RESEARCH ARTICLE OPEN ACCESS

Quality and Proportion of Sexed X and Y Spermatozoa Using Different Percoll Density Gradient Centrifugation Methods After Freezing in Friesian Holstein Bulls

Chairun Nisa Aperi Simbolon*, Anny Amaliya**, Dhanis Fitriana**, Aulia Puspita Anugra Yekti***, Nurul Isnaini***, Trinil Susilawati***
*Postgraduate Student, Faculty of Animal Science, Malang 65145, East Java, Indonesia
**Artificial Insemination Center, Singosari 65153, East Java, Indonesia
***Department of Animal Reproduction, Faculty of Animal Science, Malang 65145, East Java, Indonesia
Corresponding Author Email: tsusilawati@ub.ac.id)

--************************----------------------------------

Abstract:
The application of sexing technology represents a strategic endeavor to enhance the productivity of livestock farming by enabling the prediction of the sex of offspring, thereby aligning with the specific goals of the farm. This study focuses on evaluating the quality and distribution of sexed spermatozoa achieved through the use of the Percoll density gradient centrifugation method at varying Percoll densities. The research utilized fresh semen from three Friesian Holstein Bulls, each with an average age of 3.5 years and a body weight of 600 kg, and a minimum semen motility quality of 70%. Conducted as a laboratory experiment, this study employed two different treatments across ten replicates: T1 involved a 20%–65% Percoll density plus tris aminomethane egg yolk, while T2 utilized a 20%–60% Percoll density also combined with tris aminomethane egg yolk.The analysis incorporated a dependent t-test to assess the quality of the post-thaw sexed frozen semen, along with chi-square analysis to examine the proportion of X and Y spermatozoa. The findings revealed that varying the Percoll density within the gradient centrifugation sexing method did not affect the quality of the sexed frozen semen in terms of individual motility, viability, and abnormality rates for both the upper and lower layers. However, treatment T2 (20%–60% density) demonstrated superior outcomes in sperm concentration and total motile spermatozoa specifically in the lower layer, without significant differences observed in the upper layer. The spermatozoa proportions were as follows: in the upper layer, T1 had 19.60% X and 80.40% Y, while T2 had 18.60% X and 81.40% Y; in the bottom layer, T1 showed 78.10% X and 21.90% Y, whereas T2 had 79.20% X and 20.80% Y.In conclusion, both Percoll density treatments of 20%–65% and 20%–60% yielded high-quality spermatozoa, with individual motility percentages exceeding the SNI (Indonesian National Standard) threshold of >40%. Moreover, these treatments proved effective in separating X and Y spermatozoa, thereby demonstrating their potential utility in livestock breeding programs aimed at sex selection.

Keywords **—Percoll gradient; Semen sexing; Semen quality.**

--************************----------------------------------

I. INTRODUCTION

Sperm sexing refers to the technique of influencing the sex ratio of offspring by altering the natural balance of spermatozoa (traditionally 50:50) to a desired proportion through specific identification and sorting methods. This process leverages the known differences between X and Y chromosome-bearing spermatozoa, such as variations in size and DNA content. For instance, X chromosome spermatozoa in cattle contain approximately 3.8% more DNA than their Y

 ©IJSRED: All Rights are Reserved

chromosome counterparts [1]. Additionally, [2] noted that Y chromosome spermatozoa exhibit smaller head sizes and lower mass compared to X chromosome spermatozoa, attributes that grant them greater speed and mobility. These differences facilitate various separation techniques, including but not limited to albumin column separation, velocity sedimentation, Percoll density gradient centrifugation, electrophoresis, isoelectric focusing, H-Y antigen detection, flow cytometric sorting, and Sephadex filtration. Among these methods, Percoll density gradient centrifugation has been identified as the most prevalently utilized technique for sperm separation [3]

Variations in DNA content between spermatozoa carrying X and Y chromosomes lead to differences in their weight and density. This discrepancy facilitates the separation of spermatozoa using a Percoll gradient, as demonstrated by [4]. X spermatozoa have heads that are heavier and larger than those of Y spermatozoa. Therefore, during separation by centrifugation, the X spermatozoa will be found in the lower fraction, while the Y spermatozoa will be in the upper fraction [5].The technique of sexing spermatozoa through Percoll density gradient centrifugation is superior to other methods. One of the key advantages of using Percoll is the ease with which its density can be adjusted, allowing for the customization of the gradient to suit specific needs[2]

Building on the approach described, the author has experimented with the spermatozoa sexing technique using Percoll density gradient centrifugation, specifically by adjusting the density range. This adjustment modifies the original Percoll density range proposed [2], from 20% - 65% to a narrower range of 20% - 60% to evaluate whether this alteration in density affects the quality and proportion of the resulting spermatozoa.

