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Seasonal Variation and Age-Related Changes in Semen Quality of Limousin Bull in Indonesian Artificial Insemination Center

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ABSTRACT

The objective of this study was to determine the effect of season and age on semen quality (individual motility, mass motility, concentration, volume, pre-freeze motility, and post-thaw motility) of Limousin Bull under the tropical condition in Indonesian artificial insemination center. This study used a large data set recorded over eight years (2011 until 2018) of production period (n = 6758 ejaculates, n = 19 bulls) retrieved from Singosari National Artificial Insemination Center. Rainfall and other climatic data were collected from Malang Meteorological, Climatology, and Geophysics Agency, Indonesia. Seasonal effect was grouped into three groups, i.e. rainy season (total rainfall per month >200mm), humid season (total rainfall per month = 100-200 mm), and dry season (total rainfall per month <100). Bull age is grouped into seven groups (2, 3, 4, 5, 6, 7 and 8). Data were analyzed using Mixed Model procedure classifying effect of season and age on fixed effect and bull as a random effect. Season had a highly significant effect on individual motility, pre-freeze motility, and post-thaw motility (P<0.001), and did not have a significant effect on concentration, volume, pH, and mass motility. Individual motility, pre-freeze motility, and post-thaw motility were highest in the rainy season and declined in the dry season. Bull age had a highly significant effect on individual motility, concentration, volume, pre-freeze motility, and post-thaw motility (P<0,001). Bull age did not have a significant effect on mass motility and pH. Two- and three-years old bulls have superior individual motility and concentration. However, it is decreasing at age three until eight years old. The lowest volume was at age two and increased gradually until age eight. Meanwhile, pre-freeze motility and post-thaw motility increased at two to five years old and then declined until eight years old.

Key words: Season, Age, Semen quality, Limousin.

INTRODUCTION

Artificial Insemination is the first generation of biotechnology on reproduction that has an important role to improve the genetic quality of livestock, especially in cattle, and now has become a technology widely used commercially in many countries (Verma *et al.*, 2012; Büyükleblebici *et al.*, 2014). Artificial insemination developed rapidly and became a technology that has high economic value because it has many advantages. Advantage of artificial insemination are maximizing the use of superior bull, widely disseminating superior genetic material, increasing the efficiency of genetic selection, introducing new genetic material, reducing the transportation cost of the bull for breeding purposes, and reducing the risk of transmission of reproductive diseases (Verma *et al.*, 2012).

Frozen semen of Limousin Bull is the most widely used in Indonesia. In one year, more than two million doses of Limousin Bull's frozen semen (39% from total distribution) were distributed throughout the regions in Indonesia. Currently, almost all Limousin Bull used for the production of frozen semen are imported from a subtropical country. That can be the inhibiting factor for reproductive performance and impact on the sustainability of frozen semen production due to seasonal and climatic differences between Limousin Bull origin and production sites. Based on the author's knowledge, the study of seasonal effect on semen quality (fresh semen, liquid semen, and frozen semen) of Bos Taurus, especially Limousin Bull in tropical climate county, has not been widely published.

In addition to seasonal factors, reproductive performance is influenced by the age of bull (Jaenudeen and Hafez, 2000; Juma *et al.*, 2018). The effect of age on several parameters of sperm quality does not simultaneously increase with increasing age of the bull.

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Volume increases significantly in the onset of pubertal bull and continues to increase until 60 months. Motility increases rapidly during 10 to 20 weeks after puberty, while concentration increases slowly at that age (Pharrish *et al.*, 2018; Ondho *et al.*, 2019). The amount of frozen semen production is certainly not only influenced by one parameter of semen quality, but many parameters such as motility, semen volume, concentration also contribute to the amount of frozen semen production. Artificial insemination center that produces frozen semen certainly needs to pay attention to the optimal age range of bull to produce good quality semen with an optimal amount of frozen semen production.

Many factors can affect semen quality, it becomes a challenge for artificial insemination centre to produce frozen semen in a sustainable manner with a sufficient amount to fulfil the national demand. Therefore, to answer these challenges, a research effort is needed in studying the above factors and their relation to frozen semen production.

