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Double-Dose Artificial Insemination and Sperm Kinematics of Percoll Density Gradient-Sexed Semen in Holstein-Friesian Cattle

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ABSTRACT

This study aimed to evaluate the effect of sperm sexing using the Percoll density gradient centrifugation (PDGC) method on the kinematic, reproductive performance, and sex ratio of calves in Holstein-Friesian (HF) cattle. The study used three treatments: conventional semen (G0), PDGC sexed semen at 20%–60% (G1), and PDGC sexed semen at 20%–65% (G2). Kinematic variables were analyzed using a computer-assisted sperm analysis (CASA) system, while reproductive performance was evaluated based on non-return rate (NRR), conception rate (CR), calving rate (CvR), and sex ratio. Insemination in the sexed semen group was performed using a double-dose method to compensate for the potential decrease in viable sperm. The results showed that the PDGC method significantly affected several kinematic variables, particularly motility, distance average path (DAP), distance straight line (DSL), distance curved line (DCL), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), Wobble (WOB), amplitude of lateral head movement (ALH), and beat cross frequency (BCF) ($p<0.05$), reflecting changes in sperm movement dynamics due to the density-based separation process. However, there were no significant differences in reproductive performance variables between treatments, indicating that PDGC-based sexing does not significantly reduce in vivo fertilization capacity. The use of sexed semen increases the proportion of female offspring compared to conventional semen. Sexing sperm using the PDGC method is a relatively safe and applicable approach to increase the chances of female offspring without sacrificing reproductive efficiency, especially when combined with a double-dose insemination strategy.

Keywords: artificial insemination; Holstein-Friesian; kinematic characteristic; sexed semen; sperm quality

INTRODUCTION

Sex control of calves is an important strategy in dairy cattle production systems. In Holstein-Friesian (HF) cattle, increasing the proportion of female offspring has direct implications for milk production efficiency and the sustainability of the breeding population (Morek-kopeć *et al.*, 2021). Reproductive technologies such as artificial insemination (AI) have significantly enhanced animal production efficiency (Ayantoye *et al.*, 2025). The use of sexed semen via AI programs is becoming an increasingly relevant approach to meet the needs of the dairy cattle industry (Quelhas *et al.*, 2023).

However, the use of sexed semen in the field still faces a major challenge: a generally lower fertility rate compared to conventional semen (Pozdyshev *et al.*, 2023; Sharma *et al.*, 2024). The sexing process can cause mechanical and physical stress that affects sperm integrity and function, reducing viability by approximately 20% (Walsh *et al.*, 2021; Rasad *et al.*, 2020). These effects are often observed in changes in motility characteristics, especially kinematic parameters.

These parameters perform a key role in sperm transport, interaction with the oocyte, and the success of fertilization. Previous studies have reported that kinematic parameters such as curvilinear velocity (VCL), straight-line velocity (VSL), and beat cross frequency (BCF) are strongly associated with the probability of pregnancy in dairy cattle (Araya-Zúñiga *et al.,* 2024). Large-scale studies show that the pregnancy rate with sexed semen still fluctuates, ranging from 69%–87% in heifers and 72%–85% in lactating cows, compared to conception rate (CR) achieved through conventional semen (Butler *et al.,* 2014; Maicas *et al.,* 2019).

Percoll density gradient centrifugation (PDGC) offers a simple and economical alternative to flow cytometry for sperm sex determination (Safa *et al.,* 2025). This method separates sperm carrying the X or Y chromosome based on density (Yekti *et al.,* 2023). Evaluating the kinematic characteristics after Percoll separation is important to assessing semen suitability for AI (Tanga *et al.,* 2021). Changes in sperm quality observed *in vitro* may not predict *in vivo* fertilization success (Santolaria *et al.,* 2023). One approach to increase pregnancy chances, especially when sperm quality decreases due to separation processes, is the double-dose insemination technique, involving two inseminations during one estrus (Utami *et al.,* 2022). While promising, the effectiveness of this method, particularly with PDGC-sexed semen, remains uncertain in field conditions.