II. MATERIALS AND METHOD

This section explains the materials and method used in this research.

A. Research Setting

The research was conducted at the Laboratory of the Center for Artificial Insemination (BBIB) Singosari and the Laboratory of Animal Reproduction, Faculty of Animal Science, Brawijaya University, Malang from September 26 to December 15, 2023.

B. Research Materials

The research utilized fresh semen samples collected from 3 Friesian Holstein Bull with an average age of 3,5 years and an average body weight of 600 kg and fresh semen quality motility ≥70.For the spermatozoa sexing process, the study employed Percoll density gradient centrifugation, using Percoll combined with an egg yolk tris aminomethane diluent as the medium for separation.

C. Research Method

This laboratory experimental research was performed in 2 treatments with 10 replications. The treatments in the study were T1: 20% – 65% density percoll + tris aminomethan egg yolk and T2: 20% – 60% density percoll + tris aminomethan egg yolk.Parameters measured after freezing included the percentage of individual motility, viability, abnormalities, concentration, total motile spermatozoa and the percentage proportion of X and Y spermatozoa.

D. Percoll Density Gradient Centrifugation Sexing Method

The sperm sexing procedure utilizing the Percoll Density Gradient Centrifugation method, as outlined by [2]involved the creation of a Percoll density gradient using tris aminomethan egg yolk T1 diluent with ten gradients (ranging from 65% to 20%) and T1 diluent with ten gradients (ranging from 60% to 20%). Each treatment consisted of tubes with ten gradients of T1: 20% – 65% and T1: 20% – 60% percoll density, arranged from highest to lowest density, with each density level containing 0.5 mL. Following the formation of the gradient, 1 ml of high-quality semen was added to each tube. The tubes were centrifuged at 2250 rpm for 5 minutes, resulting in six layers. The top layer, containing seminal plasma, was then removed, and the second layer that contain Y spermatozoa was identified. The bottom layer, abundant in X spermatozoa was collected and transferred to a tube containing 3 ml of tris aminomethan egg yolk. This mixture was then centrifuged at 1500 rpm for 5

 ©IJSRED: All Rights are Reserved

minutes, and the supernatant was discarded, leaving 1-2 ml of sediment containing sorted spermatozoa

E. Cyropreservation Procedure

The cryopreservation started by gradual dilution using tris aminomethan egg yolk. The diluent semen was then equilibrated in a refrigerator at 5°C for 22 hours. The semen was put into a 0,25 mL straw using an automatic filling and sealing machine. The straw was then put into a pre-freezing machine for 9 minutes at -140°C. After that, the sample was frozen using liquid nitrogen -196°C and stored in a container containing liquid nitrogen for at least 1 x 24 hours.Thawing was later carried out using warm water 37-38°C for 30 seconds [6], from which individual motility, viability, abnormality, concentration, total motile spermatozoa and the proportion of X and Y spermatozoa were observed.

F. Assessment of Sperm Quality After Freezing

The evaluation of sperm motility was conducted using a light microscope at 400X magnification. Viability and abnormality assessments were performed by placing a drop of semen on a warm glass slide and adding one drop of dual-staining eosin negrosin solution. After gentle mixing, the slide was observed under the microscope at 400X magnification. Dead sperm cells absorbed the stain, while live sperm cells did not, and 200 sperm were examined in each sample, following the method outlined [7]. Spermatozoa concentration was calculated using the Neubauer counting chamber, as described [8] as follows.
Where:

- N : Average number of spermatozoa in chambers A and B
- 5: Correction factor because only 5 out of 25 squares are counted
- 0,25 : Correction factor because straw 0,25 mL
- FP : Dilution factor (1:100)
- 10.000: Depth of the Neubauer chamber (0.0001 ml/Neubauer chamber)

The total motile spermatozoa can be calculated by multiplying the percentage of individual sperm motility by the sperm concentration and the semen volume [9].