This study aims to determine the effect of season and age on the quality of fresh semen (individual motility, concentration, volume, mass motility, pH), liquid semen (individual motility), and frozen semen (individual motility) of Limousin Bull in Indonesian artificial insemination center in relation to the tropical condition.

MATERIALS AND METHODS

Animal, weather data and treatment

Semen quality data from Limousin Bull (n = 6758 ejaculates, n = 19 bulls) were retrieved from Singosari National Artificial Insemination Center, Indonesia (the number of ejaculates in each parameter is presented in Table 1). Data used in this study were collected from over eight years of production period (2011 until 2018). Each bull housed in a barn was under similar management, feeding, and ambient temperature conditions (climatic condition is presented in Table 2). Data on rainfall, temperature, and humidity were collected from Malang Meteorology, Climatology, and Geophysics Agency, Indonesia, which is about 5 km away from Singosari National Artificial Insemination Center.

The season and age of bulls are the treatments in this study. Bulls were categorized into seven groups (2,3,4,5,6,7 and 8). Seasonal effect was grouped into three groups, i.e. rainy season (total rainfall per month >200mm), humid season (total rainfall per month = 100-200mm), and dry season (total rainfall per month <100 mm) (the number of the season is presented in Table 3).

Semen collection

Semen was collected twice a week. All bulls were sexually stimulated using bull teaser and allowed to false mount three until five times until the bulls reach optimum libido. Semen were collected from all bulls using artificial vagina with the temperature around 40-43ºC.

Frozen semen production

Semen that had been collected was given tris egg yolk-based extender. Dilution is divided into three stages-the first step was by adding diluent A1, the second step was by adding diluent A2, and the third step was by adding B. Diluents A1 and A2 had the same composition consisting of 1.6% tris aminomethane, 0.9% citric acid, 1.4% lactose, 2.5% raffinose, 80% aquades, and 20% egg yolk. The composition of diluent B was the same as those of diluent A but was added by 13% glycerol. Equilibration was performed for 3 until 4 hours. Liquid semen (extended semen) is packaged in 0.25 ml straw, cooled quickly to reach -140℃ using IMV Technology® digitcool, and frozen in liquid nitrogen until -196℃.

Fresh, liquid, and frozen semen quality measurement

Fresh semen collected from each ejaculation were assessed for their volume, individual motility, mass motility, concentration, and pH immediately. Volume was measured with a collector tube inside an artificial vagina. Individual motility and mass motility were assessed by dripping 0.1 µl fresh semen, which had been mixed with 0.1 µl tris egg yolk-based extender on the glass object above the Minitube® Slide Warmer. Individual motility was observed with a microscope at 200x magnification and mass motility observed at 10x magnification. Concentration was evaluated by mixing 35 µl fresh semen with 3.5 mL 0.9% NaCl, then it was placed in a cuvette tube and homogenized using a stirrer. Concentration was measured using Minitube® Photometer SDM6 spectrophotometer. pH was measured directly after collecting semen using a pH meter. Pre freeze motility (liquid semen) and post-thaw motility (frozen semen) evaluation use the same method as the evaluation of individual motility on fresh semen. Pre freeze was evaluated directly after equilibration. Post-thaw motility was evaluated after frozen semen had been thawed at 37ºC for 15 seconds.

Statistical analysis

Data were analyzed using the SAS® University Edition (SAS Institute Inc., Cary, NC, USA). Quality parameter of semen was analyzed by the MIXED MODEL (SAS Institute Inc., 2015) procedure by classifying the effects of seasons and bull age as fixed effects and bull as a random effect. Interactions between season and age were assumed as negligible, so they were not tested. Tukey multiple comparisons were used when the differences among the least-square means were significant (P<0.05).

RESULTS

Effect of season on semen quality

The results of the study show that season has a significant effect on individual motility (P<0.01), pre-freeze motility (P<0.05), and post-thaw motility (P<0.01) and no significant effect on concentration, volume, mass motility, pH, pre-freeze motility and post-thaw motility (Table 4). The individual motility of fresh semen in the rainy season (66.71%) is higher than a humid season (65.72%) and dry season (65.37%). Pre freeze motility is 55.51% in rainy season, 55.38% in humid season, and 55.13% in dry season. Similar to the individual motility of fresh semen, pre-freeze motility in rainy season is higher than the humid and dry season. PTM in the rainy season (43.29%) and humid season (43.03%) are higher than dry season (42.51%).