Although sperm sexing techniques, particularly those using the PDGC method, have been extensively developed, information regarding the effects of combining this method with double-dose AI on sperm kinematic quality and reproductive performance remains limited. Previous studies have generally focused only on evaluating semen quality *in vitro* or *in vivo* fertility outcomes separately, and the integration of these two approaches has not been widely reported. Furthermore, studies on the effectiveness of PDGC methods in maintaining sperm function while improving reproductive success under field conditions remain incomplete. Therefore, research is needed to comprehensively examine the relationship between the quality of sexed sperm and the resulting reproductive performance. This study examines the kinematic characteristics of sexed semen produced by PDGC and evaluates its fertility performance following double-dose AI during the same estrus cycle in HF cows, with the aim of optimizing the use of sexed semen in dairy farming systems.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Research Ethics Committee (Animal Care and Use Committee) of Universitas Brawijaya, Malang, Indonesia, with an approval number: 180-KEP-UB-2024. Ethical approval was obtained for all procedures involving animals, including AI, pregnancy diagnosis, and monitoring. The study followed institutional and international animal welfare standards. Trained veterinarians performed all procedures using standard, non-invasive methods to minimize animal stress, pain, and discomfort. Rectal palpation was used to diagnose pregnancy. Animals were managed under normal farm conditions throughout, with free access to feed and water. No animal welfare issues were observed.

Study Period and Location

This field study was conducted between January and December 2024 on smallholder farms in Ngantang subdistrict, Malang, East Java, Indonesia (7°52′52.162″S 112°22′16.241″E). The study area is characterized by a tropical climate, with an average temperature of 25-28 °C and a temperature humidity index of 75-80 during the study period. The laboratory analysis of sexed semen was conducted at Singosari National Artificial Insemination Center (SNAIC) in Malang, East Java, Indonesia.

Experimental Animals and Study Design

A total of 60 multiparous HF cows were included in this study. The cows were aged 3 to 7 years and weighed 400 to 500 kg. Each had a body condition score (BCS) of 3-5 on a scale of 1 to 5 (Swartz *et al.,* 2025). All cows were in parity 2-5 and met the established inclusion criteria. Cows with a history of reproductive disorders, abnormal health conditions, or low body condition scores (BCS < 3) were excluded from the study.

This study was conducted in two stages: (1) evaluation of the kinematic characteristics of frozen-thawing semen, and (2) application of AI to assess reproductive performance. In the first stage, three treatments based on semen sexing methods were used: G0 = conventional semen (control), G1 = sexed semen using PDGC with a gradient of 20%-60%, and G2 = sexed semen using PDGC with a gradient of 20%-65%. Each treatment had 10 replicates. The second stage was conducted on HF cows (20 per group). Treatments were defined by semen type: T0 = double-dose AI used conventional semen (control); T1 = double-dose AI used 20%-60% PDGC sexed semen; T2 = double-dose AI used 20%-65% PDGC sexed semen.

Double-dose AI refers to two AIs performed during the same estrus period. The first AI is performed immediately after the cow is detected to be in clear estrus, while the second AI is performed 8 hours after the first AI. Each AI uses one standard dose of frozen semen. The two inseminations were performed at specific intervals, according to the timing of estrus detection and standard field AI procedures.

Semen Collection and Preparation

Semen was collected from healthy, proven fertile HF bulls using an artificial vagina (IMV Technologies, France). Semen was collected twice a week by experienced technicians. Bulls were aged 2–6 years, weighed 510–870 kg, and met reproductive eligibility

criteria based on the breeding soundness examination (BSE). Bulls were housed individually in well-maintained pens with good sanitation and ventilation in SNAIC. Semen was obtained from four bulls: Diplomasi (BET Cipelang, body weight 870 kg, age 6 years), Raja (Greenfield, body weight 600 kg, age 3 years), Rodgers (BPTU-HPT Baturaden, body weight 510 kg, age 2 years), and GW Amish (Australia, body weight 698 kg, age 2 years). A total of 40 semen samples were analyzed in this study, including 10 fresh semen samples, 10 conventional frozen semen samples, 10 sexed semen samples separated using the PDGC method with a gradient of 20%–60%, and 10 sexed semen samples with a gradient of 20%–65%. Since the ejaculate was evenly distributed across all treatments, the effect of the bull was minimized in the experimental design stage. It was therefore not included as a random factor in the analysis model.

