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PREFACE

In this edition, Volume 30 No 1, we proudly present articles from animal and veterinary sciences including genetic, reproduction; animal physiology; and veterinary from scientist all over the world. The articles published in this edition are:
“Diversity of Myostatin Gene SNPc.267G>A and SNPc.111G>C as Candidate Genetic Markers for Growth Traits in Ongole Grade Cattle”; “Determination of STAT5A gene SNPs and their association with reproductive traits in Ongole Grade cow of Rembang”; “Comparison of Two Methods for Sperm Plasma Membrane Integrity Assessment in Frozen Murrah Buffalo Semen”; “Alternative Quantitative Digital Analysis of Agarose Gel PCR Products for Detection of Molecular Markers in Livestock”; “CSN1S1 Gene Polymorphism of Indonesian Local PE, Saanen, and Sapera Goats”; “The Effects of Canola Oil, Vitamin E, and Selenium Supplementation in the Ration on Blood Metabolites Profile and Liquid Semen Quality in Fat-Tailed Rams”; “Adapted Local Feed Supplement for African Dwarf Sheep in the Rainy Season”; and “Physiological Responses of Saanen Does: A Comparative Study of Traditional Wooden and Aluminium Galvanized Iron Housing System in a Tropical Climate”.

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully, these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from scientists to contribute their papers to the Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor

Bogor, March 2025

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LIST OF CONTENT

Diversity of Myostatin Gene SNPc.267G>A and SNPc.111G>C as Candidate Genetic Markers for Growth Traits in Ongole Grade Cattle

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ABSTRAK

Naufal SM, Noor RR, Jakaria. 2025. Keragaman gen meostatin SNPc.267G>A dan SNPc.111G>C sebagai kandidat marka genetik sifat pertumbuhan pada sapi Peranakan Ongole. JITV 30(1):1-7. DOI:http//dx.doi.org/10.14334/jitv.v30.i1.3417.

Penelitian ini bertujuan untuk menganalisis keragaman gen MSTN dan asosiasinya terhadap bobot lahir, bobot sapih, dan laju pertumbuhan pada sapi PO. Sebanyak 77 sampel darah sapi PO dari Balai Perbibitan dan Pengembangan Inseminasi Buatan Ternak Sapi Perah (BPPIB-TSP) Ciamis yang terdiri dari 38 sapi betina dan 39 sapi jantan digunakan dalam penelitian ini. Keragaman SNPc.267G>A dan c.111G>C dianalisis dengan teknik PCR-RFLP menggunakan enzim restriksi berupa HaeIII dan AluI. Frekuensi genotipe, frekuensi alel, nilai heterozigositas, dan keseimbangan Hardy-Weinberg dihitung menggunakan program PopGen32. Metode uji-*t* digunakan untuk menganalisis asosiasi keragaman dari SNPc.267G>A dan c.111G>C terhadap bobot badan. Hasil penelitian menunjukan bahwa frekuensi genotipe AA (0,38), AG (0,56), and GG (0,06) pada SNPc.267G>A dengan frekuensi alel A (0,66) dan G (0,34). Pada SNPc.111G>C, frekuensi genotipe CC (0,16), CG (0,29), dan GG (0,56) dengan frekuensi alel C (0,30) dan G (0,70). Kedua SNP tersebut tidak berada dalam keseimbangan Hardy-Weinberg. SNPc.267G>A tidak berasosiasi dengan bobot badan sapi PO jantan dan betina. SNPc.111G>C berasosiasi nyata dengan bobot sapih dan laju pertumbuhan hanya pada sapi PO jantan.Hasil penelitian menunjukkan bahwa SNPc.111G>C genotipe GG dapat dijadikan kandidat marka genetik untuk seleksi bobot sapih dan laju pertumbuhan pada sapi PO jantan.

Kata Kunci: Bobot Badan, Gen Myostatin, Sapi PO, SNP

ABSTRACT

Naufal SM, Noor RR, Jakaria. 2025. Diversity of myostatin gene SNPc.267G>A and SNPc.111G>C as candidate genetic markers for growth traits in Ongole grade cattle. JITV 30(1):1-7. DOI:http//dx.doi.org/10.14334/jitv.v30.i1.3417.

This study aims to analyze the diversity of the MSTN gene and its association with birth weight, weaning weight, and growth rate in PO cattle. A total of 77 blood samples from PO cattle, including 38 cows and 39 bulls, were obtained from the Balai Perbibitan dan Pengembangan Inseminasi Buatan Ternak Sapi Perah (BPPIB-TSP) in Ciamis. The diversity of SNP c.267G>A and c.111G>C was analyzed using the PCR-RFLP technique and the restriction enzymes HaeIII and AluI. Genotype frequencies, allele frequencies, heterozygosity values, and Hardy-Weinberg equilibrium were computed using the PopGen32 program. The t-test method assessed the association of SNP c.267G>A and c.111G>C with body weight. The results indicated genotype frequencies of AA, AG, and GG at 0.38, 0.56, and 0.06, respectively, for SNPc.267G>A, with allele frequencies of A and G at 0.66 and 0.34. For SNPc.111G>C, the genotype frequencies of CC, CG, and GG were 0.16, 0.29, and 0.56, respectively, while the allele frequencies for C and G were 0.30 and 0.70. Both SNPs were not in Hardy-Weinberg equilibrium. The SNPc.267G>A did not show an association with the body weight of male and female PO cattle. However, SNPc.111G>C was significantly associated with weaning weight and growth rate in male PO cattle. These findings suggest that the GG genotype of SNPc.111G>C may be a candidate genetic marker for selecting weaning weight and growth rate in PO bulls.

Key Words: Body Weight, Myostatin Gene, PO Cattle, SNP

INTRODUCTION

The potential impact of this research on the livestock industry is significant, as it could lead to more efficient breeding programs and enhanced meat production. Domestic meat demand can be met by utilizing the genetic resources of local livestock, including Ongole grade cattle, also known as PO cattle. The PO cattle are a local dual-purpose breed used for both meat and as working animals, making them popular among farmers and livestock keepers in Indonesia (Kusuma et al. 2017). PO cattle were developed in 1930 by breeding Javanese cattle with Sumba Ongole cattle (Rohayati and Christi 2017). The Minister of Agriculture Decree Number 2841/Kpts/LB.430/8/2012 designates the Peranakan Ongole Cattle as local livestock genetic resources to be preserved in Indonesia, recognizing PO cattle as a valuable local genetic resource that should be conserved (Kepmentan 2012). According to estimates by Adinata et al. (2022), the total population of PO cattle in Indonesia was approximately 2.8 million head in 2020, accounting for about 16% of the total beef cattle population, and they can be found in nearly all provinces. Farmers favor PO cattle due to their high adaptability to tropical environmental conditions, which supports efficient growth (Kurniawan et al. 2021). Research conducted by Ngadiyono et al. (2015) indicates that the average live weight of adult PO cattle is 363 kg, with a carcass percentage of 53.83%. This high carcass percentage suggests that PO cattle possess good economic value and muscle growth.

It is stated that muscle growth is a quantitative trait influenced by various pairs of genes and environmental factors (Hilmia et al., 2019). The myostatin gene is essential for regulating muscle development and growth traits (Konovalova et al. 2021). The myostatin gene (MSTN), also referred to as growth and differentiation factor-8 (GDF-8), is part of the transforming growth factor-β (TGF-β) family, which oversees muscle development (Chen et al. 2021). The MSTN gene is located on chromosome 2, consisting of three exons and two introns, and encodes 375 amino acids (Jakaria et al. 2021). As a result, mutations or deficiencies in the MSTN gene impact muscle growth, particularly hypertrophy, which is the excessive growth of muscle tissue (Ayuti et al. 2024). This phenomenon has been applied in livestock breeding, especially beef cattle, as it can increase their body weight.

