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# The Influence of Individual Factors on the Characteristics and Production of Frozen Semen of Bali Cattle

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**ABSTRACT**

Individual difference is one of the factors that influence the semen quality during ejaculation. The study aimed to analyze the influence of individual factors on the characteristic and production of frozen semen of Bali cattle. The ejaculation was collected every morning, resulting in 92-115 ejaculates from each animal. One hundred fifteen ejaculate semen samples were collected from 10 Bali cattle using an artificial vagina. The semen color, consistency, pH, volume, abnormality, motility, concentration, total sperm (TS), and total sperm motility (TSM) were analyzed. The frozen semen was evaluated for motility before freezing (MBF), post thawing motility (PTM), total sperm motility counted form PTM (TSMP), and frozen semen production. Data were analyzed statistically using one-way analyzed variance to obtain the difference between individuals. The results showed the semen was a milky color with a thin, medium, and thick consistency. The semen pH and motility were not significantly different between individuals ($p>0.05$). In comparison, the individual differences affected ($p>0.01$) volume, abnormality, concentration, TS, and TSM. The individual differences of frozen semen also affected MBF, PTM, and TSMP. The characteristics and production of frozen semen were influenced by individual differences, which can then be used to determine superior males.



## Introduction

Bali cattle is local cattle with a wide distribution throughout Indonesia (Purwantara *et al*., 2012). The excellence of Bali cattle is resistant to extreme climate changes, easy adaptation to feed, a relatively high percentage of the carcass with weight reaching 138.68±1.77 kg, balanced ratio of meat to bone, low backfat thickness with a fat weight of 14.52±1.65 kg, small marbling with a safe level of cholesterol content (Suryanto *et al*., 2017; Tahuk *et al*., 2018). Bali cattle's value of service preconception is 1.4-1.49 (Deskayanti *et al*., 2020; Samberi *et al*., 2010), with a pregnancy rate of 86.5±5.2 % (Akma *et al*., 2016). These various advantages cause these cattle to maintain their genetic material through a breeding program strategy (Widyas *et al*., 2017).

Artificial insemination is one of the reproductive technologies to produce a high genetic potential of offspring (Isnaini *et al*., 2019). Several factors, including semen quality, influence the success of artificial insemination. The difference of individual influence the semen quality and affect the production of frozen semen (Fazrien *et al*., 2020). The previous studies reported that different individual impact semen quality (Isnaini *et al*., 2019; Fazrien *et al*., 2020; Indriastuti *et al*., 2020). Several factors cause differences, even though the analysis procedure manual and frozen semen standards have been issued (SNI 4869-1: 2017). Semen volume, pH, concentration, motility, motility before freezing (MBF), and post-thawing motility (PTM) are influenced by Bali cattle individuals (Fazrien *et al*., 2020; Indriastuti *et al*., 2020). Fresh semen, which is qualified to be produced as frozen semen, has a pH of 6.2-6.8, motility > 70%, and abnormality <10% (Indriastuti *et al*., 2018). The process and steps are crucial in the production of semen frozen due to semen damage that often occurs (Ozkavukcu *et al*., 2008). The frozen semen that meets the standard to be distributed in the market has PTM > 40%. The evaluation of semen quality is a crucial step to determine the proper condition of semen used and processed into frozen semen. Therefore, this study aimed to examine the individual influence on the characteristics and production of frozen Bali cattle semen.

## Materials and methods

*Experimental animals*

The study was conducted at the Center for Artificial In-

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semination (AI) laboratory, Singosari, Malang, East Java, Indonesia. Semen was obtained from 10 male cattle, including Ajay, Renon, Wasuki, Sanur, Uluwatu, Tabanan, Gorudo, Nabha, Bratan, and Jimbaran cattle. The animals were aged 6-11 years old and weighed 546.1-672.7 kg. The animals were fed by forage, silage, concentrate, and mineral according to the body weight and water ad libitum. The ejaculation was collected every morning, resulting in 92-115 ejaculates from each animal. Measurement of each variable was carried out from January 2019 to December 2020. Semen was collected using a bull teaser, while to increase libido, a false mount was carried out. Semen with a motility value> 70% was then processed into frozen semen according to the procedure at the AI center.

*Ethical approval*

The study procedures were done according to the operational standard (SNI ISO 9001: 2015 No. G.01-ID0139- VIII-2019) at Artificial Insemination (AI) Center, Singosari, and controlled by the veterinarian.

