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Research Article

Quality of Sexed Bali Bull Semen Post-Thawing Using Percoll Density Gradient Centrifugation with Different Gradients and AndroMed® Diluent

Aditiya Wahyudi[1], Aulia Puspita Anugra Yekti[1], Nanda Ayu Rahmawati[1], Putri Utami[1], Habib Asshidiq Syah[1], Fardha Ad Durrun Nafis[2], Sri Wahjuningsih[1], Achadiah Rachmawati[1], Nurul Isnaini[1], Trinil Susilawati[1]*

[1]Department of Animal Science, Universitas Brawijaya, Malang 65145, East Java, Indonesia; [2]Singosari National Artificial Insemination Center, Ngujung, Toyomarto, Singosari, Malang District 65153, East Java, Indonesia.

Abstract | Sexing is a reproduction technique used to modify the natural 50:50 spermatozoa ratio through specific methods to achieve a desired proportion, enabling the production of calves with the preferred sex. This study is the first to systematically investigate the use of three simplified levels of Percoll Density Gradient Centrifugation (PDGC) combined with Andromed® extender for improving both the efficiency of X- and Y-spermatozoa separation and the post-thaw quality of Bali bull semen. This dual-focused approach aims to enhance sex-specific sperm sorting while preserving key functional attributes of cryopreserved sperm, including motility, viability, and membrane integrity. This study evaluated the quality of sexed frozen semen produced using the Percoll Density Gradient Centrifugation (PDGC) method with three different gradient levels: 10, 5, and 3. Semen collection from bull was performed using an artificial vagina. The experimental treatments included T1: 10 gradients, T2: 5 gradients, and T3: 3 gradients, each with 10 replications. Statistical analysis revealed there were no significant differences (P>0.05) observed in individual motility, viability, abnormality, concentration, total motile spermatozoa, motility, progressive motility, and kinematic parameters, including VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, DAP, DSL, and DCL. In conclusion, the different gradient levels used in the sexing process did not significantly affect the quality of frozen sexed semen processed with Andromed® diluent. However, the use of 3 gradients is recommended, as it demonstrated higher values for individual motility of sexed spermatozoa.

Keywords | Andromed®, Bali bull, Percoll density gradien centrifugation, Sexing, Quality semen, CASA

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***Correspondence** | Trinil Susilawati, Department of Animal Science, Universitas Brawijaya, Malang 65145, East Java, Indonesia; **Email:** tsusilawati@ub.ac.id
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INTRODUCTION

Indonesia's rapid population growth has led to increased food demand, including beef, which continues to face challenges due to a supply-demand deficit. In 2023, beef consumption reached 816.79 thousand tons, while local production was only 524.76 thousand tons, resulting in a deficit of 374.10 thousand tons. A 2.05% decline in the beef bull population in 2022 further exacerbated the issue, with 30–40% of national beef demand still reliant on imports. On average, monthly imports of meat and feeder bulls exceed 29,000 tons (BPS, 2023). To address this

domestic beef production shortfall, one of the innovations being developed in the livestock sector is artificial insemination (AI) a reproductive biotechnology that is relatively affordable and easy to implement. AI is considered effective in producing superior offspring and its value can be further enhanced through sperm sexing technology, enabling the production of offspring of the desired sex (Susilawati, 2014).

Bali cattle (Bos javanicus/sondaicus) is a significant indigenous breed in Indonesia, comprising 32.3% of the national herd (Sudrajad *et al*., 2022). Distributed across several provinces, they are valued for high fertility, lean meat, adaptability to tropical climates, and superior carcass yield, making them vital for meat supply and rural livelihoods.

Sperm sexing is a technique used to predetermine the sex of offspring by altering the natural 50:50 ratio of X- and Y-chromosome-bearing spermatozoa to a desired proportion through the separation and identification of sperm cells. Spermatozoa with X chromosomes and Y chromosomes show differences in size and DNA content, X chromosome spermatozoa in bull have 3.8% more than Y chromosome spermatozoa (Sharma and Hadiya, 2023). On the other side, X and Y spermatozoa also have prominent physical and kinetic characteristics in terms of DNA amount, size, density, motility, and charge (Quelhas *et al*., 2021). Currently developing sperm sexing methods include H-Y antigen identification, albumin gradient, electrophoresis, sex-specific protein detection, percoll density gradient centrifugation, and flow cytometry (Manzoor *et al*., 2017). The difference in the amount of Deoxyribonucleic Acid (DNA) content between spermatozoa carrying X and Y chromosomes causes differences in weight and density, which allows the separation of spermatozoa in a percoll gradient. X spermatozoa have a larger size than Y spermatozoa because they contain more chromatin threads in their head. Percoll density gradient centrifugation method is better when compared with other media (Susilawati, 2014).