The practical implications of this research for livestock breeders are significant, as it could provide them with valuable insights for their breeding programs. One method to increase local livestock productivity is through a breeding program for beef cattle. The breeding of PO cattle is generally still based on a phenotyping approach, which has a relatively long generation interval compared to other livestock, resulting in slow genetic improvement. Today's breeding technology includes genome mapping through Single Nucleotide Polymorphism (SNP) to determine the relationship between quantitative traits and DNA variations (Sutikno et al., 2020).

This technology will enhance the exploration of genomic data, particularly in PO cattle. The PCR-RFLP method can evaluate the diversity of polymorphism within a population. This technique was chosen because it identifies genotypes, revealing the genes that encode essential traits (Jakaria et al. 2007).

A study on the genetic diversity of MSTN related to growth traits such as birth weight, weaning weight, and growth rate has been conducted. Previous research using sequencing techniques on PO cattle identified SNPc.111G>C and SNPc.267G>A in the MSTN gene within exon 1 (Jakaria et al. 2021). However, further validation through PCR-RFLP is essential to confirm these findings and evaluate their potential as candidate genetic markers for growth traits in PO cattle. Therefore, this study employed the PCR-RFLP technique to identify and analyze the diversity of SNPc.111G>C and c.267G>A in the MSTN gene within the exon 1 region of PO cattle. The observed diversity is also linked to PO cattle's birth weight, weaning weight, and growth rate.

MATERIALS AND METHODS

Phenotypic data and blood sample collection

A total of 77 blood samples from PO cattle were obtained from the Balai Perbibitan dan Pengembangan Inseminasi Buatan Ternak Sapi Perah (BPPPIB-TSP) in Ciamis, consisting of 38 females and 39 males. The PO calves in this study were produced through artificial insemination using semen collected from bulls chosen based on their weight performance, employing a purebred mating system. The phenotypic data were sourced from records at Balai Perbibitan dan Pengembangan Inseminasi Buatan Ternak Sapi Perah (BPPIB-TSP) in Ciamis. The selection criteria for the cattle were established from their recorded birth weights, weaning weights, and the dates of these measurements. Birth weight data were recorded and measured within 24 hours postpartum. Their dams raised the calves until they reached 180 to 205 days of age to ascertain each calf's weaning weight. The calves received 10% forage and 1% concentrate at this breeding station based on their total body weight. Birth weight data were collected using a digital scale, while weaning weight was measured with a Rondo measuring tape, which was subsequently converted into body weight. Blood samples of five milliliters were drawn via the jugular vein using a 21G needle, placed into a vacutainer with EDTA, and stored in a refrigerator at 4°C until analysis.

DNA extraction and genotyping of MSTN gene using PCR-RFLP Method

A blood sample was collected for DNA isolation using a gSYNCTM DNA extraction kit from GeneAid (Taiwan) following a new blood protocol at the Laboratory of Molecular Genetics, Faculty of Animal Science, IPB University. It was stored at -20°C until analysis. Primers were utilized to amplify the MSTN gene exon 1 according to the PCR procedure specified by Jakaria et al. (2021) with access number AY794986. A 608 bp fragment was amplified using the forward primer 5′-CAA GTT GTC TCT CAG ACT GG-3′ and the reverse primer 5′-CTC CTC CTT ACA TAC AAG CC-3′. A total of 15 µL of PCR amplification mix was prepared by combining 1.0 µL of DNA isolation, 6.1 µL of nuclease-free water (Qiagen, Germany), 0.2 µL of forward primer, 0.2 µL of reverse primer, and 7.5 µL of Red Mix. The reaction was performed in the Esco Swift Maxi SWT-MX-BLC-1 Thermal Cycler (Esco Technology, Inc., USA) under the following conditions: pre-denaturation at 95°C for 1 minute (1 cycle);

denaturation at 95°C for 15 seconds (35 cycles); annealing at 59°C for 20 seconds (35 cycles); extension at 72°C for 10 seconds (35 cycles); and final extension at 72°C for 5 minutes (1 cycle). The PCR product was visualized by electrophoresis in a 1.5% agarose gel (Vivantis Inc., USA), stained with Florosafe DNA (Axil Scientific Pte Ltd., Singapore), with a 100 bp marker (Geneaid, Taiwan), and viewed using a UV transilluminator (AlphaImager; Alpha Innotech, USA).

The MSTN gene was analyzed using the PCR-RFLP method. The PCR product (608 bp) of the MSTN gene was digested at 37°C for 4 hours with two restriction enzymes: *Alu*I (AG│CT) for SNPc.267G>A and *Hae*III (GG│CC) for SNPc.111G>C, as determined by the Neb-cutter program (https://nc3.neb.com/NEBcutter/). Both *Alu*I and *Hae*III used the same reaction mix volume, which consisted of 5.0 µL of PCR product, 1.0 µL of nuclease-free water (Qiagen, Germany), 0.7 µL of enzyme buffer, and 0.3 µL of restriction enzyme. The restriction products were electrophoresed on a 2% agarose gel at a voltage of 100V for 45 minutes and then visualized using a UV transilluminator. The lengths of the DNA bands that appeared were determined by comparing them to a 100 bp DNA ladder, which enabled the determination of the DNA band length and the MSTN gene genotype.

Data analysis

Data on birth weight in PO cattle were available. In addition, several weaning weight measurements were adjusted to 205 days of age using the formulas of Jakaria et al. (2019):

$$WW205=\left[\frac{(WW\text{-}BW)}{\text{actual age}}\times 205\text{ days}\right]+BW$$

where WW205 is corrected weaning weight at 205 days, WW is actual weaning weight, and BW is birth weight. The growth rate, or average daily gain from birth to weaning in kg/day, was estimated using the following formula (Amiano et al. 2020):

$$P=\frac{WW-BW}{\text{Cattle ages}}$$

where P is weight gain (kg/day), WW is weaning weight, and BW is birth weight.

Using Popgene32 software, we conducted data analysis for genotype frequency, allele frequency, heterozygosity value, and Hardy-Weinberg equilibrium. We also performed an association analysis of SNPc.111G>C and c.267G>A diversity concerning birt h weight, weaning weight, and growth rate. Genotype and allele frequencies for SNPc.111G>C and c.267G>A were analyzed using the relevant formulas (Nei & Kumar 2000):

$$X_{ii}=\frac{n_{ii}}{N} \qquad X_i=\frac{\left(2n_{ii}+\sum_{i\neq j} n_{ij}\right)}{2N}$$

where Xi refers to the frequency of allele I, Xii refers to the frequency of genotype ii, nii denotes the number of genotypes ii, nij indicates the number of genotypes ij, and N represents the total sample size. Observed and expected heterozygosity were determined using the formulas (Eugenia & Victoria 2021):

$$H_o=\sum_{i\neq j}^{N}\frac{n_{ij}}{N} \qquad H_e=1-\sum_{i=1}^{n}P_i^2$$

where Ho denotes observed heterozygosity, He represents expected heterozygosity, N signifies the number of observed individuals, nij represents the count of heterozygous individuals, q indicates the number of alleles, and Pi denotes allele frequency. The Hardy-Weinberg equilibrium was calculated using the following formulas (Láruson & Reed 2021):

$$\chi^2=\sum\frac{(O-E)^2}{E}$$

where χ^2 is chi-square value, O: number of observed genotypes; E: number of expected genotypes. The association between SNPc.111G>C and c.267G>A genotype diversity with birth weight, weaning weight, and growth rate was analyzed using a two-sample t-test and calculated using the SAS Demand for Academic program. The mean and standard deviation of birth weight, weaning weight, and growth rate were calculated for each genotype.

RESULTS AND DISCUSSION

Description of body weight and growth in PO cattle

Table 1 presents data on birth weight, weaning weight, and growth rate of PO cattle at UPTD Balai Perbibitan dan Pengembangan Inseminasi Buatan Ternak Sapi Perah (BPPIB-TSP) Ciamis, obtained through descriptive analysis.