G. The Proportion of Spermatozoa X and Y

Morphometric measurements of spermatozoa, specifically focusing on the length and width of the sperm head, were conducted using LC-Micro software to facilitate the identification of X and Y chromosome-bearing spermatozoa. In establishing a baseline for the expected natural 50:50 proportion, a total of 1000 spermatozoa from fresh semen samples were analyzed. For each treatment group subjected to the specific Percoll density gradient centrifugation conditions, 100 spermatozoa were evaluated in each sample. The differentiation between X and Y spermatozoa was based on the head size, with those exhibiting larger-than-average heads identified as X spermatozoa and those with smaller heads classified as Y spermatozoa, adhering to the methodology [10].

H. Data Analysis

The data of this research were analysed using the dependent t test to assess the quality of post-thawing frozen semen, including individual motility, viability, abnormality, concentration, total motile spermatozoa and chi square analysis for the proportion of X and Y spermatozoa.

RESULTS AND DISCUSSION

I. Fresh Semen Quality

The results of the evaluation of fresh semen quality of Friesien Holstein bulls in this study can be seen in Table I.

TABLE I
MEAN±SETHE FRESH SPERM QUALITY OF FRIESIEN HOLSTEIN BULLS

The Fresh Sperm Quality of Friesien Holstein bulls	
Volume (mL)	7.4 ± 0.42
Color	Milky white
Consistence	Moderate
Odor	Typical
pH	6.4 ± 0.02
Individual Motility (%)	79.00 ± 1.00
Concentration (10^6/mL)	1491.9 ± 96.91
Total Motile Spermatozoa (10^6/mL)	8699.45 ± 715.00
Viability (%)	85.32 ± 1.37
Abnormality (%)	6.50 ± 0.66

 ©IJSRED: All Rights are Reserved

Based on the data presented in Table I, the macroscopic evaluation of fresh semen from Holstein Friesian Bulls indicated a volume of 7.4 ± 0.42 mL, aligning with the findings of [11], who noted that the normal semen volume for male cattle ranges between 4 to 8 mL per ejaculation. The fresh semen in this study exhibited a distinctive semen like odor and a milky white coloration, consistent with observations made by [12], who reported a similar milky white semen color in Holstein Friesian Bulls. Additionally, the pH level of the fresh semen was measured at 6.4 ± 0.08, which falls within the optimal acidity range (pH 6.2 – 6.8) for semen as stated [7], indicating suitable conditions for maintaining semen quality.

The microscopic analysis of fresh semen from Holstein Friesian bulls revealed an individual motility rate of 79.00 ± 1.00%. This rate falls within the "good" category and deems the semen suitable for sexing and subsequent processing for freezing, aligning with the standards set by [3], which suggest that fresh semen with more than 70% motility is appropriate for these procedures. The total sperm concentration was found to be 1491.9 ± 96.91 10^6/mL, indicating medium semen consistency as per the classification by [9], where semen consistency correlates with the concentration of spermatozoa per ejaculation. The viability of the semen was measured at 85.32 ± 4.32%, a figure that closely matches the findings of [13] who reported an average semen viability of 85% in Holstein Friesian bulls. Furthermore, the percentage of abnormal spermatozoa was recorded at 6.50 ± 2.09%, which is comparably close to the 5.11 ± 4.98% [14], indicating a relatively low level of morphological abnormality in the semen.

J. Individual Motility Percentage Post Thawing Sexed Frozen Semen

The individual motility percentage post thawing is presented in TableII.

TABLE II
MEAN±SEMOTILITY OF INDIVIDUAL FROZEN SEMEN
SEXING CENTRIFUGATION DIFFERENT PERCOLL DENSITY GRADIENTS

Layer/Treatment	Individual Motility (%)	
	T1	T2
TOP	41.70 ± 1.37	44,30 ± 1.71
BOTTOM	43.00± 1.41	44.30 ± 2.03

Statistical analysis utilizing a paired t-test revealed that varying the densities in the Percoll density gradient centrifugation process for semen sexing did not significantly impact (P>0.05) the individual motility rates of sexed frozen semen in either the top or bottom layers. This finding suggests that the modification of densities within the Percoll density gradient centrifugation technique does not affect the motility of sexed frozen semen collected from both layers. According to the data presented in Table II, there is a general decline in the average individual motility of post-thaw sexed frozen semen compared to its fresh semen counterpart, which exhibited an individual motility rate of 79.00 ± 1.00%.