*Evaluation of fresh semen*

Fresh semen was evaluated for the semen color, consistency, pH, motility, abnormality, concentration, total sperm (TS), total sperm motility (TSM), and motility before freezing (MBF). The semen color was evaluated by directly observing the color of the semen stored in the tube. The consistency of semen was evaluated by tilting the semen container tube and then re-enforcing it. The assessment was carried out after the tube was straightened. The determination of semen pH was carried out using the Bromoltimolblue indicator.

The evaluation of motility was done using the CASA IVOS II tool. Semen was mixed with physiological NaCl (1:10), which then a drop of the mixture was dropped to object glass and covered with a cover glass. Motility observations were carried out with a 200x magnification microscope in several fields of view. Sperm abnormality was evaluated using eosin-nigrosin staining. A drop of semen was placed on an object-glass, and eosin-nigrosin staining was added. Mixed the mixture and used for semen smear, then dried. The slide was observed under the microscope with 400x magnification. Observations were made on the morphology of spermatozoa.

The concentration calculation was done using photometer SDM 6 (Minitube Germany) (Atiq *et al*., 2011). A 35 µl of fresh semen was placed in a cuvette containing 3.5 ml physiological NaCl (1:100), then homogenized using a thermomixer. The homogenate was then set to the photometer, and the concentration value was read on the screen.

The total sperm (TS) value was obtained from the multiplication of the semen volume and the concentration (Oshio *et al*., 2004).

TS=volume ×sperm concentration (1)

The total sperm motility (TSM) value was the total semen value and multiplied by the percentage of motility of liquid semen (Nikbakht and Saharkhiz, 2011).

TSM=TS×sperm motility (2)

The MBF (Motility Before Freezing) examination was the second stage of evaluation after evaluating fresh semen. This examination aimed to determine the motility of the spermatozoa before the freezing process was carried out. The assessment was done by taking one drop of semen and dropped on a glass object, then examined under a microscope with 200x magnification. Observations were made on five fields of view.

*Processing and evaluation on frozen semen*

Ejaculate semen was processed as frozen semen according to The Indonesian National Standard Number SNI:4869-1:2017 in Singosari AI Center. Semen was diluted in Trisaminomethan egg yolk (1.6% tris aminomethane, 0.9% citric acid, 1.4% lactose, 2.5% raffinose, 80% distilled water, 20% egg yolk, penicillin, streptomycin, and glycerol). Semen was preserved at 5ºC, filling in 0.25 ml straw, sealing, labelling prefreezing, and froze at temperature -196ºC.

The evaluation of PTM was done by dipping the straw in water (37-38ºC) for 30 seconds. The straw was then removed and dried using a tissue, then cut and dropped semen on the object-glass. Examination procedures were performed, such as the MBF examination.

The calculation of the TSMP value was the multiplication value between the spermatozoa concentration and the PTM (Post Thawing Motility) value multiplied by 0.25 (volume of each dose) (Rahmawati *et al*., 2015).

TSMP=sperm concentration×PTM×0.25 (3)

*Statistical analysis*

The statistical analysis was done using IBM SPSS 25.0 for windows. Individual differences between Bali cattle were obtained using a one-way analysis of variance with Duncan's multiple range test and $P < 0.05$ was considered statistically significant.

## Results

*Bali cattle fresh semen quality*

The result of macroscopic analysis of semen quality was milky semen color, with a thin consistency (Renon, Uluwatu, and Nabha cattle), medium consistency (Ajay, Wasuki, Gorudo, and Bratan cattle), and thick consistency (Sanur, Tabana, and Jimbara cattle). The semen pH showed no difference in the range of values 6.4-6.5. The study demonstrated that the individual difference influenced the volume, abnormality, concentration, TS, and TSM significantly ($p<0.01$). Whereas there was no effect against the pH and motility ($p>0.05$). The semen volume produced was 5.5-8.7 ml per ejaculate. The lowest semen volume was produced by Sanur cattle, followed by Tabanan, Bratan, Uluwatu, Ajay, and Jimbara cattle, while the highest volume of semen was produced by Nabha and Wasuki cattle.