In this study, the mean birth weight for female and male PO cattle calves was 21.75±2.81 kg and 23.39±3.33 kg, respectively. The mean weaning weights for female and male calves were 89.89±15.08 kg and 96.81±20.56 kg, respectively. The birth and weaning of PO cattle in

Table 1. Mean and standard deviation for birth weight, weaning weight, and growth rate of PO cattle

Sex	N	Birth Weight (kg)	Weaning Weight (kg)	Growth Rate (kg/day)
Female	38	21.76±2.81	89.89±15.08	0.33±0.07
Male	39	23.39±3.33	96.81±20.56	0.35±0.09

N= number of individuals

SPR Tanjungsari, which reported values of 23.81±2.01 kg and 24.27±2.56 kg for female and male birth weights, respectively, and a weaning weight of 122.40 kg (Kurniawan et al. 2021).

Generally, male cattle weigh more than female cattle due to differences in steroid hormones, which result in faster growth rates for males (Setiyono et al. 2017). Variations in genetic potential, geographic origin, management practices, feed quality, and breeding techniques can lead to differences in livestock weaning weight and overall body size (Hikmawaty et al. 2014).

Polymorphism of SNPc.111G>C and SNPc.267G>A in the MSTN gene

The diversity of the MSTN gene was analyzed using the PCR RFLP method with *Hae*III and *Alu*I restriction enzymes, which produced three genotypes (Figure 1). Two alleles and three genotypes were identified for SNPc.267G>A after digestion with the AluI enzyme, resulting in AA, AG, and GG genotypes. The AA genotype displayed three bands with 322 bp, 261 bp, and 25 bp lengths. The AG genotype showed four bands measuring 322 bp, 199 bp, 261 bp, and 25 bp. The GG genotype exhibited four bands with lengths of 322 bp, 199 bp, 62 bp, and 25 bp. Similarly, the *Hae*III enzyme, when applied to SNPc.111G>C, generated two alleles (G and C) and three genotypes: CC, CG, and GG. The CC genotype revealed three bands with lengths of 380 bp, 173 bp, and 55 bp. The CG genotype displayed three bands measuring 380 bp, 435 bp, and 173 bp. The GG genotype presented two bands with lengths of 435 bp and 173 bp. DNA bands shorter than 100 bp may be invisible due to their short lengths.

The results of the genotype frequency, allele frequency, heterozygosity, and chi-square analysis of the MSTN gene are shown in Table 2. The diversity analysis for SNPc.267G>A revealed that the AG genotype had the highest frequency at 0.56, followed by the AA and GG genotypes with frequencies of 0.38 and 0.06, respectively. The allele frequency distribution displays a higher value for allele A compared to allele G. The highest genotype frequency for SNPc.111G>C was observed for the GG genotype, which had a frequency of 0.56, followed by the CG and CC genotypes with frequencies of 0.29 and 0.16. The distribution of the G allele exceeded that of the C allele, with respective frequencies of 0.70 and 0.30. Genotype frequency represents the ratio of a genotype in a population by comparing a particular genotype to the total population. In contrast, allele frequency indicates the ratio of an allele to the total number of alleles in that population (Noor 2010).

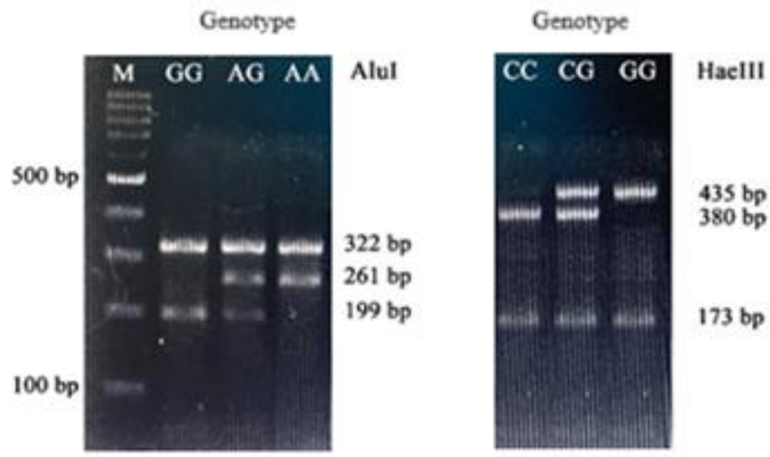

Figure 1. Visualization of PCR-RFLP for the MSTN gene exon 1 following digestion by *Alu*I and *Hae*III restriction enzymes

Table 2. Genotypic and Allelic Frequencies on c.111G>C and c.267G>A at MSTN Gene

SNP	N	Genotypes Frequencies			Alleles Frequencies		Ho	He	χ^2
267G>A	77	AA	AG	GG	A	G	0.56	0.45	4.327*
		0.38 (29)	0.56 (43)	0.06 (5)	0.66	0.34			
111G>C	77	CC	CG	GG	C	G	0.28	0.42	7.788*
		0.16 (12)	0.29 (22)	0.56 (43)	0.30	0.70			

N= Number of individual, Ho= Observed heterozygosity, He= Expected heterozygosity, χ^2= Chi-square test, χ^2 table (0.05;1)= 3.841, (*) significant differences (P<0.05)

Genetic diversity in a population is considered polymorphic when two or more alleles are present, and no single allele exceeds a frequency of 0.99, or 99 percent (Karki et al. 2015). Therefore, SNPc.267G>A and SNPc.111G>C in this study are classified as polymorphic. Extensive genetic diversity creates opportunities for selecting desirable genotypes, thus streamlining the selection process (Mardi et al. 2022).

The equality of alleles in a population, known as the Hardy-Weinberg Equilibrium, can be assessed using the chi-square (χ^2) value, which is calculated based on the differences between observed and expected genotype frequencies (Putra et al. 2017). The results of the population equilibrium analysis are presented in Table 2. The research findings indicated that SNPc.267G>A had a χ^2 value of 4.327, while SNPc.111G>C had a χ^2 value of 7.788. Both SNPs have χ^2 values greater than the critical value presented in the chi-square table, indicating that the observed frequency significantly differs ($P<0.05$) from the expected genotype frequency (Hartwig 2014). Abramovs et al. (2020) noted that several factors, including mutation, natural selection, non-random mating, genetic drift, and gene migration, can lead to deviations from Hardy-Weinberg equilibrium. However, a population can achieve Hardy-Weinberg equilibrium if mating occurs randomly, allowing allele and genotype frequencies to remain constant in the subsequent generation (Smith and Baldwin 2015). The observed heterozygosity and expected heterozygosity values serve as indicators for estimating the level of inbreeding and explaining genetic diversity within a livestock population (Agustina et al. 2021). The study results revealed that SNPc.267G>A had Ho > He values of 0.56 and 0.42, whereas SNPc.111G>C had Ho < He values of 0.28 and 0.42 (Table 2). Similar findings were reported in the study by Jakaria et al. (2021), where the average Ho and He values were 0.42 and 0.44. These Ho values suggest that the genetic variation in PO cattle is high, which is beneficial for fostering genetic development. (Chesnokov and Artemyeva 2015) explained that Ho and He values approaching 0 indicate a lack of heterozygosity, while values nearing 1 imply a more significant presence of alleles with balanced frequencies or heterozygous allele variation.

Association of the MSTN gene with body weight and growth traits

The association of SNPc.111G>C and c.267G>A in the MSTN gene with birth weight, weaning weight, and growth rate are examined. The associations of SNPc.267G>A in MSTN exon 1 with birth weight, weaning weight, and growth rate in PO cattle for both male and female calves are presented in Tables 3 and 4. In contrast, the association of SNPc.111G>C can be found in Tables 5 and 6. Table 3 indicates that female calves with the AA genotype have higher birth weight, weaning weight, and growth rate than those with the AG and GG genotypes. Conversely, male calves with the AG genotype display greater birth weight, weaning weight, and growth rate than the AA and GG genotypes (Table 4). However, the analysis shows that SNPc.267G>A in female and male PO cattle has no significant association ($P>0.05$) with birth weight, weaning weight, and growth rate.