Table 1: Total sample in each parameter

Semen Type	Parameters	n
Fresh Semen	Individual Motility	6758
	Concentration	6758
	Mass Motility	6758
	Volume	6758
	pH	6758
Liquid Semen	Pre-freeze Motility (%)	4968
Frozen Semen	Post-Thaw Motility (%)	4722

Table 2: Season classification during study

Season classification	Dry season	Humid Season	Rainy season
Rainfall per month (mm)	<100	100-200	>200
Total Month (n)	38	21	34

Table 3: Climatic condition during study

Season	THI Max*	Temperature (ºC)		Humidity (%)	
		Average	Max	Average	Min
Dry	69.74±1.12	23.09±1.73	29.10±1.40	70.72±6.56	36.73±11.75
Humid	69.70±1.14	24.21±0.67	29.04±1.43	74.55±6.89	46.18±9.53
Rainy	69.44±0.51	24.07±0.75	28.72±0.64	81.39±5.42	50.88±7.00

*THI Max = 0.8 x Maximum Temperature (ºC) + (Minimum Humidity%/100) x: (Maximum Temperature-14.4+46.4) (Sabes-Alsina *et al*., 2018)

Effect of age on semen quality

There was a highly significant effect caused by bull age on fresh semen motility (P<0.01). Individual motility decreases with age (Table 5). Individual motility of two years old bull is the highest among the other age groups (68.45%). Individual motility decline begins at the age of 4 to 5 years old. The individual motility is stagnant or not significantly different (P>0.05) from 5 to 6 years old. Then the decrease in individual motility occurs gradually starting from the age of 6, 7, and 8 years old (P<0.01).

Bull age had a highly significant effect on concentration (P<0.01). This is similar to the case with fresh semen motility, where the concentration of spermatozoa decreases periodically with age (Table 5). The decrease in spermatozoa concentration gradually starts at age 4 to 7 (P<0.01), after stagnating (not significant) at age 2, 3, and 4. Then, at the age 7 and 8, the concentration again stagnated (P>0.05). Concentrations at age 7 to 8 were not significantly different.

Bull age also had highly significant effect on semen volume (P<0.01). Volume increases with age (Table 5). This is inversely proportional to the individual motility of fresh semen and the concentration, which declines in quality with age. Meanwhile, there is no significant effect caused by age on mass motility and pH.

Bull age had a highly significant effect on pre-freezing motility (P<0.01; Table 5). Pre freezing motility increases from age 2 to 5, then decreases on age 8. There is a highly significant difference by bull age on post-thaw motility (P<0.01; Table 5). Post-thaw motility has the same biological pattern as pre-freeze motility. Post-thaw motility increases from age 2 to 5, then decreases on age 8.

DISCUSSION

The season had a highly significant impact on the individual motility, pre-freeze motility, and post-thaw motility. These results are similar to previous studies where season has a significant effect on the motility of fresh semen of Thapkar Bull (Perumal *et al*., 2017) and Karan Fries Bull (Bhakat *et al*., 2014). Meanwhile, Murphy *et al*. (2018) show a decrease in post-thaw motility in Friesian Bull during the summer compared to other seasons. Decreased motility tends to occur in seasons with higher temperature-humidity index (THI), higher temperatures (Majic-Balic *et al*., 2012; Sabes-Alsina *et al*., 2018) and low humidity (Biniova *et al*., 2017).

The factor that may be the cause of motility changes in different seasons is the climatic conditions, which also differ in each season. Dry season tends to have higher THI, higher maximum temperatures and lower humidity compared to the rainy season (Table 2). It causes heat stress that can decrease motility. Bouraoui *et al*. (2002) state that for cow dairy, the increase in THI from 69 to 70 decreases milk production by 9% as a result of heat stress. In reproduction, heat stress reduces the motility through the mechanism of increasing gene expression from Heat Shock Protein (HSP) (Cheng *et al*., 2016).