The process of sexing spermatozoa undertaken in this study is believed to contribute to the reduction in individual motility observed. Individual motility rates for thawed frozen semen that had not undergone sexing were recorded at 62.6% [15]. The centrifugation step involved in separating and washing the spermatozoa is suspected to cause damage to the sperm membranes. This aligns with findings of [16] who noted that the act of centrifugation generates friction among the spermatozoa and between the spermatozoa and both the separating medium and the walls of the centrifuge tubes, leading to structural damage to the sperm membranes and metabolic disruptions. The separation process via centrifugation effectively isolates the spermatozoa from the seminal plasma, which in turn compromises the integrity of the sperm's outer membrane or acrosome. In such a state, the spermatozoa are deprived of supportive materials and nutrients essential for their vitality and motility.

The decline in spermatozoa quality observed in the study can be attributed not only to the separation process but also to the cooling and freezing procedures involved in preparing frozen semen. The cooling, freezing, and thawing processes induce drastic temperature changes, leading to damage to the spermatozoa cell membrane [11]. Furthermore, [9] emphasized that these procedures impose physical and chemical stress on the spermatozoa membrane. It was elaborated by [15] that the storage of frozen semen

 ©IJSRED: All Rights are Reserved

compromises spermatozoa quality due to cold shock and ice crystal formation. Cold shock primarily affects spermatozoa by reducing motility and viability, altering membrane permeability, and changing the composition of lipid components such as phospholipids and cholesterol, which are crucial for maintaining the structural integrity of the plasma membrane. This concept is supported by [18], who stated that phospholipids and cholesterol are vital for sustaining membrane stability.

K. Percentage of Post Thawing Viability of Sexed Frozen Semen

TABLE III
MEAN±SETHE PERCENTAGE OF VIABILITY OF SEXED FROZEN SEMEN WITH DIFFERENT PERCOLL DENSITY GRADIENT CENTRIFUGATION

Layer/Treatment	Viability (%)	
	T1	T2
TOP	62.60 ± 3,05	64.77 ± 2.99
BOTTOM	63.92 ± 3.10	64.06 ± 3.13

The findings from the paired t-test analysis reveal that altering the densities in the Percoll density gradient centrifugation sexing method does not significantly impact the viability of sexed frozen semen in both the top and bottom layers (P>0.05). This suggests that the spermatozoa viability is comparable across both treatment densities. Data presented in Table III highlight a reduction in the overall viability of post-thaw frozen sexed semen when compared to the viability of fresh semen, which was initially 85.32 ± 1.37%. This decrease in viability post-thaw aligns with the research findings [6], which indicate that about30% of spermatozoa succumb during the freezing process.

The observed decline in sperm viability percentages is believed to result from the centrifugation steps involved in sperm separation and washing. According to [19], the centrifugal separation of spermatozoa induces mechanical friction, potentially leading to membrane damage. This observation is supported by [20], who explained that the change in color indicating dead spermatozoa results from damage to the sperm cell's plasma membrane, allowing eosin dye to penetrate the cells. Such membrane damage compromises the membrane's semipermeable nature, preventing it from selectively regulating the influx and efflux of substances [21]. Consequently, when the eosin negrosin stain test is performed, the dye is able to enter the sperm cell, indicating compromised viability.

The centrifugation process integral to sperm separation can result in the release of seminal plasma from spermatozoa, consequently diminishing the protective stability of the sperm membrane. Sperm separation process detrimentally affects sperm quality, as spermatozoa are subjected to a series of procedures ranging from separation to washing, all of which demand significant energy to maintain physiological condition [22]. Furthermore, [23] highlight that the sexing process via centrifugation can lead to the loss of certain sperm membrane phospholipids due to the centrifugal force applied. This loss of membrane phospholipids disrupts the integrity of the sperm membrane, thereby adversely impacting the viability of the spermatozoa.

The storage of semen in a frozen state is identified by [24] as another significant factor that can affect the quality of spermatozoa. The freezing process exposes spermatozoa to a critical temperature point at which they must endure to survive, making them susceptible to cold shock. Cold shock is known to cause both structural and biochemical damage to cells, leading to membrane damage and ultimately resulting in the death of spermatozoa [25]. The cooling, freezing, and thawing processes induce physical and chemical stress on the spermatozoa membrane [9]. This stress compromises the membrane's integrity, potentially reducing the viability of the spermatozoa and their ability to fertilize.