The results of the association analysis of SNP c.111G>C diversity in female cattle presented in Table 5 indicate that cattle with the GG genotype had the lowest birth weight, weaning weight, and growth rate compared to those with the CC and CG genotypes. However, the analysis showed that the SNP c.111G>C diversity did not

Table 3. Association of SNPc.267G>A MSTN Gene on female PO calves

Trait	Genotypes (N)		
	AA (16)	AG (19)	GG (3)
BW (kg)	22.42±2.54	21.24±3.19	21.51±0.88
WW (kg)	93.38±11.30	87.96±18.16	83.45±9.43
GR (kg/day)	0.35±0.05	0.32±0.09	0.30±0.04

N= Number of individual, BW= birth weight, WW= weaning weight, GR= growth rate

Table 4. Association of SNPc. 267G>A MSTN Gene on male PO calves

Trait	Genotypes (N)		
	AA (13)	AG (24)	GG (2)
BW (kg)	22.85±2.40	23.78±3.85	22.27±0.38
WW (kg)	93.85±21.39	99.47±20.60	84.17±14.38
GR (kg/day)	0.34±0.09	0.37±0.09	0.30±0.07

N= Number of individual, BW= birth weight, WW= weaning weight, GR= growth rate

Table 5. Association of SNPc.111G>C MSTN Gene on female PO calves

Trait	Genotypes (N)		
	CC (7)	CG (8)	GG (23)
BW (kg)	22.58±1.64	21.57±1.74	21.56±3.37
WW (kg)	90.76±15.77	96.25±13.54	87.41±15.33
GR (kg/day)	0.33±0.08	0.36±0.07	0.32±0.08

N= Number of individual, BW= birth weight, WW= weaning weight, GR= growth rate

Table 6. Association of SNPc.111G>C MSTN gene on male PO calves

Trait	Genotypes (N)		
	CC (5)	CG (14)	GG (20)
BW (kg)	20.32±1.53^{a}	24.75±3.17^{b}	23.21±3.30^{ab}
WW (kg)	69.60±10.31^{a}	99.43±19.76^{b}	101.78±18.16^{bc}
GR (kg/day)	0.24±0.05^{a}	0.36±0.09^{b}	0.38±0.08^{b}

N= number of individuals, BW= birth weight, WW= weaning weight, GR= growth rate. Superscripts with different letters on the same line signify significant differences (P<0.05)

significantly differ among the three genotypes. Additionally, the results indicated a significant association (P<0.05) between SNP c.111G>C and the ability of male calves to reach weaning weight and growth rate (Table 6). Therefore, SNP c.111G>C could be a genetic marker for selecting superior bulls, significantly enhancing early growth efficiency in PO cattle. Moreover, the GG genotype exhibited a higher weaning weight and growth rate than the CG genotype. Still, there was no significant difference in body weight between the CG and GG genotypes.

CONCLUSION

The MSTN gene variation at SNPc.267G>A and SNPc.111G>C in PO cattle was polymorphic, and the allele frequencies were not in Hardy-Weinberg equilibrium. SNPc.267G>A displays three genotypic variations: AA, AG, and GG, while SNPc.111G>C has three genotypic variations: CC, CG, and GG. The diversity of SNPc.267G>A among female and male calves did not correlate with birth weight, weaning weight, or growth rate. In female calves, SNPc.111G>C was not associated with growth traits but was significantly linked to weaning weight and growth rate in males. Therefore, the GG genotype of SNPc.111G>C can be considered a potential genetic marker for selecting weaning weight and growth rate in PO bulls.

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Determination of STAT5A gene SNPs and Their Association with Reproductive Traits in Ongole Grade Cow of Rembang

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ABSTRAK

Indahwati A, Kurnianto E, Setiatin ET, Samsudewa D, Lestari DA. 2025. Penentuan SNP gen STAT5A dan hubungannya dengan sifat reproduksi pada sapi Peranakan Ongole di Rembang. JITV 30(1):8-18. DOI:http://dx.doi org/jitv.v30i1.3484.

Sapi Peranakan Ongole (PO) dikenal memiliki efisiensi reproduksi yang baik. Hingga saat ini belum dikonfirmasi melalui penelitian yang lebih mendalam terkait gen yang memiliki kontribusi besar dan dapat dijadikan sebagai penciri genetik sifat reproduksi. Gen Signal Transducer and Activator of Transcription 5A (STAT5A) diduga berperan sebagai mediator dalam jalur sinyal hormon reproduksi yang berpengaruh besar terhadap sifat reproduksi. Penelitian ini bertujuan mengetahui asosiasi antara gen STAT5A sebagai penanda seleksi sifat reproduksi *service/conception* (S/C), *calving interval* (CI), *days open* (DO), dan *estrus post partus* (EPP). Sampel penelitian adalah 80 sapi PO betina berdasarkan rangking sesuai SNI Sapi PO Bibit Betina Tahun 2015. Metode penelitian meliputi isolasi *deoksiribonukleat acid* (DNA), *polymerase chain reaction* (PCR) dan sekuensing DNA menggunakan metode *Sanger* serta menggunakan *primer* GAGAAGTTGGCGGAGATTATC (*Forward*) dan CCGTGTGTCCTCATCACCTG (*Reverse*). Hasil sekuensing didapatkan pita DNA sepanjang 820 base pairs (bp) sesuai dengan hasil *blasting primer* di NCBI pada ekson ke delapan. Analisis data menggunakan metode multivariat principal component analysis (PCA) dan non-parametrik kontingensi lamda. Hasil penelitian menunjukkan terdapat asosiasi antara gen STAT5A dengan S/C, CI, DO dan EPP pada mutasi g.482 G>A. Temuan ini menunjukkan bahwa gen STAT5A memiliki peran penting dalam meningkatkan kualitas reproduksi sebagai penanda untuk dasar seleksi sapi PO unggul di Kabupaten Rembang.

Kata Kunci: Asosiasi, Ekspresi Gen, Sapi PO Betina, Kabupaten Rembang, Parameter Reproduksi, Gen STAT5A

ABSTRACT

Indahwati A, Kurnianto E, Setiatin ET, Samsudewa D, Lestari DA. 2025. Determination of STAT5A gene SNPs and their association with reproductive traits in Ongole grade cow Rembang. JITV 30(1):8-18. DOI:http://dx.doi org/jitv.v30i1.3484.

The Ongole Grade cows are known for their good reproductive efficiency. However, it has not been confirmed through more in-depth research regarding the genes that have significant contributions and can serve as genetic markers for reproductive traits. The Signal Transducer and Activator of the Transcription 5A (STAT5A) gene is suspected to mediate in the reproductive hormone signaling pathway, significantly influencing reproductive traits. This study aims to determine the association between the STAT5A gene as a selection marker for reproductive traits service/conception (S/C), calving interval (CI), days open (DO), and estrus post partus (EPP). The sample used was 79, based on the rank that was determined according to the SNI Ongole Grade cow in 2015. The research method was carried out by isolating DNA, PCR, and DNA sequencing using the Sanger method and with primers GAGAAGTTGGCGGAGATTATC (Forward) and CCGTGTGTCCTCATCACCTG (Reverse). The sequencing results showed an 820 bp DNA band according to the results of primary blasting in NCBI in the eighth exon. Data analysis used multivariate principal component analysis (PCA) and non-parametric contingency lambda analysis. The results showed a significant association between the STAT5A gene and reproductive parameters in the g.482 G>A mutation; this indicates that the STAT5A gene is important in improving reproductive quality and can be used as a marker for selecting superior Ongole Grade cows in the Rembang Regency.

