

Correa J. R., Pace M. M., Zavos P. M., 1997 Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. Theriogenology 48:721-731.

Dalton J. C., 2011 Semen quality factors associated with fertility. Proceedings, Applied Reproductive Strategies in Beef Cattle – Northwest, Boise, pp. 265-281.

Evenson D. P., 2016 The sperm chromatin structure assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim Reprod Sci 169:56-75.
Gabor D. V., Roman M., Bogdan A. T., Vintila I., Tăpăloagă D., Tăpăloagă P. R., 2011 Analysis of the distribution of the frozen semen made by Semtest BVN Targu Mures in 2010. Scientific Papers: Animal Science and Biotechnologies 44(1):216-218.
Gliozzi T. M., Turri F., Manes S., Cassinelli C., Pizzi F., 2017 The combination of kinetic and flow cytometric semen parameters as a tool to predict fertility in cryopreserved bull semen. Animal 11(11):1975–1982.
Kastelic J. P., 2013 Male involvement in fertility and factors affecting semen quality in bulls. Animal Frontiers 3(4):20-25.
Kordan W., Fraser L., Wysocki P., Strzeżek R., Lecewicz M., Mogielnicka-Brzozowska M., Dziekońska A., Soliwoda D., Koziorowska-Gilun M., 2013 Semen quality assessments and their significance in reproductive technology. Pol J Vet Sci 16(4):823-833.
Kumar U., Gawande A. P., Sahatpure S. K., Patil M. S., Lakde C. K., Bonde S. W., Borkar P. L., Poharkar A. J., Ramteke B. R., 2015 Assessment of semen quality in pure and crossbred Jersey Bulls. Veterinary World 8 (10):1266-1272.
Morado S., Pereyra V., Breininger E., Sara R., Cetica P., 2015 Study of sperm evaluation parameters to estimate cryopreserved bovine semen fertility. Austin J Vet Sci & Anim Husb 2(1): id1005.
Morrell J.M., Valeanu A. S., Lundeheim N., Johannisson A., 2018 Sperm quality in frozen beef and dairy bull semen. Acta Vet Scand 60(41):1-10.
Nagy Á., Polichronopoulos T., Gáspárdy A., Solti L., Cseh S., 2015 Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis. Acta Vet Hungarica 63(3):370–381.
Onaciu G., Jurco E., 2011 Semen quality analysis for a group of five bulls used for artificial insemination and disseminated by SC Semtest BVN from Mures County. Bulletin UASVM Animal Science and Biotechnologies 68(1-2):429.
Rabidas S. K., Talukder A. K., Alam M. G. S., Bari F. Y., 2012 Relationship between semen quality parameters and field fertility of bulls. J Emb Trans 27(1):21-28.
Rodriguez-Martinez H., Barth A. D., 2007 In vitro evaluation of sperm quality related to in vivo function and fertility. Soc Reprod Fertil 64:39-54.
Saacke R. G., 2006 Semen quality factors associated with fertility. Proceedings, Applied Reproductive Strategies in Beef Cattle, St. Joseph, pp. 271-280.
Serafini R., Romano J. E., Varner D. D., Di Palo R., Love C. C., 2015 Sperm DNA assays and their relationship to sperm motility and morphology in bulls (*Bos taurus*). Anim Reprod Sci 159:77-86.
Takeda K., Uchiyama K., Kinukawa M., Tagami T., Kaneda M., Watanabe S., 2015 Evaluation of sperm DNA damage in bulls by TUNEL assay as a parameter of semen quality. J Reprod Dev 61(3):185.
Thundathil J. C., Dance A. L., Kastelic J. P., 2016 Fertility management of bulls to improve beef cattle productivity. Theriogenology 86(1):397-405.
Utt M. D., 2016 Prediction of bull fertility. Anim Reprod Sci 169:37-44.
Vianna F. P., Papa F. O., Zahn F. S., Melo C. M., Dell'Aqua J. A. Jr., 2009 Thermoresistance sperm tests are not predictive of potential fertility for cryopreserved bull semen. Anim Reprod Sci 113(1-4):279-82.

Received: 29 October 2018. Accepted: 04 December 2018. Published online: 12 December 2018.
Authors:
Marius Zăhan, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Department of Animal Reproduction, Romania, Cluj-Napoca, 3-5 Mănăştur Street, 400372, e-mail: mzahan@usamvcluj.ro
Emoke Pall, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Institute of Life Sciences, Romania, Cluj-Napoca, 3-5 Mănăştur Street, 400372, e-mail: emoke.pall@usamvcluj.ro
Mihai Cenariu, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Department of Reproduction and Obstetrics, Romania, Cluj-Napoca, 3-5 Mănăştur Street, 400372, e-mail: mihai.cenariu@usamvcluj.ro
Ileana Miclea, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Department of Animal Reproduction, Romania, Cluj-Napoca, 3-5 Mănăştur Street, 400372, e-mail: ileana.miclea@usamvcluj.com
Alexandru Sergiu Dascăl, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Department of Animal Reproduction, Romania, Cluj-Napoca, 3-5 Mănăştur Street, 400372, e-mail: dascalsergiu90@yahoo.com

How to cite this article:
Zăhan M., Pall E., Cenariu M., Miclea I., Dascăl A. S., 2018 Relationship between *in vitro* semen parameters and bull fertility. ABAH Bioflux 10(2):156-163.