L. Percentage of Post Thawing Abnormality of Sexed Frozen Semen

Spermatozoa abnormality refer to morphological irregularities in one or more components of the sperm cell. Such abnormalities can significantly impact fertility, as sperm with normal morphology are essential for successful fertilization. The variation in the percentage of abnormalities among spermatozoa, processed through sex-sorting frozen

 ©IJSRED: All Rights are Reserved

semen via different Percoll density gradient centrifugation methods, is detailed in Table IV.

TABLE IV
MEAN±SE THE PERCENTAGE OF ABNORMALITY OF SEXED FROZEN SEMEN WITH DIFFERENT PERCOLL DENSITY GRADIENT CENTRIFUGATION

Layer/Treatment	Abnormality (%)	
	T1	T2
TOP	9.67 ± 0.87	8.43 ± 0.58
BOTTOM	9.31 ± 0.71	8.82 ± 0.59

The paired t-test analysis indicated that utilizing varying densities in Percoll density gradient centrifugation for sperm sexing does not significantly influence (P>0.05) the occurrence of abnormalities in frozen semen, across both upper and lower layers. This suggests that alterations in density during Percoll density gradient centrifugation for sexing do not affect sperm morphology detrimentally. A sperm morphology abnormality rate of 8–10% does not significantly impact fertility [26]. However, abnormalities exceeding 25% are associated with a decline in fertility.

The abnormality observed in this study predominantly fell into the category of secondary abnormalities, including severed heads and tails, tails folding upwards towards the neck, and numerous bent tails. The rise in spermatozoa abnormality rates could be attributed to the centrifugation process involved in sexing and washing. Such abnormalities arise from the mechanical impact against the tube walls during the separation process, which compromises sperm morphology [22].

M. Spermatozoa Concentration Post Thawing in Sexed Frozen Semen

Spermatozoa concentration in frozen semen is measured to verify that each straw contains a minimum of 25 million spermatozoa. This requirement is in accordance with the Indonesian National Standard 4869 – 1: 2017, which stipulates that frozen bovine semen, when packaged in mini straws of 0.25 mL, must have at least 25 million spermatozoa. The concentrations of spermatozoa in frozen semen, processed through various Percoll density gradient centrifugation methods for sexing, are presented in Table V.

TABLE V
MEAN±SE CONCENTRATION OF FROZEN SEMEN SEXING CENTRIFUGATION DIFFERENT PERCOLL DENSITY GRADIENTS

Layer/Treatment	Concentration (Million/Straw)	
	T1	T2
TOP	27.66 ± 2.33	32.66 ± 3.11
BOTTOM	20.58 ± 1.46[a]	28.53 ± 2.01[b]

Different superscripts within rows indicate differences at P<0.05

Table V reveals that the paired t-test analysis of density variations in Percoll density gradient centrifugation for sperm sexing showed no significant differences (P>0.05) in the sperm concentration of the upper layer post-sexing, whereas the lower layer exhibited significant differences (P<0.05), with the concentration in T2 being higher than in T1. The observed variance in sperm concentrations between T1 and T2 in the lower layer can be attributed to the precision in micropipette use and the researcher's proficiency in collecting semen (pipetting) following separation. This aligns with [2] observation that the sexing process utilizing the Percoll density gradient centrifugation technique demands a high level of skill in micropipette handling. Suboptimal pipetting techniques in sperm collection post-sexing can significantly impact sperm concentration [27].

N. Total Motile Spermatozoa Post Thawing of Sexed Frozen Semen

Total motile spermatozoa is the number of motile spermatozoa in 0.25 mL/straw. Total motile spermatozoa are strongly influenced by the percentage of progressive motility [28]. Total motile spermatozoa frozen semen sexing different percoll density gradient centrifugation can be seen in Table VI.