Key Words: Association, Gene Expression, Ongole Grade Cow, Rembang Regency, Reproduction Paramaters, STAT5A Gene

INTRODUCTION

Ongole Grade is widespread in Indonesia and is developing in almost every district with a high livestock population (Subiharta et al. 2012). According to the Ministry of Agriculture Decree, Ongole Grade Strain cattle in Kebumen Regency have been designated Kebumen Ongole Grade cattle (358/Kpts/PK.040/6/2015). Rembang Regency has just received the determination of the Ongole Grade Breeding Area. In Rembang Regency, the Ongole Grade population over eight years (2013–2021) decreased from 117180 heads, representing a decline from 94.03% to 91.10%. In the last two years (2021-2023), 152721 heads decreased to 33.78%. However, Ongole Grade is still in great demand as selected livestock. The population decline occurred due to suboptimal development strategies and a lack of research on practical breeding and selection programs.

Sudaryanto et al. (2018) and Lestari et al. (2023) have researched genetic diversity and phylogenetic studies of Ongole-grade cattle in Rembang Regency. The community highly values the Ongole Grade in Rembang Regency due to its good reproductive efficiency (Indahwati et al. 2021). However, this has not been thoroughly validated through in-depth research, so it is necessary to have targeted selection in qualitative, quantitative, reproductive, and genetic aspects. Information on reproductive efficiency has been carried out through research on Ongole Grade in Rembang Regency (Pratiwi et al. 2022; Panjono et al. 2022).

Reproductive efficiency is closely related to gene expression that affects reproductive potential (Pratiwi et al. 2022). The gene influencing reproductive efficiency, including conception rate (CR) and service per conception (S/C), is the Signal Transducer and Activator of Transcription 5A (STAT5A) (Cochran et al. 2013). This gene has been identified in dairy cattle (Michel et al. 2020) and beef cattle (Paramitasari et al. 2015). Observation of genes related to reproductive traits has not been carried out in Ongole Grade in Rembang Regency, so further research is needed to determine the genetic characteristics related to reproduction that are expressed phenotypically.

The main objective of the selection program in cattle farming is to enhance productivity by improving genetic quality. Utilizing molecular biology technology as a selection marker greatly assists livestock selection and development. The method to determine the molecular markers is to identify polymorphism or gene diversity. Genetic quality improvement through the selection of production and reproductive traits has traditionally been carried out using conventional methods (Prihandini et al. 2020), which require a long time to produce high quality breeding livestock. Genomic technology in developed countries has been used in cattle selection programs. It has succeeded in increasing selection accuracy, reducing the cost of lineage testing, shortening generation intervals, and making it possible to identify early undesirable recessive traits in livestock (Sudrajad et al. 2021). The discovery of genetic markers can potentially increase selection accuracy, especially economically (Sutiyono et al. 2018).

The application of molecular genetic technology to improve the genetic quality of cattle is through a selection approach based on phenotypic data information paired with genetic markers related to economic traits according to desired productivity and will be inherited (Archana 2013). Genomic selection can use genetic markers (marker assisted selection), which are significantly associated with highly economic-value livestock traits. This technology can increase accuracy, shorten generation intervals, and allow selection to be carried out earlier (Putri et al. 2021; Sudrajad et al. 2021). The expected novelty in the research is to determine the genetic characteristics expressed phenotypically (qualitatively and quantitatively), such as the STAT5A gene related to reproductive traits with qualitative and quantitative class grouping based on the Indonesian National Standard (SNI). Therefore, this study aims to determine the association between the STAT5A gene as a selection marker for reproductive traits in Ongole Grade cattle.

MATERIALS AND METHODS

Materials

Samples

The samples used in this study were 200 Ongole-grade cows for reproductive traits and whole blood samples of 80 Ongole-grade cows in Rembang Regency. The materials used were DNA isolated from blood and DNA isolation kits in the form of GeneJet from Thermo Scientific, 70% alcohol, agarose gel, tris acetate EDTA (TAE), loading dye, floro-safe DNA stain, absolute ethanol, a pair of primers, Taq polymerase Bioline fast ready mix + dye, PCR water, ddH2O, DNA ladder, and phosphate buffer saline (PBS).

Equipment

The equipment used was a syringe, cotton, EDTA tube, ice box, ice gel, centrifuge, micropipette, pipette tips, microcentrifuge tube, parafilm, label, marker, hot plate, Vortex, spin-down, polymerase chain reaction (PCR) machine, PCR tube, Erlenmeyer flask, analytical balance, plastic wrap, aluminum foil, bubble wrap, microwave, gel mold, comb, stopwatch, electrophoresis machine, and gel doc.

Methodes

Reproductive traits

A total of 200 Ongole Grade cows that have been identified have given birth. The research unit is classified into 4 SNI classes based on morphometric measurements in the study's second phase. Samples with Grade I, Grade II, Grade III, and non-grade each consist of 80, 40, 30, and 50 Ongole Grade cows. Secondary data surveys were conducted from farmers and officers, and then the rankings were ranked according to the order of S/C, CI, DO, EPP, and estrus duration (ED).

The data were analyzed using descriptive statistics and principal component analysis (PCA). The percentage of variance criteria were determined by looking at the cumulative percentage of variance, where the component with the more significant percentage of variance would be taken. The analysis showed a relationship between the studied variables and their interrelatedness.

Blood samples

The sampling technique was carried out by selecting 20 cows from each class based on the ranking (Class I, II, III, and non-class) based on Service per Conception (S/C), Calving Interval (CI), Days open (DO) and Estrus Post Partum (EPP). Blood sampling was done by cleaning the neck of the cow using cotton with 70% alcohol. Blood was taken through the jugular vein as much as 3 ml using a vacuum tube with EDTA and put into an ice box containing ice gel. The blood sample was used to obtain genomic DNA.

DNA isolation

For mammalian DNA blood samples, isolation was based on the GeneJET™ isolation kit protocol (Thermofisher ScientificTM). Blood samples were taken with a tube containing EDTA. Mixing 200 µl of blood, inserting an Eppendorf tab with 20 µl of proteinase and 400 µl of K solution using a micropipette, then vortexing/pipetting at a speed of 1500 rpm. The mixture was incubated at 56^0 C, then Vortex occasionally for 10 minutes or a shaking water bath, rocking platform, or thermomixer was used until the cells were completely lysed. Then 200 µl Ethanol (96–100%) was added and mixed by pipetting or vortexing. Transfer the prepared lysate to a GeneJET™ Genomic DNA Purification Column inserted in a collection tube. The column was centrifuged at 6,000 × *g* for 1 minute. Discard the collection tube containing the flow-through solution, and place the GeneJET™ Genomic DNA Purification Column into a new 2 mL collection tube. Then 500 µl of wash buffer I and 30 ml of ethanol were added and centrifuged at 12,000 rpm for 1 minute. The liquid was discarded, and the purification column was put back into the collection tube. Wash buffer II 500 and 30 ml ethanol were added to the GeneJET™ Genomic DNA Purification Column, centrifuged at 12,000 rpm for 3 minutes, and discarded to a sterile 1.5 mL microcentrifuge tube. Elution buffer 200 was added to the center of the GeneJET™ Genomic DNA Purification Column membrane to elute genomic DNA, incubated for 2 minutes at room temperature, and centrifuged for 1 minute. The filter was removed and stored on a shelf at an optimum temperature of 56ºC. The results of DNA isolation are stored at -20ºC and can be known by conducting qualitative tests using the electrophoresis method with agarose gel.