Percoll Density Gradient Centrifugation (PDGC) sexing has a very complex process including gradient level, centrifugation duration and speed, dilution, and freezing (Yekti *et al*., 2023). The success of the sex separation process used gradient centrifugation depended on various factors, including the quality of fresh semen, the type of diluent used, the duration and speed of centrifugation, the composition of the density gradient, and the number of gradient layers (Muratori *et al*., 2019). PDGC is widely used for sperm sexing in Indonesia due to its simplicity and cost-effectiveness (Susilawati, 2014; Safa *et al*., 2025). In general, the sexing process of the PDGC method is carried out using 10 gradients with density variations of 1.036, 1.038, 1.047, 1.052, 1.055, 1.057, 1.060, 1.065, and 1.070 (Rumende *et al*., 2021). Percoll separation media is used because it is easy to form gradient variations and can separate X and Y spermatozoa (Paes, *et al*., 2019).

The sexing process can reduce the quality of frozen semen, so diluent media is needed to serve as a living medium for spermatozoa and maintain semen quality from sexing to freezing. Diluent media plays an important role as a buffer that provides essential nutrients, protects cell membranes from mechanical damage, and maintains optimal pH and osmolarity. Components such as lipoproteins, antioxidants, and cryoprotectants in the diluent media serve to minimize damage due to lipid peroxidation and free radical formation. AndroMed® diluent was used because it has the advantage of being an animal protein-free commercial diluent based on vegetable lecithin from soybean extract (Arif *et al*., 2022). Its yolk-free composition avoids the risk of microbial contamination and quality variability often encountered in conventional diluents. AndroMed® contains phospholipids, fructose, glycerol, citric acid, buffers, and various minerals that work synergistically to maintain sperm quality. The antioxidants in AndroMed® effectively protect spermatozoa from oxidative damage that increases during the sexing and freezing process (Piaček, *et al*., 2024).

Recent studies in Bali bulls have shown that reducing the number of Percoll gradients can maintain acceptable semen quality while improving operational efficiency (Rahmawati *et al*., 2025). According to Simbolon *et al*. (2024), the quality of sexed semen processed using Percoll Density Gradient Centrifugation (PDGC) with Andromed® diluent varied depending on the gradient level. The 10-gradient PDGC method produced superior results in terms of individual motility, abnormality rate, concentration, and total motile spermatozoa (TMS) compared to the 5-gradient method. However, the proportion of X- and Y-chromosome-bearing spermatozoa was comparable between the 10-gradient and 5-gradient treatments, indicating similar effectiveness in sperm separation. Despite these advantages, the 10-gradient PDGC method is less efficient and more complex, requiring greater time and material consumption for processing.

The use of gradient variation is done to increase the effectiveness and efficiency of the separation process of X and Y chromosomal spermatozoa, considering that the success of the sexing process is highly dependent on the accuracy of separation and the efficiency of X and Y spermatozoa cell removal. The use of 10 gradients provides a finer level of separation and high precision (Susilawati, 2014), thus increasing the purity of the resulting spermatozoa fraction, but requires longer processing time and more intensive labor. Meanwhile, the use of 5 gradients offers a balance between a better level of purity and time efficiency while

maintaining an adequate level of separation for practical applications. The use of 3 gradients was developed to speed up the separation process with a focus on improving time efficiency and reducing the resources required. This study aims to identify the optimal combination of gradient number and processing time to enhance semen quality, with the goal of developing a more affordable and field-applicable commercial-scale semen sexing technology.

MATERIALS AND METHODS

Ethical Approval

This study was exempted from ethical review under the institutional guidelines for animal research, as determined by the Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya, Indonesia. The exemption letter No. 67/EC/KEPK/03/2025.

Materials

Semen sample: This study was conducted at the Singosari National Artificial Insemination Center (SNAIC) in Malang, East Java, Indonesia, from December 2023 to February 2024. Semen collection was performed weekly in the morning from a seven-year-old Bali bull named Sabala (bull code: 116132). Semen was collected using an artificial vagina (Minitube, Germany). Semen quality evaluation included macroscopic and microscopic assessments. Macroscopic evaluation involved the assessment of color, consistency, pH, volume, and odor, while microscopic evaluation examined motility, viability, concentration, abnormality, mass movement, and total motile spermatozoa (TMS).