TABLE VI
MEAN±SE TOTAL MOTILE SPERMATOZOA FROZEN SEMEN SEXING CENTRIFUGATION DIFFERENT PERCOLL DENSITY GRADIENTS

Layer/Treatment	Total motile spermatozoa (Million/Straw)	
	T1	T2
TOP	11.37 ± 1,2	15.22 ± 2.02
BOTTOM	9.22 ± 0.67[a]	13.23 ± 1.05[b]

Different superscripts within rows indicate differences at P<0.05

 ©IJSRED: All Rights are Reserved

Table VI shows that the paired t-test analysis assessing the impact of density variations in Percoll density gradient centrifugation for sperm sexing did not reveal significant differences (P>0.05) in the total motile spermatozoa count of frozen semen in the top layer post-sexing. However, significant differences were observed in the bottom layer (P<0.05), where the total number of motile spermatozoa in T2 was higher than in T1. Furthermore, Table VI demonstrates that the average concentration of spermatozoa in frozen sexed semen for each treatment was higher in the top layer compared to the bottom layer. Notably, the total count of motile spermatozoa in the lower T1 layer was the lowest, which is believed to be due to the reduced sperm concentration in T1 at the bottom layer, given that the total number of motile spermatozoa is determined by both the percentage of individual motility and sperm concentration. This finding is consistent with the observations made by [29], who noted that the percentage of motility and sperm concentration are directly related to the overall count of motile spermatozoa.

O. The Proportion of X and Y Spermatozoa

The effectiveness of X and Y spermatozoa separation is reflected in the resultant proportions of each sperm type. In this study, identification of X and Y spermatozoa was based on measuring the dimensions of the sperm heads, specifically their length and width, using a light microscope equipped with an ocular micrometer [27]. Measurements were taken for 100 spermatozoa from each treatment across both the upper and lower layers. Spermatozoa exhibiting head sizes larger than the control average were classified as X spermatozoa, whereas those with head sizes below the average were classified as Y spermatozoa [10]. The distribution percentages of X and Y spermatozoa obtained through different density gradient centrifugation processes for sexed frozen semen are detailed in Table VII.

TABLE VII
MEAN±SE PERCENTAGE OF SPERMATOZOA PROPORTION X AND Y FROZEN SEMEN SEXING PERCOLL DENSITY GRADIENT CENTRIFUGATION

Treatment	Layer	X & Y Spermatozoa Proportion		Total
		X (%)	Y (%)	
Fresh Semen		50.00 ± 0.00	50.00 ± 0.00	100
T1	Top	19.60 ± 0.89	80.40 ± 0.89	100
	Bottom	78.10 ± 1.39	21.90 ± 1.39	100
T2	Top	18.60 ± 0.89	81.40 ± 0.89	100
	Bottom	79.20 ± 0.94	20.80 ± 0.94	100

The separation of spermatozoa aims to alter the natural balance of X and Y spermatozoa, which is traditionally 50:50, through specific methodologies [30]. Table VII demonstrates that the application of the Percoll density gradient centrifugation technique in this research successfully adjusted this natural ratio. Initially, both X and Y spermatozoa constituted 50% of the total. However, following the application of this method, the proportions shifted to align with the expectations delineated by [27], who predicted a sexing efficiency of 80% for one sperm type and 20% for the other. This finding is corroborated by [1], who noted that the expected outcomes for the separation of Y spermatozoa (from the top layer) should be 80% Y and 20% X, and vice versa for X spermatozoa (from the bottom layer), indicating an 80% concentration of X and 20% Y.

III. CONCLUSIONS

The utilization of Percoll density gradient centrifugation for sperm sexing, employing gradients ranging from 20% to 65% and 20% to 60%, demonstrated comparable quality outcomes post-freezing in terms of visual individual motility, viability, and abnormalities. However, the 20% to 60% density gradient exhibited superior results regarding sperm concentration and total motile spermatozoa. Both Percoll density gradients, 20%–65% and 20%–60%, have proven to be effective in the separation of X and Y spermatozoa..

ACKNOWLEDGMENT

Gratitude is expressed to the Faculty of Animal Science, UniversitasBrawijayathat has grantedHibah Guru Besar 2023 under letter number: 2044.15/UN10.F05/PN/2023.Additionally, heartfelt

thanks are extended to the Singosari National Artificial Insemination Center (BBIB) for its valuable collaboration with the Faculty of Animal Science at Brawijaya University.