Polymerase Chain Reaction

The primers used for the STAT5A gene sequencing stage (Katib et al. 2008) were in the sequence GAGAAGTTGGCGGAGATTATC (Forward) and CCGTGTGTCCTCATCACCTG (Reverse) in the eighth exon with a base pair size of 820 base pairs (bp). Primer genes in bovines were designed based on sequences from the gene bank using BLAST. PCR mixture including 3µl DNA template, 25µl PCR Master Bioline MyTaq HS Red Mix, 1µl each forward and reverse primers, and 20µl PCR water was put into a PCR tube. Samples were inserted into the PCR machine with the following conditions: initial denaturation stage at 95ºC for 1 minute, denaturation stage at 95ºC for 15 seconds, annealing stage at 51.8ºC for 15 seconds, and extension stage at 72ºC for 55 seconds. The results of PCR analysis were stored in a freezer at -20ºC.

Elektrophoresis

Electrophoresis was performed after DNA isolation and PCR analysis. The results of DNA isolation, as much as 4 µL were added with 1 µl of loading dye and inserted into the wells on a 1% agarose gel using a micropipette. Electrophoresis was performed at a voltage of 100 volts for 30 minutes. Electrophoresis of the PCR analysis results was performed with a DNA ladder. The DNA ladder was inserted into the end well, while the results of the PCR analysis were inserted into the well after it. Electrophoresis was performed at a voltage of 100 volts for 30 minutes. The electrophoresis results were visualized using UV light on the Gel Documentation System (GDS).

DNA sequencing

Sample sequencing was conducted at PT using the Sanger method. Sanger sequencing uses oligonucleotide primers to amplify specific DNA regions. The sequencing stage begins with the denaturation of double-stranded DNA. Then, the oligonucleotide primers attach to the single-stranded DNA and are extended using a mixture of deoxynucleotide triphosphates (dNTPs).

Data analysis

The data analysis to identify mutations from the sequencing results used MEGA11 (Molecular Evolutionary Genetics Analysis version 11) from https://www.megasoftware.net/ (Tamura et al. 2021). Association data analysis between S/C, CI, DO, and EPP with the STAT5A gene was performed using lambda contingency analysis. The diversity of each individual's genotype was determined from the DNA bands of the genes found. Allele frequency (Xi) is the ratio of an allele to all alleles at a locus in the sample population. Gel electrophoresis or sequencing-based methods were used to determine genotype diversity, and chi-square software tools were applied for band pattern analysis. Allele and genotype frequencies are described and used to calculate Hardy-Weinberg equilibrium using chromatography from sequencing results. Each sample was compared based on the same size (marker), and the allele frequency was calculated based on the formula of Nei & Kumar (2000):

Alele frequency → $Xi = \frac{(2n_{ii} + \sum n_{ij})}{2N}$

where Xi is alele of frequency i, nii is number of genotyped individuals ii (homozygote), nij is number of genotyped individuals ij (heterozygote), and N is number of individuals.

Genotype frequency → $Xii = \frac{n_{ii}}{N}$

where Xii is frequency of genotype ii, nii is number of genotyped individuals ii, and N is number of individuals.

Statistical analysis

Statistical analysis used principal component analysis (PCA) and non-parametric Contingency Lambda. The PCA is a statistical procedure used to observe a variable that may be correlated into an uncorrelated value.

RESULTS AND DISCUSSION

Average reproductive parameters of Ongole Grade cow in Rembang Regency based on class of Indonesian nasional standard is showed in the Table 1. From these five parameters, PCA analysis was carried out to determine the relationship between the variables being studied and their interrelationships, and the results are presented in Table 2. The best reproductive parameters of Ongole Grade cows in Class I and Class II and the differentiating factors of the reproductive parameters are S/C, CI, DO, and EPP. These parameters are used in further analysis to determine their association with the STAT5A marker gene.

Identification of DNA quality

The formation of bands near the wells depicts the results of DNA isolation and PCR electrophoresis. The bright and thick bands indicate a sufficient concentration of isolated DNA. The image of the results of electrophoresis of DNA isolation is shown in Figure 1, and the electrophoresis of PCR results in Figure 2. The sequencing results show that a DNA band of 820 bp is obtained according to the results of the primary blasting at NCBI..

Table 1. The average reproductive parameters of Ongole Grade cows in Rembang Regency are based on the class of Indonesian national standards

Reproductive Traits	Class I	Class II	Class III	Non Class
S/C	1.85	1.89	1.90	1.94
CI (day)	395.57	392.04	401.56	395.69
DO (day)	115.69	114.11	122.95	124.43
EPP (day)	97.78	95.20	106.89	107.39
ED (hour)	19.49	19.11	21.19	19.85

S/C= service/conception; CI= calving interval CI; DO= days open; and EPP= estrus post partus; ED= estrus duration

Table 2. Principal componen analysis of reproductive performance of Ongol grade cow in Rembang Regency

	Component	
Reproductive Traits	1	2
S/C	0.831	-0.300
CI	0.964	0.031
DO	0.989	0.052
EPP	0.827	0.340
ED	-0.121	0.946

S/C= service/conception; CI= calving interval CI; DO= days open; and EPP= estrus post partus; ED= estrus duration