Andromed® diluent: The Andromed® diluent was prepared by mixing Andromed® (Minitube, Germany) and aquabidest (Onemed, Indonesia) in a 1:4 ratio. The solution was then homogenized and transferred into a container, which was subsequently placed in a water bath (Memmert, Germany) at 38°C (Susilawati *et al*., 2022).

Research Methods

This study was designed as a laboratory experiment utilizing a group randomized design, replicates (n = 10) were assigned as groups. The primary objective was to assess the effects of sperm sexing and cryopreservation using the PDGC method combined with Andromed® diluent, under controlled centrifugation conditions of 541 × g for 5 min (Susilawati, 2014).

The experimental treatments were categorized based on the number of gradient layers used during the sexing process:

T1: 10 gradient layers (20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, and 65%).
T2: 5 gradient layers (20%, 30%, 40%, 50%, and 60%).
T3: 3 gradient layers (20%, 40%, and 60%).

This process resulted in the upper fraction containing a higher proportion of Y-chromosome-bearing spermatozoa, while the lower fraction was enriched with X-chromosome-bearing spermatozoa.

Sexing Procedure

Semen was diluted at a 1:1 ratio, using 0.5 mL of fresh semen and 0.5 mL of Andromed® diluent (Minitube, Germany). The Percoll Density Gradient Centrifugation (PDGC) sexing process followed the protocol described by Susilawati (2014). A Percoll density gradient (Sigma-Aldrich, U.S.A) was prepared using Andromed® diluent for each treatment according to the designated concentration. The gradient layers were arranged in Pyrex test tubes, from the highest to the lowest density, with volumes allocated as follows: T1 (0.5 mL per layer), T2 (1 mL per layer), and T3 (1.5 mL per layer). After forming the gradient, 1 mL of semen (motility ≥70%) was added to each treatment and centrifuged at 541 × g for 5 min using a centrifuge (Thermo Scientific, U.S.A.). After the first centrifugation process, the sample was divided into four layers. The first layer consists of dead spermatozoa and seminal plasma. The second layer was dominated by spermatozoa carrying the Y chromosome. The third layer contains a mixture of X and Y spermatozoa in relatively equal proportions (about 50:50). Meanwhile, the fourth layer contains more spermatozoa carrying the X chromosome compared to Y spermatozoa. Next, the sample underwent a second centrifugation at 286 × g for 5 min. The supernatant was discarded, leaving 1–2 mL of sediment containing spermatozoa, then tested for quality and sex ratio proportion. Following quality testing, the sexed semen was processed for cryopreservation, following the frozen semen production protocol at SNAIC. The sample volume was adjusted, and the temperature was gradually reduced for 18–22 hours at 5°C. A pre-freezing motility test was performed, with semen eligible for freezing if sperm motility exceeded 55%. The sample was then transferred into cool tubes for sealing. The freezing process consisted of two stages: a) Initial cooling with liquid nitrogen vapor for 12 minutes, reaching −140°C. b) Final freezing by immersion in liquid nitrogen at −196°C for 24 hours.

Post Thawing Procedure

Thawing was performed at 37°C for 30 seconds, following the method described by Ramadhani *et al*. (2022).

Evaluation of Sexed Semen Quality

The evaluation of motility of sexed semen was performed using a binocular light microscope (Olympus CX 23, Japan) at 400× magnification, assessing an av-

erage of five fields of view. Viability and abnormality tests were conducted using eosin-nigrosine staining, with observations made under a binocular light microscope at 400× magnification (Susilawati *et al.*, 2022). The viability of spermatozoa was calculated using the following formula (Susilawati *et al.*, 2022):

$$Viability\ of\ Spermatozoa\ (\%) = \frac{Total\ live\ spermatozoa}{Total\ observed\ spermatozoa} \times 100\%$$

Spermatozoa concentration was determined using a Neubauer hemocytometer following the method described by Mahendra *et al.* (2018) and Yekti *et al.* (2024). The concentration of sperm was calculated using the following formula:

$$The\ number\ of\ sperm/ml = N \times 5 \times FP \times 10^4$$

Notes:
N: Average number of spermatozoa in chamber A and B.
5: Correction factor for only counting 5 boxes out of 25 boxes.
FP: Dilution factor (1:100).
10^4: depth of neubauer chamber 0.0001 ml/neubauer chamber.

motility by the sperm concentration (Susilawati, 2013).