REFERENCES

[1] Grant, V.J., and L.W. Chamley. 2007. Sex-Sorted Sperm and Fertility: an Alternative View. *Biology of Reproduction*. 76 (2) : 184 – 188.
[2] Susilawati, T. 2014. *Sexing Spermatozoa*. Malang: UB Press. ISBN: 978-602-203-711-8
[3] Wahjuningsih, S., T. Susilawati, Suyadi, M.N. Ihsan, W. Busono, N. Isnaini dan A.P.A. Yekti. 2019. *Teknologi Reproduksi Ternak*. Malang: UB Press. ISBN: 978-602-432-843-6.
[4] Machado, G.M., J.O. Carvalho, E. S. Filho, E.S. Caixeta, M.M. Franco, R. Rumpf and M.A.N Dode. 2009. Effect of *Percoll* Volume Duration and Force of Centrifugation on in Vitro Production and Sex Ratio of Bovine Embryos. *Theriogenology*. 71 (8) : 1289 – 1297.
[5] Kusumawati, E.D., N. Isnaini, A. Yekti, M. Luthfi, L. Affandhy, D. Pamungkas, Kuswati, A. Ridhowi, H. Sudarwati, S. Rahadi dan T. Susilawati. 2019. The Motility and Ratio of X and Y Sperm Filial Ongole Cattle Using Different Sexed Semen Methods. *American Journal of Animal* and *Veterinary Sciences*. 14 (2) : 111 – 114.
[6] Susilawati, T., N. Isnaini, A.P.A. Yekti, I. Nurjanah, Errico dan N. da. Costa. 2016. Keberhasilan Inseminasi Buatan Menggunakan Semen Beku dan Semen Cair pada Sapi Peranakan Ongole. *Jurnal Ilmu – Ilmu Peternakan*. 26 (3) : 14 – 19.
[7] Susilawati, T., Suyadi, N. Ihsan, S. Wahjuningsih, N. Isnaini, A. Rachmawati, A.P.A. Yekti dan P. Utami. 2022. *Manajemen Reproduksi dan Inseminasi Buatan*. Malang: UB Press. ISBN: 978-623-296-624-6.
[8] Mahendra, H.C., D. Samsudewa, and Y.S. Ondho. 2018. Evaluation of Semen Quality of Bufallo Frozen Semen Produced by Artificial Insemination Center. *Journal of the Indonesian Tropical Animal Agriculture*. 43 (1) : 26 – 34.
[9] Susilawati, T. 2013. *Pedoman Inseminasi Buatan pada Ternak*. Malang: UB Press. ISBN: 978-602-203-458-2.
[10] Takdir, M., Ismaya dan S. Bintara. 2017. Proporsi Spermatozoa X dan Y, Viabilitas dan Motilitas Spermatozoa Domba sesudah Pemisahan dengan Putih Telur. *Buletin Peternakan.* 41 (1) : 1 – 7.
[11] Arifiantini, R.I. 2012. TeknikKoleksidanEvaluasi Semen padaHewan. Bogor: IPB Press. ISBN: 978-979-493-418-0.
[12] Komariah, I. Arifiantini dan F.W. Nugraha. 2013. Kaji Banding Kualitas Spermatozoa Sapi Simmental, Limousin dan *Friesian Holstein* terhadap Proses Pembekuan. *Buletin Peternakan.* 37 (3) : 143 – 147.
[13] Samik, A., Y. Oktanella, T. Hernawati, N.M.R. Widjaja dan I.P. Dewanti. 2014. Penambahan Osteopontin dalam Pengencer Semen Beku Sapi Perah *Friesian Holstein* Meningkatkan Ekspresi *B-Cell Cll/Lymphoma-2* Spermatozoa *Post Thawing*. *Jurnal Veteriner*. 15 (4) : 461 – 466.
[14] Santoso, M.Y.P. 2021. Korelasi Lingkas Skrotum terhadap Kuantitas dan Kualitas Semen Pejantan Sapi Perah *Friesian Holstein* di BBIB Singosari Kabupaten Malang. *Jurnal Ilmu Peternakan*. 16 (1) : 31 – 41.
[15] Yekti, A.P.A., S. Rahayu, G. Ciptadi, T. Susilawati. 2023. The Quality and Proportion of Spermatozoa X and Y in Sexed Frozen Semen Separated with *Percoll* Density Gradient Centrifugation Method on *Friesian Holstein* Bull. *Advance in Animal* and *Verterinary Science.* 11 (3) : 371 – 378.