After collection, semen was immediately placed in a water bath at 37°C. Evaluation included ejaculate volume, sperm concentration, mass motility, individual motility, percentage of abnormalities, and sperm kinematic profile. Only ejaculates with mass motility of at least +2 (++), individual motility of at least 70%, and sperm abnormalities below 20% were used for further processing. The semen was either processed as conventional semen or underwent sexing using the PDGC method as per treatment. The semen was cryopreserved following standard cryopreservation protocols with several modifications, including the cooling, packaging, and freezing stages. The cooling process involved gradually lowering the temperature to 5 °C, followed by the addition of a diluent and cooling for 22 hours. Next, a diluent + 13% glycerol was added during the glycerolization stage. Prior to freezing, semen quality was evaluated (before freezing), with an individual motility of ≥55% as the standard for suitability for freezing. Semen meeting the criteria was then packaged into 0.25 mL straws (packaging) and sealed in a cool tub, followed by an equilibration stage. The freezing process begins with a pre-freezing stage at −140 °C for 9 minutes, followed by freezing in liquid nitrogen at −196 °C and storage. Frozen semen was thawed at 37 °C for 30 seconds before post-thaw evaluation or AI. Post-thaw kinematic profile was assessed using a computerized assisted sperm analysis (CASA; Hamilton Thorne IVOS II, USA). The sperm kinematic parameters analyzed include: Motile, Progressive, distance average path (DAP), distance straight line (DSL), distance curved line (DCL), VCL, VSL, VAP, linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head movement (ALH), and beat cross frequency (BCF) (Yusuf *et al.,* 2026).

Semen Sexing Process

Sperm sexing was performed using PDGC with gradients of 20%-60% and 20%-65%. The selection of these two gradient ranges is based on the difference in density between X- and Y-chromosome-carrying sperm, which is influenced by DNA content. X sperm have a slightly higher DNA content and thus tend to have a slightly higher DNA content and therefore a higher density than Y sperm. Gradients within these ranges have been widely used to separate sperm subpopulations based on their physical and functional characteristics (Safa *et al.,* 2025). Furthermore, the use of two gradient ranges (20%-60% and 20%-65%) in this study was intended to assess the extent to which density variations affect separation outcomes and the quality of the resulting sperm. Percoll is a suspension of colloidal silica particles coated with polyvinylpyrrolidone (PVP) (Cai & Yang, 2023). First, Percoll medium (Sigma-Aldrich, St. Louis, MO, USA) was layered in a 10-level gradient of 20%-60% and 20%-65% in a centrifuge tube, with 0.5 mL of each concentration, starting with the highest at the bottom and the lowest at the top, resulting in a total volume of 5 mL. Next, 2 mL of semen is placed on top of the gradient and centrifuged (Hettich, Germany) at 291×*g* (relative centrifugal force) for 7 min. After centrifugation, the upper fraction (containing seminal plasma) is discarded. This process separates the sample into two fractions: 1.5 mL of the upper layer (containing Y sperm) and 1.5 mL of the lower layer (containing X sperm). Each fraction was then mixed with 3 mL of egg yolk Tris-aminomethane (Tris) diluent, followed by a second centrifugation at 148.1 ×*g* for 5 min to wash the cells. Afterward, the supernatant is discarded, leaving the sperm pellet. Finally, the sperm pellet is resuspended and processed for cryopreservation (Safa *et al.,* 2025; Utami *et al.,* 2025).

Artificial Insemination and Pregnancy Diagnosis Procedures

All inseminations were performed by a single licensed veterinarian using each 0.25 mL semen straw to ensure procedural consistency. AI was performed during the estrus phase. The estrus phase was determined by observing visual signs such as clear mucus, vulval swelling and reddening, and behavioral changes such as increased restlessness, and these signs were confirmed by rectal palpation and a manual examination of the cervix and uterus (Gaude *et al.,* 2021). AI is performed using a double-dose protocol: the first insemination is administered at the onset of estrus, followed by a second 8 hours later. This approach aims to maximize the chances of fertilization by covering both the early and late phases of estrus.

Pregnancy diagnosis was conducted 60 days after AI using rectal palpation, using rectal palpation to assess reproductive status. Pregnant cows were subsequently monitored until parturition (birth) to record non-return rate (NRR) 1 and 2, which refer to animals that do not come back into heat at approximately 21 days and 42 days (Susilawati *et al.,* 2022), CR (the percentage that became pregnant after 60 days post AI) (Firdaus *et al.,* 2024; Utami *et al.,* 2022), calving rate (number of live births, CvR), and sex ratio outcomes (male to female offspring ratio) (Susilawati *et al.*, 2023).

Statistical Analysis

Semen kinematic characteristics data were analyzed using one-way analysis of variance (ANOVA) to evaluate differences between treatments. If significant differences were obtained ($p<0.05$), the analysis was continued with a post hoc test to compare the means between groups. Principal component analysis (PCA) was performed to identify patterns of relationships between kinematic parameters and to determine the main components contributing to the total data variation. Reproductive performance data were analyzed using Fisher's Exact Test on a 3×2 contingency table to compare the proportions of success between treatments. Where appropriate, pairwise comparisons were performed in pairs using Fisher's Exact Test (2×2). All analyses were performed using R (version 4.4.1) (R Core Team, Vienna, Austria) with a significance level of 5% ($p<0.05$).