Based on microscopic analysis, the most abnormal semen was in Uluwatu cattle (6.2%), and the lowest abnormality value was in Wasuki and Sanur cattle (4.0%). The semen concentration was in the range of 876.2±225.3-1464.6±338.7million/ml, with the highest mean values were recorded in Tabanan, Jimbara, and Sanur cattle (1464.6; 1459.6; 1405.9 million/ml, respectively), and the lowest was in Nabha cattle (Table 1). The highest TS was in Gorudo cattle (11726.8±2931.9 million/ml), and the lowest was observed in Uluwatu cattle (6426±2412.3 million/ml) (Table 2). Garudo cattle was showed also the highest TSM (9796.4±2563.3 million/ml), while the lowest TSM was in Uluwatu cattle (5249.6±1975.2 million/ml) (Table 2).

*Bali cattle frozen semen quality*

The evaluation of frozen semen quality included MBF, PTM, and TSMP. Individual variations affected the MBF, PTM, and TSMP variables ($p<0.01$). The highest mean MBF values were recorded in Nabha and Jimbara (59.0±46.8%), where the lowest mean values were in Ajay and Uluwatu cattle

(56.0±0.04%; 56.0±0.03%, respectively) (Table 3). Besides, the highest PTM score was in Renon cattle (47.3±0.03%), and the lowest was observed in Ajay cattle (41.3±0.04%). The highest TSMP was obtained in Tabanan cattle (66.1±53.9 million / ml), while the lowest was in Uluwatu and Nabha (99.5±47.6; 99.3±29.9 million/ml, respectively). The TSMP value was used to calculate the number of motile spermatozoa on each frozen semen straw.

*Bali cattle frozen semen production*

The highest frozen semen production was obtained in Gorudo cattle (461.8±112.1 straw/ejaculate), while the lowest was in Ajay, Uluwatu, Bratan, Nabha, and Renon with each value of 271.6±100.4; 272.4±89.2; 283.3±96.6; 289.6±94.5; 300.6±108.3 straw/ejaculate, respectively (Table 3).

## Discussion

A milky white color indicates the quality of cattle semen. The color of the good quality of Bali cattle semen is milky white (Dwinofanto *et al*., 2018; Suhardi *et al*., 2020), where it is related to the concentration and consistency of semen (Santoso *et al*., 2021). The semen consistency affects spermatozoa concentration, and normally, the semen will coagulate shortly after ejaculation and thaw after 15-20 minutes (Vasan, 2011).

In this study, the pH of semen did not show any differences. This may be attributed to the lack of differences in supplied rations during maintenance. According to Salas-Huetos

Table 1. Bali cattle fresh semen quality

| Cattle | Color | Consistency | pH | Volume (ml) | Abnormality (%) | Motility (%) |
|---|---|---|---|---|---|---|
| Ajay | Milky | Medium | 6.5±0.2$^{a}$ | 6.3±1.7$^{ab}$ | 4.4±0.00$^{bc}$ | 79.7±0.09$^{a}$ |
| Renon | Milky | Thin | 6.6±0.2$^{a}$ | 7.2±1.8$^{bc}$ | 4.2±0.07$^{ab}$ | 85.6±0.08$^{a}$ |
| Wasuki | Milky | Medium | 6.5±0.2$^{a}$ | 8.6±2.1$^{cd}$ | 4.0±0.01$^{bc}$ | 82.5±0.09$^{a}$ |
| Sanur | Milky | Thick | 6.4±0.2$^{a}$ | 5.5±1.1$^{a}$ | 4.0±0.03$^{bc}$ | 83.6±0.06$^{a}$ |
| Uluwatu | Milky | Thin | 6.4±0.2$^{a}$ | 6.2±1.4$^{a}$ | 6.2±1.50$^{d}$ | 82.6±0.06$^{a}$ |
| Tabanan | Milky | Thick | 6.4±0.2$^{a}$ | 5.5±1.1$^{a}$ | 4.5±0.03$^{bc}$ | 81.2±0.07$^{a}$ |
| Gorudo | Milky | Medium | 6.4±0.2$^{a}$ | 8.5±1.4$^{bc}$ | 4.7±0.02$^{cd}$ | 83.6±0.06$^{a}$ |
| Nabha | Milky | Thin | 6.4±0.2$^{a}$ | 8.7±1.5$^{d}$ | 4.1±0.07$^{a}$ | 84.1±0.08$^{a}$ |
| Bratan | Milky | Medium | 6.5±0.2$^{a}$ | 5.6±1.5$^{a}$ | 4.6±0.02$^{cd}$ | 83.7±0.08$^{a}$ |
| Jimbara | Milky | Thick | 6.4±0.2$^{a}$ | 6.4±1.3$^{a}$ | 4.1±0.03$^{c}$ | 84.5±0.06$^{a}$ |

Different superscripts of letters in the same column indicate significantly different (P<0.01).