[16] Susilawati, T. 2005. Tingkat Keberhasilan Kebuntingan dan Ketepatan Jenis Kelamin Hasil Inseminasi Buatan Menggunakan SemeBeku *Sexing* pada Sapi Peranakan Ongole. *Animal Production*. 7 (3) : 161 – 167.
[17] Susilawati, T., dan A.P.A. Yekti. 2018. *Teknologi Inseminasi Buatan Menggunakan Semen Cair (Liquid Semen)*. Malang: UB Press. ISBN: 978-602-432-553-4
[18] Situmorang, P. 2002. Pengaruh Kolesterol terhadap Daya Hidup dan Fertilitas Spermatzoa Sapi. *JITV*. 7 (4) : 251 – 258.
[19] Diliyana, Y.F., T. Susilawati dan S. Rahayu. 2014. Keutuhan Membran Spermatozoa Disekuensing Sentrifugasi Gradien Densitas *Percoll* Berpengencer Andromed dan CEP-2 yang Ditambahkan Kuning Telur. *Jurnal Veteriner*. 15 (1) : 23 – 30.
[20] Damayanti, R., A.L. Toleng dan M. Yusuf. 2017. Pengaruh Albumin Putih Telur Medium *Sexing* terhadap Motilitas, Persentase Hidup dan Abnormalitas Spermatozoa Y setelah Pembekuan Sapi Bali. *Jurnal Sains dan Teknologi*. 17 (1) : 96 – 102.
[21] Danang, D.R., N. Isnaini dan P. Trisunuwati. 2012. Pengaruh Lama Simpan Semen terhadap Kualitas Ayam Kampung dalam Pengencer Ringer's pada Suhu 4°C. *Jurnal Ternak Tropika.* 13 (1) : 47 – 57.
[22] Anwar, N. Solihati dan S.D. Rasad. 2019. Pengaruh Medium dan Lama Inkubasi dalam Proses Sexing Spermaterhadap Kualitas Semen Kambing Boer. *Jurnal Ilmu Ternak.* 19 (1) : 53 – 61
[23] Putri, R.D.A., M. Gunawan dan E.M. Kaiin. 2015. Uji Kualitas Sperm *Sexing* Sapi *Friesian Holstein* Pasca Thawing. *Prosiding Nasional Masyarakat Biodiversitas Indonesia*. 1 (8) : 2057 – 2061
[24] Hanifi, H., M.N. Ihsan, dan T. Susilawati. 2016. Pengaruh Lama Equilibrasi pada Proses Pembekuan terhadap Kualitas Semen Sapi Wagyu Menggunakan Pengencer Andromed. *Jurnal Ternak Tropika*. 17 (1) : 1 – 11.
[25] Bebas, W. dan D. I. Laksmi. 2014. Pengaruh Berbagai Konsentrasi *Dimethylsulfoxide* terhadap Kualitas Semen Beku Ayam Hutan Hijau *Post Thawing. Jurnal Ilmu Kesehatan dan Hewan*. 2 (2) : 105 – 115.
[26] Pasyah, B.I., B. Rosadi dan Darmawan. 2021. Pengaruh Penyimpanan pada Suhu 5°C terhadap Motilitas, Persentase Hidup (Viabilitas) dan Abnormalitas Semen Sapi Simmental. *Jurnal Ilmiah Ilmu – Ilmu Peternakan*. 24 (1) : 11 – 18.
[27] Mardi, I., A.P.A.Yekti, Kuswati, M. Luthfi dan T. Susilawati. 2020. Kualitas Semen Beku Sexing Sapi Perenakan Ongole Menggunakan Volume Awal Semen yang Berbeda. *Jurnal Ilmu dan Teknologi Peternakan Tropis*. 7 (3) : 238 – 346.
[28] Rahmawati, M.A., T. Susilawati dan M.N. Ihsan. 2015. Kualitas Semen dan Produksi Semen Beku pada Bangsa Sapi dan Bulan Penampungan yang Berbeda. *Jurnal Ilmu – Ilmu Peternakan.* 25 (3) : 25 – 36.
[29] Ratnawati, D.,N. Isnaini and T. Susilawati. 2018. Characteristic Motility of Liquid Semen on Ongole Crossbreed (PO), Bali, and Madura Bulls with Different Diluents at Cold Storage. *Asian Jr. of Microbiol. Biotech. Env. Sc*. 20 (1) : 21 – 28.
[30] Afiati. F. 2004. Proporsi dan Karakteristik Spermatozoa X dan Y Hasil Separasi Kolom Albumin. *Media Peternakan*. 27 (1) : 16 – 20.