RESULTS

Fresh Semen Quality of Holstein-Friesian Bulls

Fresh semen from HF bulls showed good quality based on macroscopic, microscopic, and kinematic evaluations (Table 1). Macroscopically, the average semen volume was 7.56 ± 1.20 mL with a milky white color, characteristic odor, and pH of 6.5 ± 0.10, which is still within the normal physiological range for bovine semen. Overall, the combination of macroscopic, microscopic, and kinematic parameters indicates that the fresh semen used in this study is of excellent quality and meets the standards for further semen processing.

Table 1. Fresh semen quality of Holstein-Friesian bulls

Variables	Mean ± SD
Macroscopic	
Volume (mL)	7.56 ± 1.20
Colour	Milky white
pH	6.5 ± 0.10
Odor	Specific
Microscopic	
Individual motility (%)	78.7 ± 3.2
Concentration (106/mL)	1,546 ± 336.2
Viability (%)	86.3 ± 4.8
Abnormality (%)	6.4 ± 2.1
Kinematics	
Motile (%)	90.5 ± 10.3
Progressive (%)	78.6 ± 10.2
DAP (μm)	53.2 ± 9.4
DSL (μm)	48.4 ± 8.5
DCL (μm)	74.0 ± 8.7
VAP (μm/s)	144.9 ± 22.2
VSL (μm/s)	133 ± 20.9
VCL (μm/s)	202.6 ±19.4
STR (%)	91.4 ±1.5
LIN (%)	65.5 ± 6.15
ALH (μm)	6.3 ± 0.7

Note: Values are presented as mean ± standard deviation (SD). DAP: distance average path, DSL: distance straight line, DCL: distance curved line, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head movement, BCF: beat crossing frequency.

Kinematic Characteristics of Post-Thawed Sperm

The data showed that PDGC sexing treatment had a significant effect on several sperm kinematic parameters ($p<0.05$): motility, DAP, DSL, DCL, VSL, VCL, LIN, ALH, and WOB. Progressive, VAP, STR, and BCF did not differ significantly between treatments ($p>0.05$). Motility was highest in G0 and decreased significantly in G1 and G2. However, the velocity parameters showed a different pattern: G1 had the highest VSL and VCL values, while G2 showed the lowest. The ALH also increased significantly in G1, suggesting increased sperm movement dynamics. LIN and WOB were higher in G0 than in G1. Overall, the 20%-60% PDGC treatment (G1) increased the speed parameters and movement dynamics of sperm, while higher concentrations (G2) tended to decrease total motility and several kinematic parameters (Table 2).

Principal Component Analysis (PCA) of Post-Thawed Spermatozoa

PCA of the kinematic characteristics showed that the first two principal components explained 70.7% of the total data variation (Dim1 = 42%; Dim2 = 28.7%). The first dimension (PC1) mainly reflects parameters related to speed and movement dynamics, including VCL, VSL, VAP, and ALH. In contrast, the second dimension (PC2) represents movement patterns and frequencies related to parameters, such as BCF and trajectory characteristics. The biplot (Figure 1) illustrates that G1 is more closely associated with parameters describing speed and amplitude. Meanwhile, G0 and G2 are more closely related to linearity parameters such as LIN, STR, and WOB. Overall, these results suggest that the 20%-60% PDGC treatment produced a more dynamic kinematic, compared to other treatment groups.

Reproductive Performance

The Exact Test (Table 3) showed no statistically significant differences among treatments for reproductive performance parameters ($p>0.05$). Although descriptively, T2 showed higher CR and CvR (75%) than T0 (60%) and T1 (50%), these differences were not statistically significant. Paired comparisons for CR (Table 4) also showed no significant differences between treatment groups ($p>0.05$). The estimated odds ratios indicated a higher probability of pregnancy in T2 compared to T1 (OR = 0.34).