Table 2. Sperm concentration, total sperm (TS), and total sperm motility (TSM)

| Cattle | Concentration (million/ml) | TS (million/ml) | TSM (million/ml) |
|---|---|---|---|
| Ajay | 1148.5±260.6$^{c}$ | 7275.5±2724.6$^{bc}$ | 2208.0±110$^{a}$ |
| Renon | 1013.8±269.3$^{b}$ | 7451.8±2852.7$^{bc}$ | 2442.0±108$^{b}$ |
| Wasuki | 1060.4±296.1$^{b}$ | 8847.3±2356.7$^{e}$ | 2356.7±112$^{c}$ |
| Sanur | 1405.9±262.6$^{de}$ | 7863.7±2535.2$^{cd}$ | 2125.1±115$^{b}$ |
| Uluwatu | 1029.0±281.9$^{b}$ | 6426.3±2412.3$^{a}$ | 1975.2±112$^{a}$ |
| Tabanan | 1464.6±338.7$^{e}$ | 8037.6±2381.6$^{d}$ | 1985.2±96$^{b}$ |
| Gorudo | 1365.7±238.9$^{d}$ | 11726.8±2931.9$^{f}$ | 2563.2±92$^{e}$ |
| Nabha | 876.2±225.3$^{a}$ | 7636.2±2330.0$^{cd}$ | 2055.5±109$^{b}$ |
| Bratan | 1209.0±197.2$^{c}$ | 6832.6±2216.7$^{ab}$ | 1962.7±95$^{a}$ |
| Jimbara | 1459.6±294.1$^{e}$ | 9339.8±2672.1$^{e}$ | 2339.9±102$^{d}$ |

Different superscripts of letters in the same column indicate significantly different (P<0.01).

Table 3. Motility before freezing (MBF), post thawing motility (PTM), total sperm motility counted from post thawing motility (TSMP)

| Cattle | Value | | | |
|---|---|---|---|---|
| | MBF (%) | PTM (%) | TSMP (billion/ml) | Frozen semen procution (straw/ejaculate) |
| Ajay | 56±0.04a | 41.5±0.04$^{a}$ | 107.7±43.5$^{ab}$ | 271.6±100.4$^{a}$ |
| Renon | 58±0.03$^{bc}$ | 47.3±0.03$^{d}$ | 109.2±45.1$^{ab}$ | 300.6±108.3$^{abc}$ |
| Wasuki | 57±0.03$^{b}$ | 46.0±0.04$^{c}$ | 115.9±45.9$^{b}$ | 351.4±108.8$^{d}$ |
| Sanur | 58±0.03$^{bc}$ | 46.6±0.04$^{cd}$ | 143.9±61.1$^{ef}$ | 330.7±130.1$^{cd}$ |
| Uluwatu | 56±0.03$^{a}$ | 43.2±0.03$^{b}$ | 99.5±47.6$^{a}$ | 272.4±89.2$^{a}$ |
| Tabanan | 58±0.03$^{bc}$ | 47.2±0.04$^{d}$ | 166.1±53.9$^{g}$ | 319.3±103.6$^{bc}$ |
| Gorudo | 58±0.03$^{bc}$ | 46.5±0.03$^{cd}$ | 155.9±36.4$^{e}$ | 461.8±112.1$^{e}$ |
| Nabha | 59±0.03$^{c}$ | 46.6±0.04$^{cd}$ | 99.3±29.9$^{d}$ | 289.6±94.5$^{ab}$ |
| Bratan | 58±0.03$^{b}$ | 45.9±0.03$^{c}$ | 128.0±45.0$^{c}$ | 283.3±96.6$^{a}$ |
| Jimbara | 59±0.02$^{c}$ | 46.8±0.04$^{cd}$ | 157.0±54.9$^{fg}$ | 356.2±104.1$^{d}$ |

Different superscripts of letters in the same column indicate significantly different (P<0.01).

*et al*. (2018), differences in feeding can affect the quality of semen pH. The pH level of the research results was in the range of 6.4-6.5. This was in line with the previous research that the pH of Bali cattle semen was 6.4-6.8 (Fazrien *et al*., 2020). The absence of pH and motility influence was due to the same treatment for all individual Bali cattle, such as the same feed and standard treatment given during the maintenance process. The males used as superior males have been set the same standard of genetic and phenotypic performance (Fazrien *et al*., 2020). Spermatozoa can be affected by the pH of the semen, and the acidic environment has a big influence on the viability of spermatozoa. Changes in semen pH levels can indicate physiological conditions and diseases (Okazaki *et al*., 2010).