Increasing temperature has an impact on the process of spermatogenesis. Januskauskas *et al*. (1995) reveal a decrease in the quality of spermatozoa (Post-thaw Motility and viability) in the middle and final stages of the spermatogenesis process (16-51 days after testicular insulation). In addition, Vogler *et al*. (1991) explain that an increase in temperature causes a decrease in semen motility when spermatozoa is in the epididymis and in the rete of the testis (3-9 days after testis insulation).

The individual motility and spermatozoa concentration decrease with age. This is consistent with the results of research by Isnaini *et al*. (2019) which explains that there is a decrease in individual motility and concentration in Simental cattle from the age of 2 years, 5 years, up to 12 years. Asad *et al*. (2004) state the same thing with the decrease in individual motility and concentration from the age of 4 to 12. However, Majic-Balic *et al*. (2012) in their study explain individual motility is lower in the age group of 5 to 10 compared to the age of 2 to 4 during spring and autumn.

Motility has a negative correlation with increasing age (Carreira *et al*., 2017). Decreased motility with increasing age is a result of decreased antioxidant activity (glutathione peroxidase and peroxidase dismutase), which leads to an increase in oxidative damage. Oxidative damage is known as one of the causes of decreased motility. In addition, increasing age reduces lipid content in semen plasma. Lipids play an important role in supplying energy for the movement of spermatozoa (Kelso *et al*., 1997).

In this study, the highest spermatozoa concentration was found in age 2 and the lowest at the age of 8. Mahmood *et al*. (2014) reveal that concentration is negatively correlated with increasing age. Vince *et al.* (2018) mention the spermatozoa concentration in young Simental cattle (age 3.1±0.58 years) is 1.300×10^6 and decreases in old age (age 7.4±1.91 years) to 1.140×10^6. Decreased spermatozoa concentration indicates a decrease in the function of the testes in the process of spermatogenesis. The results of the study by Rajak *et al*. (2014) show that increasing age causes a decrease in the percentage of primary spermatocytes in Friesian Holstein and Thapkhar crosses. In addition, the percentage of spermatogonia and spermatid cells also tends to decrease in old age, although it does not show statistically significant differences.

Table 4: Effect of dry, humid, and rainy season on quality fresh, liquid, frozen semen of Limousin bull

Semen	Quality	Dry Season		Humid Season		Rainy Season		P-Value
		lsm	se	lsm	se	lsm	se	
Fresh Semen	Individual motility (%)	65.37[b]	0.17	65.72[b]	0.21	66.71[a]	0.17	<0.01
	Concentration ($x10^6$)	1422	8.12	1422	9.85	1400	7.87	0.1678
	Volume (ml)	6.16	0.037	6.13	0.05	6.15	0.03	0.9368
	Mass Motility	2.0005	0.0007	2.0016	0.0009	2.0006	0.0007	0.4817
	pH	6.26	0.04	6.13	0.05	6.21	0.04	0.1658
Liquid Semen	Pre-Freeze Motility (%)	55.13[b]	0.07	55.38[ab]	0.09	55.51[a]	0.07	0.0137
Frozen Semen	Post-Thaw Motility (%)	42.51[b]	0.12	43.03[a]	0.14	43.29[a]	0.12	<0.01

*lsm = least square means, se=standard error: *Different letters (a,b) between rows indicate significant difference.