DISCUSSION

The quality of fresh semen was good, with motility exceeding 70%. This reflects good reproductive function in bulls as well as a plasma composition that supports sperm viability (Syarif *et al.*, 2026). A slightly acidic pH contributes to membrane stability and metabolic

Table 2. Sperm kinematic variables of conventional and sexed frozen semen of Holstein-Friesian bulls

Variables	Treatments			F value	p value	Sig.
	G0	G1	G2			
Motile (%)	86.0 ± 3.3^{a}	78.7 ± 6.4^{b}	71.7 ± 6.6^{c}	16.85	<0.001	S
Progressive (%)	42.6 ± 4.6	40.2 ± 5.5	45.0 ± 9.2	1.78	0.192	NS
DAP (µm)	33.4 ± 5.1b	45.1 ± 7.5^{a}	35.9 ± 2.9^{b}	12.53	<0.001	S
DSL (µm)	25.7 ± 4.4b	32.1 ± 4.8^{a}	27.2 ± 3.6^{b}	6.02	0.007	S
DCL (µm)	66.3 ± 11b	93.1 ± 9.7^{a}	67.6 ± 5.2^{b}	28.16	<0.001	S
VSL (µm/s)	74.6 ± 6.5^{ab}	83.3 ± 9.6^{a}	68.6 ± 8.7^{b}	7.86	0.002	S
VCL (µm/s)	187.0 ± 20.9^{b}	239.1 ± 17.2^{a}	167.0 ± 18.2^{c}	52.41	<0.001	S
VAP (µm/s)	95.3 ± 10.7	112.2 ± 8.5	93.0 ± 9.3	2.41	0.109	NS
LIN (%)	42.0 ± 4.2^{a}	37.1 ± 4.1^{b}	41.4 ± 4.8^{ab}	3.92	0.034	S
STR (%)	78.3 ± 3.6	74.8 ± 4.8	78.7 ± 4.6	2.17	0.134	NS
ALH (µm)	9.34 ± 1.2^{b}	11.28 ± 1.1^{a}	8.88 ± 0.9^{b}	18.72	<0.001	S
BCF (Hz)	23.5 ± 2.4	24.7 ± 2.8	23.1 ± 2.6	0.88	0.423	NS
WOB (%)	53.7 ± 2.4^{a}	48.9 ± 3.1^{b}	53.4 ± 2.9^{a}	8.54	0.002	S

Note: Values are presented as mean ± standard deviation (SD) with n = 10 per group. Different superscript letters (a-c) on the same row indicate significant differences based on Tukey's post hoc test at a significance level of 5% (p<0.05). G0: conventional semen (control), G1: sexed semen using Percoll density gradient centrifugation (PDGC) with a gradient of 20%-60%, G2: sexed semen using PDGC with a gradient of 20%-65%, S: significant, NS: non-significant, PDGC: Percoll density gradient centrifugation, DAP: distance average path, DSL: distance straight line, DCL: distance curved line, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head movement, BCF: beat crossing frequency.

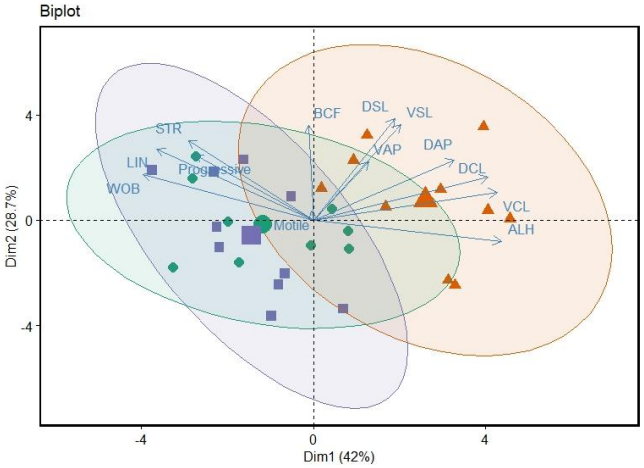

Figure 1. Principal component analysis (PCA) of post-thawed sperm kinematics in frozen-thawed Holstein Friesian bull semen. G0: conventional semen (control), G1: sexed semen using Percoll density gradient centrifugation (PDGC) with a gradient of 20%-60%, G2: sexed semen using PDGC with a gradient of 20%-65%, S: significant, NS: non-significant, PDGC: percoll density gradient centrifugation, DAP: Distance Average Path, DSL: Distance Straight Line, DCL: Distance Curved Line, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head movement, BCF: beat cross frequency. G0 ●; G1 ▲; G2 ■.

activity. High sperm concentration and good viability, with a low rate of abnormalities, indicate optimal spermatogenesis and minimal cellular damage (Fallon *et al.*, 2023). In terms of kinematics, high total and progressive motility indicate effective flagellar activity and sufficient energy. Trajectory parameters (DAP, DSL, DCL) indicate active, stable translational motion. High velocity values (VAP, VSL, VCL) reflect strong propulsion capacity (Benito Diaz *et al.*, 2025). High STR values with moderate LIN indicate mostly straight and balanced movement. Balanced ALH values indicate effective propulsion without excessive hyperactivation (Yusuf *et al.*, 2026). These characteristics confirm that the semen is suitable for further processing.