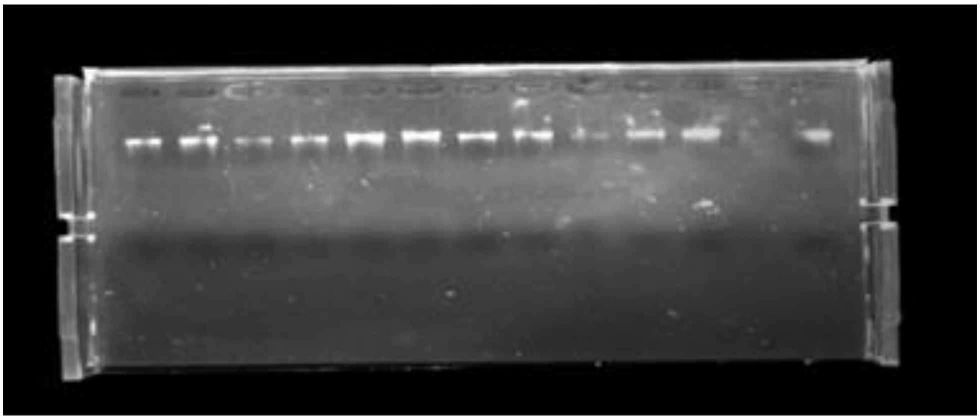


Figure 1. Electrophoresis Image of STAT5A Gene DNA Isolation Results

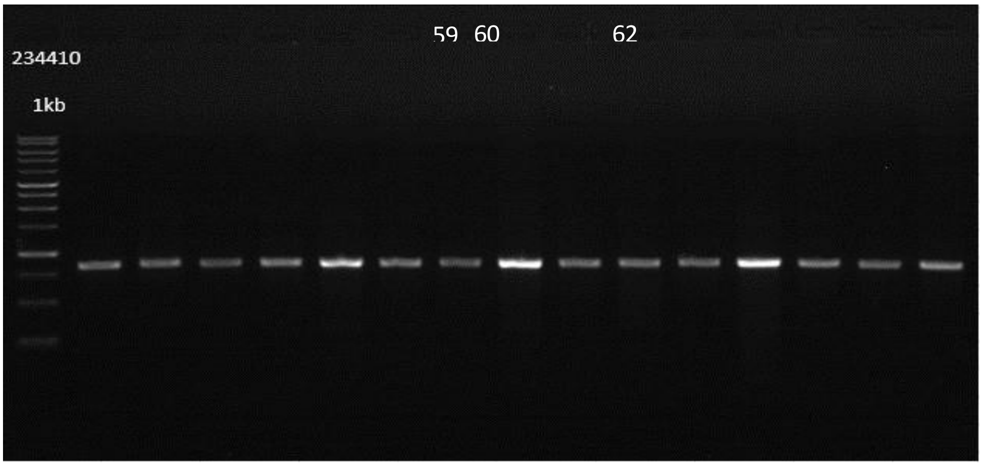


Figure 2. Electrophoresis Image of STAT5A gene PCR Results

SNPs of STAT5A gene in Ongole Grade cow in Rembang Regency

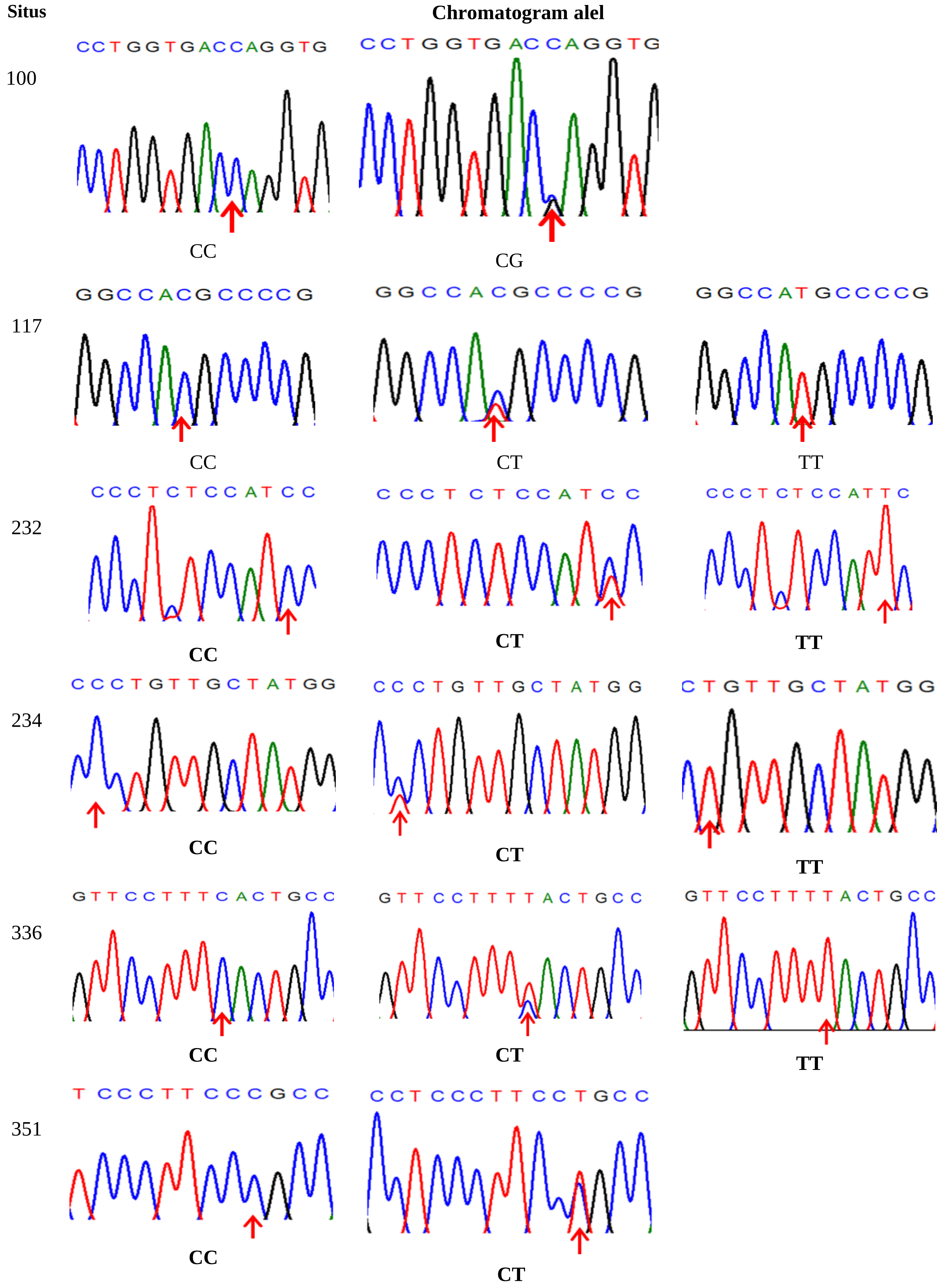
The 80 samples taken were 20 of the best from each class and analyzed for molecular test. One sample was unsuitable for further sequencing, so the sequencing data obtained in this study were 79 samples of the best Ongole Grade cow reproduction in Rembang. The results of DNA sequencing along 820 bp were alligated to 713 bp. After examining the chromatogram waves, SNP points were found at nine sites (100, 117, 232, 234, 336, 351, 372, 482, and 589) dominated by transition mutation. The mutation revealed in this study was forming 9 SNPs (g.100 C>G; g.117 C>T; g.232 C>T; g.234 C>T; g.336 C>T; g.351 C>T; g.372 T>G; g.482 G>A; g.589 C>T). At each SNP point, there are 2-3 genotypes (CC, CG, CT, GG, TG, TT, AG, and AA). Data of SNP found in the STAT5A gene is presented in Figure 3. The SNP that is associated with reproductive traits is site 482.

Genetic diversity of STAT5A gene in Ongole grade cow in Rembang Regency

The data from the observation of the STAT5A gene SNP points were tabulated for genetic diversity analysis and to determine their association with reproductive traits. The genotype and allele frequencies of the STAT5A gene are presented in Table 3.

Table 3 shows the diversity of STAT5A genes in Ongole Grade cows in Rembang Regency. Then, each allele in the analysis results has a frequency value of less than 0.95, so it can be said to be polymorphic, except the C allele in SNP g.100 C>G. This is in accordance with the opinion of Nei & Kumar (2000), which states that if one allele has a frequency of less than 99%, then the gene is said to be polymorphic or diverse; otherwise, it is said to be monomorphic or uniform.

The data in the table above showed that x^2 count $<$ x^2 table, so there is no deviation or show balance based on the Hardy-Weinberg law, except the C allele in SNP g.336 C>T. Most of the SNPs were not significantly different in the Chi-square (X^2) results, which means that the population was in HWE conditions. The HWE condition assumes that random mating occurs in the population and that no migration, mutation, or new alleles exist. The heterozygosity value in a population ranges from zero to one, indicating genetic diversity in a population. The heterozygosity value is influenced by the number of samples, alleles, and the frequency of alleles (Asmarasari et al. 2014).


Situs
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CC
CCTGGTGACCAGGTG
CG
117
GGCCACGCCCCG
CC
GGCCACGCCCCG
CT
GGCCATGCCCCG
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232
CCCTCTCCATCC
CC
CCCTCTCCATCC
CT
CCCTCTCCATTC
TT
234
CCCTGTTGCTATGG
CC
CCCTGTTGCTATGG
CT
CTGTTGCTATGG
TT
336
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CC
CCTCCCTTCCTGCC
CT