Computer-Assisted Sperm Analysis (CASA)

The Computer-Assisted Sperm Analysis (CASA) system (IVOS II, USA) functions by detecting sperm head movement patterns and analyzing the spermatozoa trajectory. The quality assessment using CASA was conducted according to the Singosari National Artificial Insemination Center (SNAIC) laboratory guidelines, following the procedure: A 4-µL of thawed semen was placed onto a glass slide, covered with a cover slip, and subsequently introduced into the CASA system (IVOS II, USA). The sample was examined across five distinct fields of view, and an auto-capture feature was utilized to record and store the analysis results.

The identification of X- and Y-chromosome-bearing spermatozoa was conducted through morphometric analysis of the sperm head, utilizing the eosin-nigrosin staining method and microscopic observation with an Olympus CX-33 microscope (Japan). The staining procedure involved preparing slides with eosin-nigrosin solution, enhancing contrast to improve the visualization of sperm head structures. The stained samples were then examined using an Olympus CX-33 microscope, connected to an LC Micro device, enabling high-resolution imaging for precise measurements. At this stage, 1,000 spermatozoa from fresh semen were analyzed to establish the natural 50:50 proportion of X- and Y-bearing spermatozoa. For each experimental treatment, 100 spermatozoa per sample were examined. The identification of X- and Y-chromosome-bearing spermatozoa was based on head size, calculated as the product of head length and width. Spermatozoa with measurements exceeding the average head size were classified as X-bearing spermatozoa (Susilawati, 2014).

Data Analysis

Statistical analyses were conducted using R Studio (Version 4.4.1). Data were analyzed through Analysis of Variance (ANOVA) to determine significant differences among treatments. When significant differences were detected ($P < 0.05$), comparisons between means were further evaluated using the Duncan's Multiple Range Test (DMRT) to assess pairwise differences and rank treatment effects. This analytical approach ensured a robust evaluation of the impact of different treatments on spermatozoa quality.

RESULTS AND DISCUSSION

Evaluation of Fresh Semen Quality in Bali Bulls

The evaluation of fresh semen quality showed an average semen volume of 8.44 ± 2.31 mL and a pH of 6.5 ± 0.13. Previous studies have reported that fresh semen from Bali bulls typically has an average volume of 6.32 ± 0.07 mL (Indriastuti *et al.*, 2020). Susandani *et al.* (2021) reported that the production of fresh semen in cattle is influenced by multiple factors, including age that has a significant effect on ejaculate volume, genetic background, ambient temperature, seasonal changes, frequency of ejaculation, nutritional status, and body weight. The mass motility of the semen was observed to be at an average rating of ++, while the individual motility averaged 82.71%. According to Susilawati *et al.* (2022), fresh semen with individual motility of ≥70% is considered viable for further processing. The average viability of the spermatozoa was 89.00%, while the abnormality rate was 5.41%, both of which fell within the normal range. The minimum viability threshold for semen to proceed to the next processing stage is ≥70%, while the maximum allowable abnormality is <20% (Susilawati, 2013). The semen concentration of Bali bulls in this study was recorded at 944.78 million/mL. Several factors influence semen quality, including individual physiological conditions, reproductive organ health, management systems, nutritional intake, breed characteristics, and age (Susandani *et al.*, 2021). These factors collectively impact sperm production, motility, and overall semen viability, determining its suitability for use in reproductive technologies such as AI and sperm sexing techniques.

Quality of Sexed Frozen Semen

The success of sperm sexing is evaluated based on the quality of the sexed semen after freezing (post-thawing), ensuring that it maintains high viability while effectively separating

Table 1: Quality of sexed frozen semen of bali bulls.

Parameter	Treatments							
	Top				Bottom			
	T1	T2	T3	P Value	T1	T2	T3	P Value
Motility (%)	17.20±8.74	22.19±5.14	23.33±10.22	0.42	55.82±7.42	52.89±12.47	50.59±16.41	0.610
Individual Motility (%)	18.46±8.56	20.50±11.84	24.28 ± 7.01	0.281	45.96±4.13	42.04±5.66	44.08±7.90	0.130
Viability (%)	22.33±12.58	21.78±10.81	22.01±11.34	0.130	45.95±13.52	37.17±14.05	38.95±13.23	0.257
Abnormality (%)	8.30±2.65	7.43±3.00	6.27±2.55	0.192	3.67±1.18	3.64±1.22	4.19±2.99	0.682
Concentration (10^6/ml)	13.34±5.35	11.31±3.95	11.12±4.25	0.083	21.90±6.64	19.78±5.64	20.69±7.02	0.483
Total Motile Spermatozoa (10^6/ml)	2.49±2.11	2.35±1.56	2.65±1.16	0.590	8.82±3.06	7.95±2.94	5.64±1.85	0.080

Description: T1: Sexed PDGC 10 gradient; **T2:** Sexed PDGC 5 gradient; **T3:** Sexed PDGC 3 gradient; **Top:** the layer containing many Y spermatozoa; **Bottom:** the layer containing many X spermatozoa.