The PDGC method for sexing sperm relies on the principle of separating cells based on differences in density and molecular mass (Rahmawati *et al.*, 2025). Sperm carrying the X chromosome have approximately 3%-4% higher DNA content than sperm carrying the Y chromosome (Pozdyshev *et al.*, 2023). They have a density difference that allows separation through concentration gradients. However, this density difference is relatively small, so the centrifugation process not only selects based on chromosomes but also has the potential to affect the physiological quality of sperm due to mechanical stress and changes in the microenvironment during the separation process.

The results showed that PDGC significantly reduced motility compared to conventional semen. This reduction was likely caused by centrifugal force, which increased mechanical stress on the plasma membrane and flagellum structure. This stress could trigger an increase in the production of reactive oxygen species (ROS) (Aitken, 2017), which is known to cause membrane lipid peroxidation, membrane fluidity disorders, and mitochondrial dysfunction (Abdelnour *et al.*, 2022). Because sperm movement is highly dependent on ATP production through oxidative phosphorylation in the midpiece, mitochondrial dysfunction will directly affect the proportion of cells capable of active movement (Amaral, 2022). However, motility in the PDGC group remains within a range suitable for fertilization.

The results of this study align with Safa *et al.* (2025), showing that density gradient based sexing can preserve sperm motility. However, there are differences compared to Syarif *et al.* (2026), who reported higher motility, likely due to variations in the sexing protocol, specifically the use of the albumin sedimentation method, the type of diluent, and the freezing process.

Table 3. Reproductive performance variables and Fisher's exact test results of Holstein Friesian cows among three artificial insemination treatments

Variables	Treatments			p-value	Sig.
	T0 (success/n)	T1 (success/n)	T2 (success/n)		
NRR1 (%)	95 (19/20)	80 (16/20)	85 (17/20)	0.5054	NS
NRR2 (%)	80 (16/20)	65 (13/20)	75 (15/20)	0.6672	NS
CR (%)	60 (12/20)	50 (10/20)	75 (15/20)	0.3038	NS
CvR (%)	60 (12/20)	50 (10/20)	75 (15/20)	0.3038	NS

Note: Percentage values are calculated based on the number of pregnant or calving cattle divided by the total number of cows per group (n = 20). The numbers in parentheses indicate the number of success/n. A p-value < 0.05 indicates a statistically significant difference between treatments. T0: double-dose AI used conventional semen (control), T1: double-dose AI used 20%-60% PDGC sexed semen, T2: double-dose AI used 20%-65% PDGC sexed semen, NRR1: non-return rate 1, NRR2: non-return rate 2, CR: conception rate, CvR: calving rate, Sig: significant, NS: non-significant.

Table 4. Pairwise exact test for conception rates f Holstein Friesian cows among three artificial insemination treatments using conventional and PDGC-sexed frozen-thawed semen

Comparison	Odds Ratio	95% CI	p-value	Sig.
T0 vs T1	1.48	0.36-6.29	0.7512	NS
T0 vs T2	0.51	0.10-2.32	0.5006	NS
T1 vs T2	0.34	0.07-1.51	0.1908	NS

Note: Values are presented, including the Odds Ratio (OR), 95% Confidence Interval (95% CI), and p-value. The OR indicates the relative odds of pregnancy in the first group compared to the comparison group. The p-value indicates the test's significance level; p<0.05 is considered statistically significant. T0: double-dose AI used conventional semen (control), T1: double-dose AI used 20%-60% PDGC sexed semen, T2: double-dose AI used 20%-65% PDGC sexed semen, Sig: significant, NS: non-significant.