The semen volume average of previous studies was 5.5-6.9 ml (Fazrien *et al*., 2020); 4.56-8.50 ml (Indriastuti *et al*., 2020); 4.43-6.40 ml (Nugraha *et al*., 2019); 5.3 ml (Suhardi *et al*., 2020), this indicated that the volume of semen of this study still has the same average as previous studies. The difference in semen volume was due to the influence of different secretions from the seminal plasma fluid. The volume of semen released during ejaculation was part of the seminal plasma secreted by semilunar vesicles (70%), prostate (20%), and <10% from bulbourethral glands and epididymis (Ricardo, 2018; Indriastuti *et al*., 2020).

The sperm abnormalities in the obtained results were still in the same range, 4.15-7.80%; 3.00% (Indriastuti *et al*., 2020). The sperm abnormality determines the number of abnormal spermatozoa, thereby determining eligibility for AI. The analysis of abnormalities is done by assessing the number of abnormal and normal morphologies. The spermatozoa motility in Bali cattle was 80.0%; 70% (Suhardi *et al*., 2020). In the present study, sperm motility between individuals showed insignificant changes (P> 0.05) due to standardization in the male selection and the same treatment during maintenance and semen storage.

The concentration of sperm is the number of spermatozoa/ml of semen produced in one ejaculation. The mean spermatozoa concentration was in the same range as several previous studies, 941.42-1492.67 million / ml (Indriastuti *et al*., 2020); and 749 million/ml (Suhardi *et al*., 2020). The difference in concentration values may be caused by differences in the scrotum size and testes (Guerra *et al*., 2013). The size of the testes affects the number of spermatozoa because of their function as a place for the production of androgen hormones and the process of spermatogenesis (Smith and Walker, 2014).

The TS values depend on ejaculatory volume and concentration (Kondracki *et al*., 2014). This study illustrated that TS and TSM affected the amount of frozen semen production. The higher the TS and TSM values caused an increase in frozen semen production. The daily frozen semen production of Limousin cattle was also influenced by the total spermatozoa and the total sperm motilityatozoa produced (Zamuna *et al*., 2016). The quality of fresh semen has a significant influence on the production of frozen semen produced (Singh *et al*., 2015). The study results obtained a higher TSM value compared to previous studies, such as 3990-4060 million/ml pf TSM (Setiawan *et al*., 2020); and 3136 ± 653.4 million/ml of TSM (Yekti *et al*., 2018).

Motility before freezing is one of the evaluations that need to be done before semen freezing. This value determines the feasibility of semen so that it can be processed into frozen semen because liquid semen is more easily oxidized. The intracellular antioxidant capacity of spermatozoa will decrease following the processes and stages of storage and freezing (Tuncer *et al*., 2010). Isnaini *et al*. (2019) showed that the frozen semen production of Bali cattle was 181 straw/ejaculate.

The ability of each animal to produce spermatozoa with a PTM value above the SNI standard indicates that the spermatozoa originating from that animal can survive during the freezing process. During freezing, the spermatozoa will experience a very drastic decrease due to changes in pressure and temperature to experience cold shock (Hezavehei *et al*., 2018). This study's PTM scores were lower than Indriastuti *et al*. (2020), such as 69.37±0.41% (Table 4). However, it was higher than the score of the Indonesian National Standard for frozen semen (number 4869.1:2017), which stated that the post-thawing motility value must be > 40% to be suitable for use during artificial insemination. The clotting process causes damage to spermatozoa due to osmotic stress, changes in fluidity, and membrane permeability (Yeste, 2016). Thawing of frozen semen can damage the spermatozoa, which caused by the changes in temperature and stress due to differences in osmotic pressure (Hezavehei *et al*., 2018). The TSM counted after post-thawing is a value that indicates the number of spermatozoa that can move after the clotting process. This value is important because it shows the number of motile spermatozoa available for insemination (Branigan *et al*., 2017).

## Conclusion

In this study, it was found that individual differences do not influence the pH and motility of fresh semen, but other characteristic values such as volume, abnormality, concentration, TS, TSM, MBF, PTM, TSMP, and frozen semen production are influenced by individual differences. Individual factors can be used as information for determining the superior bull of Bali cattle.

## Conflict of interest

The author declares that there is no conflict of interest.

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