Table 2: Quality of Sexed Frozen Semen of Bali Bulls Assessed by Computer-Assisted Sperm Analysis (CASA).

Variable	Treatment							
	Top				Bottom			
	T1	T2	T3	P Value	T1	T2	T3	P Value
Progressive Motility	14.06 ± 7.82	17.59 ±15.02	18.92 ±9.42	0.57	48.67 ± 6.84	44.48 ±10.57	43.75 ±13.59	0.51
DCL (µm)	65.39 ± 11.42	58.20 ± 18.23	65.47±11.99	0.21	64.31 ± 9.35	67.44 ± 5.75	64.51 ± 5.65	0.31
DSL (µm)	30.68 ± 4.58	28.37 ± 8.51	31.20 ± 5.21	0.41	31.68 ± 3.50	31.42 ± 3.78	31.41 ± 2.65	0.98
DAP (µm)	35.51 ± 5.31	32.88 ± 9.59	35.83 ± 5.80	0.43	36.95 ± 4.37	37.35 ± 3.20	36.79 ± 2.76	0.93
VCL (µm/sec)	152.60±23.67	148.47±44.05	152.46±23.49	0.91	180.34±24.75	183.97±17.99	184.02±10.70	0.79
VSL (µm/sec)	71.55 ± 9.18	71.29 ± 21.36	72.17 ± 11.05	0.98	89.63 ± 10.56	86.82 ± 7.40	91.25 ± 5.55	0.42
VAP (µm/sec)	83.50 ± 10.73	83.75 ± 23.77	83.00 ± 12.17	0.99	104.52±12.85	103.27 ± 8.29	106.11 ± 5.25	0.74
LIN (%)	49.84 ± 5.01	52.49 ± 8.01	50.46 ± 2.75	0.36	50.41 ± 5.21	48.95 ± 5.29	51.10 ± 3.53	0.50
STR (%)	85.78 ± 4.99	85.63 ± 6.13	86.80 ± 2.89	0.74	85.61 ± 1.93	83.78 ± 3.81	85.07 ± 2.35	0.22
WOB (%)	57.09 ± 3.16	59.96 ± 5.89	56.97 ± 2.27	0.07	59.29 ± 3.37	57.55 ± 3.97	59.24 ± 2.77	0.24
BCF (beats/sec)	25.35 ± 1.58	26.85 ± 6.33	25.14 ± 1.95	0.59	23.87 ± 1.52	24.25 ± 1.50	24.46 ± 1.89	0.93
ALH (µm)	7.01 ± 1.12	6.80 ± 1.72	7.00 ± 0.85	0.88	8.11 ± 0.97	8.41 ± 0.97	8.21 ± 0.71	0.56

Description: T1: Sexed PDGC 10 gradient; **T2:** Sexed PDGC 5 gradient; **T3:** Sexed PDGC 3 gradient; **Top:** the layer containing many Y spermatozoa; Bottom: the layer containing many X spermatozoa. DCL: Distance Curve Line; **DSL:** Distance Straight Line; **DAP:** Distance Average Path; **VCL:** Velocity Curvilinear; **VSL:** Velocity Straight-Line; **VAP:** Velocity Average Path; **LIN:** Linearity; **STR:** Straightness; **WOB:** Wobble; **BCF:** Beat Cross Frequency; **ALH:** Amplitude of Lateral Head Displacement.

X- and Y-chromosome-bearing spermatozoa. The quality parameters of the sexed frozen semen are summarized in Table 1. The ability of spermatozoa to move plays a critical role in successful fertilization. According to Kogan *et al.* (2021), spermatozoa move forward in the female reproductive tract in a straight trajectory, a characteristic known as progressive motility. Motility and progressive motility were evaluated using CASA, as showed in Tables 1 and 2. The highest motility and individual motility were recorded in the T1, with motility values of 55.82 ± 7.42% and individual motility of 45.96 ± 4.13%. Statistical analysis using ANOVA indicated that different gradient levels (T1, T2, and T3) did not significantly affect ($P > 0.05$) motility, progressive motility, or individual motility in both the upper and lower layers.