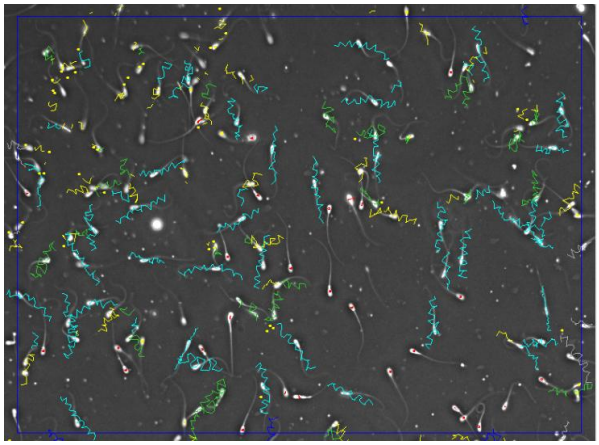

Figure 2. Representative visualization of sperm motility analysis using the Computer-Assisted Sperm Analysis (CASA) system in frozen-thawed Holstein Friesian bull semen. The movement paths of sperm are displayed using color codes. Green: indicates motile sperm, light blue: (cyan) indicates progressive sperm, red: indicates static sperm (not moving), dark blue: indicates border crossers, yellow: indicates late tracks, and gray: indicates late entries. Colored lines depict the movement trajectory of each sperm during the recording period.

These differences underscore that the method's effectiveness depends on the technical conditions used.

DAP represents the average length of the path, DSL describes the straight-line distance traveled, and DCL indicates the total curved path (O'Meara *et al.*, 2022; van der Horst, 2020). The increase in these three parameters in G1 indicates longer, more active trajectories, reflecting more optimal flagellar propulsion. This showed that a 20%-60% gradient is more effective at maintaining sperm with good translational motility. Conversely, a decrease in G2 values (20%-65%) indicates that a higher density gradient may disrupt cellular structural integrity or energy function, leading to shorter, less dynamic trajectories (Figure 2). The PCA results indicate that treatments that enhance speed parameters tend to produce sperm with more dynamic movement, whereas treatments related to linearity parameters promote more directed movement. The balance between these two aspects is a key factor in determining sperm fertility potential (Syarif *et al.*, 2026).

Significant increases in VCL and ALH, especially in the PDGC 20%-60% group, indicate a change in sperm motility, making it more dynamic and energetic. High VCL reflects high actual trajectory speed, while ALH describes increased amplitude of lateral head movement. The combination of these two parameters is physiologically associated with a pattern of hyperactivation, a change in motility that occurs during the capacitation process in the female reproductive tract. Molecularly, hyperactivation is associated with increased intracellular Ca^{2+} flux and activation of cAMP–PKA signaling pathways during capacitation (Cordero-Martínez *et al.*, 2022). This movement pattern plays an important role in penetrating the zona pellucida and achieving successful fertilization. Therefore, the increase in these parameters may reflect the selection of a subpopulation of sperm with greater metabolic and energy capacity during the PDGC process. However, hyperactivation that occurs too early can also reduce sperm viability before they reach the fertilization site (Zafar *et al.*, 2021). The absence of a decrease in reproductive performance indicates that these changes are still within adaptive physiological limits.

Changes in LIN and WOB, which decreased in the PDGC 20%-60% group, indicate a change in movement pattern towards hyperactivation. A low LIN indicates a less straight trajectory, while a decreased WOB value reflects an imbalance between average velocity and curvilinear velocity (Rotar *et al.*, 2026; Wahyudi *et al.*, 2025). This pattern aligns with the movement pattern required when approaching the oocyte. However, if hyperactivation occurs too early due to the pressure from the sex determination process, sperm may lose energy before reaching the fertilization site. The parameters of progressive motility, VAP, STR, and BCF did not show significant differences. This indicates that although changes in certain movement dynamics, the

ability of sperm to maintain progressive movement direction and flagellar beat frequency remained stable. The PDGC sex determination method does not disrupt the overall control of flagellar movement coordination. This stability is important because fertilization requires a balance between speed, direction of movement, and metabolic endurance (Zhang & Xu, 2025).

Although several motion parameters showed significant changes, analysis of NRR1, NRR2, CR, and CvR showed no significant differences between treatments. This indicates that the changes that occurred at the kinematic level were not large enough to interfere with the *in vivo* fertilization process. Successful fertilization requires sperm with intact DNA, acrosome stability, and the ability to activate oocytes by releasing factors such as phospholipase C zeta (PLCζ), which triggers cytoplasmic Ca^{2+} oscillations (Gonzalez-Castro & Carnevale, 2023). If PDGC causes severe chromatin damage or acrosomal disruption, a decrease in pregnancy rates or an increase in early embryo loss should be observed. Since this did not occur, it can be concluded that the structural and molecular integrity of sperm is maintained.