Table 5: Effect of age on quality fresh, liquid, frozen semen of Limousin bull

Bull age	Fresh Semen										Liquid Semen		Frozen Semen	
	Individual motility (%)		Concentration ($x10^6$)		Volume (ml)		Mass motility		pH		Pre-freeze motility		Post thaw motility	
	lsm	Sd	lsm	sd	lsm	sd	lsm	sd	lsm	sd	lsm	sd	Lsm	sd
2	68.45[a]	0.49	1653 [ab]	23.34	5.92[cd]	0.11	2.0038	0.0022	6.49	0.11	55.33[bd]	0.2	41.61[d]	0.31
3	67.59[a]	0.25	1679[a]	11.96	5.96[c]	0.05	2.0021	0.0012	5.95	0.05	55.72[b]	0.1	43.91[b]	0.17
4	66.25[b]	0.25	1547[b]	11.93	6.2[abc]	0.05	2.0001	0.0011	6.2	0.05	55.97[a]	0.12	43.47[b]	0.19
5	65.71[c]	0.22	1440[c]	10.73	6.0[bc]	0.05	1.9992	0.001	5.88	0.05	56.14[a]	0.1	44.11[a]	0.15
6	65.61[c]	0.24	1261[d]	11.76	6.21[ad]	0.05	1.9992	0.0011	6.19	0.05	55.26[b]	0.1	42.93[b]	0.19
7	64.43[d]	0.26	1188[e]	12.42	6.32[a]	0.06	2.0002	0.0012	6.31	0.06	54.32[d]	0.12	41.21[d]	0.19
8	63.48[e]	0.24	1133[e]	11.51	6.39[a]	0.05	2.0018	0.0011	6.4	0.05	54.61[c]	0.11	42.36[c]	0.18
P value	<0.01		<0.01		<0.01		0.1668		0.0683		<0.01		<0.01	

*lsm = least-square means, se=standard error: *Different letters (a-e) between columns indicate a significant difference.

The results of this study show an increase in volume with increasing male age. Murphy *et al*. (2018) reveal in their study that the increase in volume has an impact on increasing the semen volume for Friesian Holstein Cattle. Similar case also happens to Czech Fleckvieh Cattle (Paldusová *et al*., 2016).

Increased volume in older bulls is associated with increased body weight along with testicular growth. An increased body weight naturally follows testicular growth in the amount of semen produced (Majic-Balic *et al*., 2012). This statement is supported by the results of Mahmood *et al*. (2014) who explain that increasing age is positively correlated with scrotal circumference (r=0.590) and semen volume (r=0.928). The increase in volume with increasing age is also associated with an increase in testosterone activity along with the development of the vesicular gland and prostate gland, which increases rapidly from the age of 26 weeks (Chandolia *et al*., 1997).

Pre-freeze motility and post-thaw motility increases until the age of five and reaches the highest value at that age then decreases until the age of eight years. Low pre-freeze motility and post-thaw motility values under age five can be related to spermatozoa membrane integrity. Rehman *et al*. (2016) mention that there are significant differences in the strength of spermatozoa membrane integrity in different age groups. The 1-3 years old age group has the lowest membrane integrity, then increases in the 3-5 years old age group, and the highest membrane integrity is found in the age group of more than 5 years old. Membrane integrity is a major part of spermatozoa that is vulnerable to damage caused by the freezing process (Amal *et al*., 2015). Membrane damage is associated with the decreased kinematic activity of spermatozoa and disrupts the regulation of calcium ion exchange, therefore affecting motility (Khalil *et al*., 2019). In addition to the freezing process, membrane damage also occurs during dilution (liquid semen). The extender contributes to damaging the structure of spermatozoa and disrupting the homeostasis in the semen, therefore affecting membrane damage and decreasing motility (Gączarzewicz *et al*., 2010). Lower pre-freeze motility and post-thaw motility at the age below 2, 3, and 4 compared to the age of 5 are related to membrane integrity, which is also lower in that age.

Pre-freeze motility and post-thaw motility decrease gradually from age five until the age eight, it can be caused by a decrease in antioxidant activity at that age, which occurs in the motility of fresh semen. Kelso *et al*. (1997) mentions a significant decrease in antioxidant activity at the age of more than nine when compared to age 5-6. Antioxidant activity is needed in the freezing process to minimize the formation of reactive oxygen status (ROS). The freezing process is known to increase the level of ROS, so it affects the functional and structural damage of spermatozoa (Majic-Balic *et al*., 2012; Mostek *et al*., 2017).

Conclusion

In conclusion, the best quality fresh, liquid, and frozen semen is obtained in the rainy season. Bull age has a highly significant effect on motility, volume, concentration, pre-freeze motility, and post-thaw motility. Maximum quality of fresh semen based on individual motility and concentration is at the ages of 2 and 3 years old. Five is the maximum age for quality liquid and frozen semen.

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