A decrease in motility occurs in spermatozoa post-freezing, primarily due to temperature fluctuations that impact sperm quality. Sperm motility is influenced by the metabolic capacity of spermatozoa, which is dependent on environmental conditions such as temperature, lifespan, and the composition of the diluent medium (Iranpour *et al.*, 2019). The centrifugation process during sexing separates spermatozoa from seminal plasma, which can cause damage to the outer membrane or acrosome, leading to the loss of buffering components and nutrients essential for sperm metabolism (Yekti *et al.*, 2023). The freezing process induces structural and functional damage in spermatozoa due to cold stress, leading to cell death, excessive water loss, ice crystal formation, and the accumulation of electrolytes and other solutes (Bebas *et al.*, 2023).

The low quality of post-thawing frozen semen remains a limiting factor in the success of AI. According to Indonesian National Standards, frozen semen with a post-thawing individual motility of ≥40% is considered suitable for AI programs. Significant differences were observed between upper and lower layers in the sexed semen treatments, where motility and individual motility were higher in the lower layer, which contained a greater proportion of X-bearing spermatozoa, compared to the upper layer, which had a higher proportion of Y-bearing spermatozoa.

Spermatozoa viability is a key factor in determining the success of fertilization. The viability percentage of sexed frozen semen processed with different gradient levels using Andromed® diluent showed no significant differences ($P > 0.05$) between the upper and lower layers across treatments. However, T1 exhibited the highest viability percentage in both upper and lower layers. The viability percentage is closely related to individual motility, though the results of this study showed that viability percentages were lower than individual motility percentages. The decrease in viability in PDGC-processed frozen semen is influenced by processing time, environmental temperature, medium composition, and the freezing process. According to Nur *et al*. (2022), the decrease in viability in sexed spermatozoa is due to energy depletion during sexing, alongside additional environmental and medium composition factors. Andromed® diluent contains only one energy source, fructose, which may limit spermatozoa survival post-processing.

The reduction in sperm viability is attributed to multiple factors, including the centrifugation-based separation process, which may cause mechanical damage to spermatozoa, the smearing preparation procedure, which can lead to cellular stress. Environmental conditions, particularly the freezing process, where drastic temperature fluctuations (cold shock) reaching 0°C contribute to sperm damage.

This study showed significant differences in viability between the lower and upper layers in the sexing treatments. The lower layer, which contained a higher proportion of X-bearing spermatozoa, exhibited higher viability compared to the upper layer, which had a greater proportion of Y-bearing spermatozoa. This difference is likely due to the higher susceptibility of Y-bearing spermatozoa to cell damage and death compared to X-bearing spermatozoa.

Spermatozoa abnormalities arise from various factors, including environmental conditions, temperature fluctuations, humidity, improper handling post-collection, and genetic influences, all of which contribute to reduced sperm fertility. Statistical analysis indicated that the gradient levels (T1, T2, and T3) did not significantly affect ($P > 0.05$) the percentage of sperm abnormalities in either the upper or lower layers. The percentage of abnormalities in sexed frozen semen was classified as acceptable, as it complied with Indonesian National Standards for frozen semen, which sets a maximum sperm abnormality of 20%.

According to Susilawati (2013), primary abnormalities occur during spermatogenesis, while secondary abnormalities develop post-spermatogenesis and during ejaculation or semen processing. Solihati *et al*. (2019) reported that cold shock during the cooling and freezing process, as well as imbalanced osmotic pressure, contribute to spermatozoa abnormalities. These factors emphasize the importance of optimized cryopreservation protocols to maintain sperm integrity and minimize abnormalities in sexed frozen semen.

A high concentration of spermatozoa is essential for ensuring successful fertilization. According to Susilawati *et al*. (2022), sperm concentration reflects the number of viable spermatozoa contained in each straw, which directly influences the fertilization potential. The Indonesian National Standard (SNI 4869-1:2017) specifies a minimum concentration of 25 million spermatozoa per straw. A higher sperm concentration increases the likelihood of successful fertilization, as not all spermatozoa retain their motility post-thawing (Nomura *et al*., 2018). The results of analysis of variance (ANOVA) indicated that different gradient levels (T1, T2, and T3) did not significantly affect ($P > 0.05$) sperm concentration in either the upper or lower layers.

The results showed that the lower layer exhibited a higher sperm concentration compared to the upper layer. This is attributed to the greater density of X-bearing spermatozoa, which allows them to penetrate the gradient more effectively than Y-bearing spermatozoa. The difference in density between X and Y spermatozoa is a key factor influencing sperm separation efficiency PDGC sexing.

Total motile spermatozoa (TMS) represents the number of viable and motile spermatozoa in a semen sample, determined by sperm concentration and progressive motility (Susilawati *et al*., 2022). A higher motility rate and sperm concentration result in a higher TMS value, which is crucial for successful fertilization. The analysis of variance (ANOVA) revealed that different gradient levels (T1, T2, and T3) did not significantly affect ($P > 0.05$) the TMS.