One factor that contributes to maintaining reproductive performance in sexed semen groups is the use of the double-dose method during AI. Sexed semen typically has a lower concentration of viable sperm due to the selection process and potential cell loss during centrifugation (Barros Mothé *et al.*, 2018; Priyanto *et al.*, 2023). The use of double-dose AI aims to compensate for the potential reduction in the number of viable sperm reaching the fertilization site (the ampulla of the oviduct). By increasing the number of cells inseminated, the probability of interaction between viable sperm and the oocytes is maintained. This strategy aligns with the principle that fertilization success is determined not only by the quality of individual sperm but also by the number of cells that can survive, migrate, and achieve capacitation on time within the reproductive tract (Van de Hoek *et al.*, 2022).

Physiologically, only a small fraction of sperm successfully reach the site of fertilization due to natural selection in the cervix, uterus, and fallopian tubes. Therefore, the double-dose approach is rational to minimize the risk of fertilization failure by reducing the initial sperm population following the sexing process. The fact that reproductive performance does not differ significantly indicates that the combination of the PDGC method and the double-dose strategy maintains a balance between the quality and quantity of sperm required to achieve optimal reproductive performance.

The discussion of sex ratios yields additional strategic insights from a production management perspective. Figure 3 showed that sexed semen groups produced a higher proportion of female calves than conventional semen. While male calves still dominate in the control group, the sexing group showed a significant shift towards female calf dominance (around 67%-70%). The results of this study show a higher proportion of female calves born compared to that reported by Mendes *et al.* (2022) who reported a percentage of 47.1% female and 52.9% male calves in insemination using

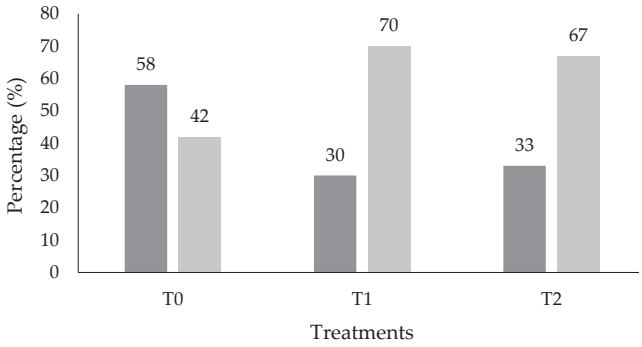

Figure 3. Percentage of male and female calf sex ratios produced from Holstein Friesian cows inseminated with frozen-thawed semen under three artificial insemination treatments. T0: double-dose AI used conventional semen (control), T1: double-dose AI used 20%-60% PDGC sexed semen, T2: double-dose AI used 20%-65% PDGC sexed semen. Sex ratio male = male calves, sex ratio female = female calves. ■ Sex ratio male (%), ■ sex ratio female (%).

sexed semen, and 51.3% female and 48.7% male calves in the use of conventional semen. In addition, Shabirah *et al.* (2025) reported a 85.2% proportion of female calves. This confirms that the density-based PDGC method can increase the chances of obtaining offspring of the desired sex, even though the success rate does not reach 100%.

The increase in the proportion of female calves reflects relative success in enriching X chromosome-carrying sperm in certain gradient fractions. Although the difference in DNA content between the X and Y chromosomes is relatively small, the multi-stage centrifugation process allows for the distribution of fractions with dominance of one population. Thus, although this method is not as precise as flow cytometry-based technology, PDGC still demonstrates practical effectiveness in significantly increasing the chances of female births compared to conventional semen, which naturally produces a ratio close to 50:50. However, this study has several limitations, particularly regarding the relatively small sample size and field conditions that could potentially influence fertility outcomes. Therefore, future studies are recommended to use a larger sample size and more controlled conditions to enhance the validity of the results.

CONCLUSION

Sexing sperm using the PDGC method significantly alters several kinematic variables, including motility, DAP, DCL, DSL, VSL, VCL, LIN, ALH, and WOB, suggesting changes in movement dynamics driven by the density-based separation process. However, these changes do not reduce *in vivo* reproductive performance, as NRR, CR, and CvR values remain comparable to those of conventional semen, especially when a double-dose insemination strategy is used to compensate for a potential reduction in viable sperm. The use of sexed semen increased the proportion of female offspring compared to conventional semen, indicating the effectiveness of this method in enriching

X chromosome-carrying sperm. Overall, PDGC-based sexing represents a practical and relatively safe approach to increase the chances of female offspring.

CONFLICT OF INTEREST

The authors declare that there are no financial or commercial relationships that could be construed as a potential conflict of interest in relation to this research.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the writing process, the authors used Grammarly software to assist with grammar and sentence structure editing. All edits have been reviewed by the author, who is responsible for the final content of the published manuscript.

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