According to the Indonesian National Standard (SNI 4869-1:2021), frozen bovine semen packaged in mini straws must contain at least 10 million motile spermatozoa with a progressive motility of at least 40%. The total progressive motile spermatozoa in a straw of frozen semen directly influences pregnancy rates, as higher numbers of motile spermatozoa enhance the probability of successful fertilization. This study demonstrated a significant differ-

ence between the upper and lower layers in the sexed semen treatments. The lower layer, which contained a greater proportion of X-bearing spermatozoa, exhibited a higher TMS compared to the upper layer, which had a higher proportion of Y-bearing spermatozoa. This difference is attributed to the higher mortality rate of Y-bearing spermatozoa compared to X-bearing spermatozoa, leading to a greater loss of motile spermatozoa in the upper layer.

Computer-Assisted Sperm Analysis (CASA)

The use of CASA as a tool for assessing sperm motility and kinematic parameters has become increasingly widespread. CASA offers several advantages in sperm motility analysis, including speed, objectivity, repeatability, accuracy, and efficiency (Gliozzi *et al*., 2017). CASA is particularly beneficial in motility testing as it eliminates the subjectivity of manual sperm motility assessment, ensuring more reliable and standardized results. The application of CASA in motility evaluation and its impact on sexed frozen semen quality is presented in Table 2.

Velocity Curvelinier (VCL) is the speed of spermatozoa movement following a curved path expressed in µm/s. Velocity Straight Line (VSL), is the average speed of the spermatozoa head along a straight line from the starting point to the end point expressed in µm/s. velocity average path (VAP), to measure the average speed of spermatozoa along the calculated path dividing the length of the groove by the travel time expressed in µm/s. The analysis of variance (ANOVA) indicated that different gradient levels (T1, T2, and T3) did not significantly affect (P > 0.05) VCL, VSL, and VAP in either the upper or lower layers.

The decrease in VCL, VSL, and VAP values is thought to be caused by various factors, such as the sexing process, freezing, and limited energy sources in the diluent. Perumal *et al*. (2014) stated that the speed of movement and motility of spermatozoa is influenced by the availability of energy sources, diluent viscosity, osmolarity, and pH. The decrease in speed parameters may be related to the use of Andromed diluent which only contains one type of energy source, namely fructose, so it is less than optimal in supporting spermatozoa survival. The availability of this energy source is closely related to the production of adenosine triphosphate (ATP) as a support for spermatozoa cellular activity.

Distance Average Path (DAP) refers to the average distance traveled by spermatozoa per second along their trajectory. Distance Curved Line (DCL) represents the distance covered by spermatozoa per second along a curved path, whereas Distance Straight Line (DSL) measures the distance traveled per second in a straight-line motion (Valverde *et al*., 2020). The analysis of variance (ANOVA) indicated that different gradient levels (T1, T2, and T3) did not significantly affect (P > 0.05) DCL, DSL, and DAP in either the upper or lower layers.

Results showed that DCL values were higher than DAP values. According to Maulana and Said (2019), a high DCL value correlates with a high VCL (Curvilinear Velocity), meaning that an increase in VCL is accompanied by an increase in DCL. Furthermore, a higher DCL value corresponds with a reduction in DAP, indicating a more curvilinear motion rather than a linear trajectory. A lower DCL value suggests that spermatozoa are moving more efficiently, as sperm velocity is positively correlated with travel distance (Afriani *et al*., 2023). Additionally, DAP is correlated with VAP (Average Path Velocity), and an increase in DCL leads to a reduction in DAP (Maulana and Said, 2019). These kinematic parameters provide insights into sperm movement efficiency and fertilization potential.

Linearity (LIN), Straightness (STR), and Wobble (WOB) are key parameters that describe spermatozoa swimming patterns. Linearity (LIN) represents the degree of straightness in a spermatozoon's curvilinear trajectory, while Straightness (STR) measures the straightness of its average path. Wobble (WOB) quantifies the oscillatory movement of sperm along its trajectory. Statistical analysis showed that different gradient levels (T1, T2, and T3) did not significantly affect (P > 0.05) LIN, STR, or WOB in either the upper or lower layers.

LIN and STR values are often used as indicators of progressive motility and sperm swimming efficiency, while fertilization ability correlates with VSL (Straight-Line Velocity) and VCL (Curvilinear Velocity), which contribute to sperm functionality (Raafi *et al*., 2021). WOB, which measures the oscillatory motion of spermatozoa, helps distinguish hyperactivated sperm from progressively motile sperm, as hyperactivated sperm exhibit erratic movements with high amplitude but lack progressive, linear motion (Susilawati *et al*., 2018).

Amplitude of Lateral Head Displacement (ALH) is a key indicator of hyperactivated sperm movement, while Beat Cross Frequency (BCF) measures the frequency of sperm flagellar oscillations per second. The results of analysis of variance (ANOVA) showed that different gradient levels (T1, T2, and T3) did not significantly affect (P > 0.05) ALH or BCF in either the upper or lower layers.

A higher ALH value is associated with reduced sperm quality, as excessive lateral head movement can hinder forward progression (Amal *et al*., 2019). ALH and BCF are key parameters analyzed using CASA, as they provide insights into sperm motility characteristics, velocity, trajectory oscillation, and flagellar beat frequency (Bravo *et al*., 2011). ALH and BCF values are strongly influenced by

VAP (Average Path Velocity), though different CASA systems may calculate VAP differently, leading to variations in ALH and BCF measurements. The ideal ALH range for optimal fertility is 2.5 - 6.5 µm, while BCF values above 20 Hz indicate good sperm motility and fertilization potential (Belala *et al*., 2019). The BCF parameter is particularly useful for identifying variations in flagellar movement patterns, where higher BCF values suggest greater flagellar stability and efficient sperm motion (Kathiravan *et al*., 2011). Conversely, low BCF values combined with high ALH values result in impaired sperm movement and reduced fertilization potential (Ratnawati *et al*., 2020).

Among the various parameters measured by CASA, progressive motility and VCL are recognized as critical indicators of fertilizing potential. Progressive motility reflects the ability of spermatozoa to move in a linear and purposeful direction, which is closely associated with intact mitochondrial function and efficient energy metabolism. Utami *et al*. (2025) highlighted that progressive motility is a key determinant of fertilization success.

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrates that the 3-gradient Percoll Density Gradient Centrifugation (PDGC) method is a reliable, efficient, and practical technique for sperm sexing in Bali bulls. Although the 10-gradient method showed slightly higher separation precision, the 3-gradient approach effectively maintained key sperm quality parameters particularly progressive motility while significantly reducing processing time and resource usage. The sorting efficiency achieved with the 3-gradient method exceeded 70%, surpassing the threshold for successful separation of X- and Y-chromosome-bearing spermatozoa. Due to its technical simplicity, time efficiency, and economic viability, the 3-gradient PDGC protocol is well-suited for commercial artificial insemination (AI) programs, especially in field conditions typical of tropical livestock systems.

Future research should focus on evaluating the fertility outcomes of sexed semen produced using the 3-gradient PDGC method, particularly with regard to conception rates, calving success, and offspring sex ratios under practical field conditions. Molecular validation of sorting accuracy using PCR or flow cytometry is also recommended to confirm cytological observations. Additionally, further refinement of centrifugation parameters and extender formulations may enhance both efficiency and consistency. Increasing the number of experimental replicates and the sample size in future studies will also improve statistical power and the generalizability of findings. Expanding the application of this method to other indigenous cattle breeds could further promote the adoption of sexed semen technologies in sustainable livestock breeding programs.

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NOVELTY STATEMENT

The novelty of this study lies in the establishment of sexed semen using three- and five-gradient PDGC combined with AndroMed® diluent an approach that has not been extensively explored in semen processing. The findings demonstrate that this methodology preserves high-quality sexed frozen semen, as evidenced by kinematic parameters measured through Computer-Assisted Sperm Analysis (CASA), with a sorting yield exceeding 70%. This represents a significant advancement in reproductive and livestock biotechnology, offering a more efficient and effective technique for improving both the quality and quantity of sperm sexing outcomes.

AUTHOR'S CONTRIBUTIONS

Aditiya Wahyudi: conceptualization, drafting the original manuscript, collecting data, analyze statistics, drafting and revisions.
Aulia Puspita Anugra Yekti: conceptualization, supervision.
Nanda Ayu Rahmawati: collecting data.
Putri Utami and Habib Asshidiq Syah: conceptualization, drafting and revisions.
Fardha Ad Durrun Nafis: collecting data.
Sri Wahjuningsih, Achadiah Rachmawati, Nurul Isnaini and Trinil Susilawati: conceptualization, supervision, and review the final manuscript.

Each author has reviewed and approved the manuscript's published form.

Conflict of Interest

The author declares that there is no conflict of interest with stakeholders related to the material written in this manuscript.

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