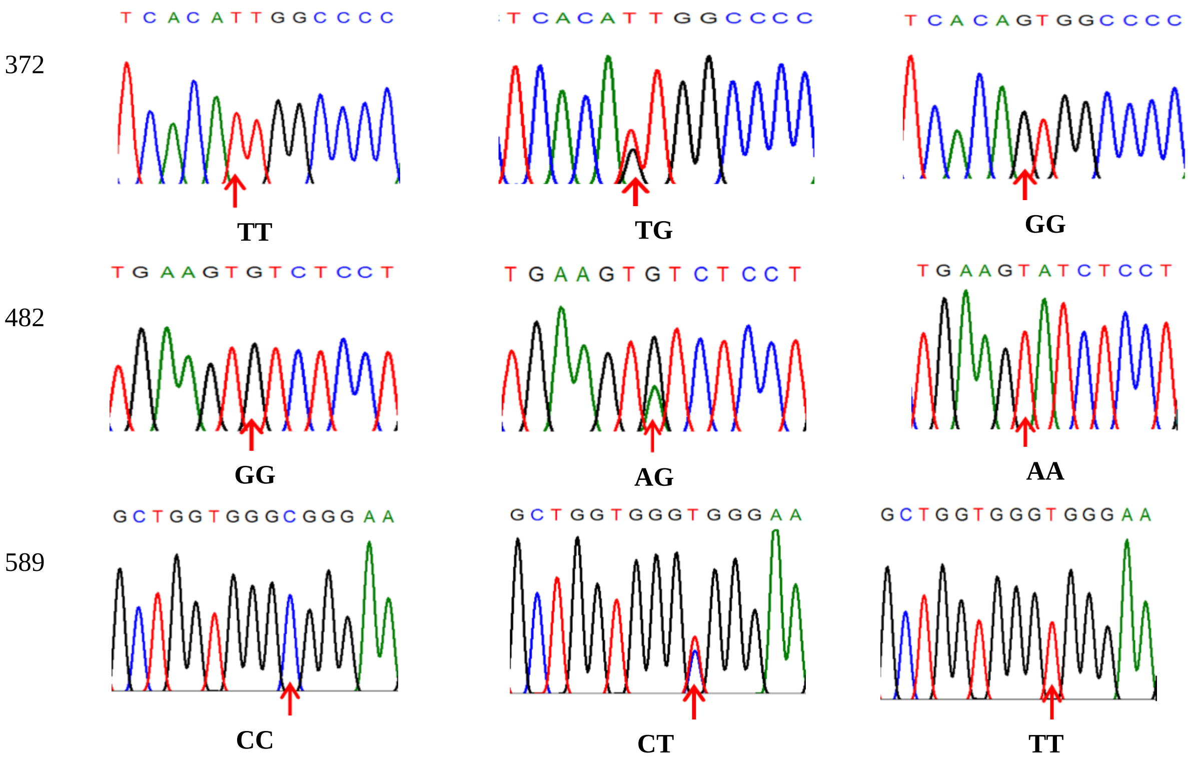

Figure 3. Chromatogram results and SNPs found in the STAT5A gene in PO cattle in Rembang Regency

Table 3. Genotype and allele frequencies of STAT5A gene SNP

SNP	Freq Genotipe (n = 79)			Freq Allele (n = 79)		Ho	He	X^2 Result	X^2 Table 0.05
	CC	CG		C	G				
g.100 C>G	0.987	0.013	0.000	0.994	0.006	0.013	0.013	0.003	5.99
	CC	CT	TT	C	T				
g.117 C>T	0.747	0.228	0.025	0.861	0.139	0.228	0.240	0.193	5.99
	CC	CT	TT	C	T				
g.232 C>T	0.671	0.304	0.025	0.823	0.177	0.304	0.292	0.138	5.99
	CC	CT	TT	C	T				
g.234 C>T	0.671	0.304	0.025	0.823	0.177	0.304	0.292	0.138	5.99
	CC	CT	TT	C	T				
g.336 C>T	0.873	0.101	0.025	0.924	0.076	0.101	0.140	6.129*	5.99
	CC	CT		C	T				
g.351 C>T	0.873	0.127	0.000	0.937	0.063	0.127	0.119	0.361	5.99
	TT	TG	GG	T	G				
g.372 T>G	0.620	0.291	0.089	0.766	0.234	0.291	0.359	2.801	5.99
	GG	AG	AA	G	A				
g.482 G>A	0.785	0.190	0.025	0.880	0.120	0.190	0.212	0.832	5.99
	CC	CT	TT	C	T				
g.589 C>T	0.785	0.190	0.025	0.880	0.120	0.190	0.212	0.831	5.99

SNP= single nucleotide polymorphism; n= number of samples. Degree of freedom is 2; X2r > X2t (0.05)= significant (*)

Most of the SNPs in the population were in HWE conditions; this may be because there is no accumulation of genotypes, selection, mutation, migration, and the same mating in the population studied so that in the population, there is a balance in the frequency of genotypes or alleles. If selection, migration, mutation, and genetic drift are not found, then the genotype frequency of a large enough population will always be balanced. The occurrence of random gamete fusion causes the genotype and allele frequencies to remain constant from the previous generation to the next generation, so it can be said that the population is in Hardy-Weinberg's law equilibrium (Yurnalis & Sarbaini 2014).

Association of STAT5A gene with reproductive traits

Based on the PCA analysis of five reproductive parameters (S/C, CI, DO EPP, and estrus duration), the results showed that the differentiating factors were S/C, CI, DO, and EPP, while the duration of estrus is not a differentiating factor. The four parameters were subjected to lambda contingency analysis to find associations between reproductive parameters in the genotype SNP sequencing results (Table 4). Lambda contingency analysis would be beneficial for finding the association or relationship between two device attributes. Each SNP can form 2-3 genotypes consisting of homozygotes and heterozygotes. For example, at site 100, there are 2 homozygous and heterozygous genotypes, while at site 117, there are 3 genotypes with 2 homozygotes and 1 heterozygous.

Based on the results of the analysis, it was found that there was an association between the STAT5A gene and the reproductive traits S/C, CI, DO, and EPP at SNP g.482 G>A with the strongest associations in GG, AG, and AA respectively; this is by the results of the study by Khatib et al. (2009), that STAT5A affects embryo survival at the development stage. The reproductive traits S/C determine the development stage and affect the value of CI, DO, and EPP. The study's results stated that mutation 153137 (G/C) in exon 8 of the STAT5A gene was significantly associated with the fertilization rate and embryo survival in embryonic compared to other SNPs tested. In other hand, no significant associations were found between reproduction traits and any of the studied polymorphisms, apart from age at first calving, for which STAT5A polymorphism (Oikonomu et al. 2011)

According to Liu et al. (2020), STAT5A is expressed in adipose tissue (lipids). Lipids play a role in synthesizing steroid hormones, including hormones that regulate reproduction, namely androgen, estrogen, and progesterone. These hormones, especially progesterone, function to maintain pregnancy. So, the association of the STAT5A gene with reproductive parameters is related to hormonal mechanisms.

The study by Paramitasari et al. (2015) showed that the STAT5A AvaI locus has a monomorphic C allele. The heterozygosity value in the STAT5A gene can be ascertained by the presence of the AvaI restriction site (C|CCGAG). SNP analysis showed that mutations in the STAT5A gene promoter region could be an early candidate for selection markers for reproductive traits in Bali cattle. Further validation studies are needed to prove the role of the STAT5A gene in genomic selection (Juniarti 2015). Selvaggi et al. (2013) stated that STAT5A is an important mediator in the lactogenic hormone response and a candidate marker for lactation traits in cattle.

Table 4. Association analysis of SNPs STAT5A gene with reproductive traits S/C, CI, DO and EPP

Mutation	Traits	P Value	Mean±SEM		
			CC (n=78)	CG (n=1)	
g.100 C>G	S/C	0.996	1.35±0.47	1.50±0	
	CI (day)	1	379.36±25.16	370.00±0	
	DO (day)	1	100.60±22.42	90.00±0	
	EPP (day)	0.991	95.77±22.19	90.00±0	
			CC (n=59)	CT (n=18)	TT (n=2)
g.117 C>T	S/C	0.968	1.36±0.49	1.36±0.49	1.00±0
	CI (day)	0.979	380.92±22.47	374.78±15.99	370.00±0
	DO (day)	0.905	102.56±24.00	94.78±15.99	90±0
	EPP (day)	0.901	98.16±24.17	88.33±12.49	90±0
			CC (n=53)	CT (n=24)	TT (n=2)

Mutation	Traits	P Value	Mean±SEM		
g.232 C>T	S/C	0.991	1.37±0.50	1.34±0.43	1.00±0
	CI (day)	0.999	379.68±25.66	379.04±25.03	370.00±0
	DO (day)	0.996	100.70±22.41	100.83±23.29	90±0
	EPP (day)	0.648	95.66±22.06	96.25±23.37	90±0
			CC (n=53)	CT (n=24)	TT (n=2)
g.234 C>T	S/C	0.991	1.31±0.50	1.34±0.43	1.00±0
	CI (day)	0.999	379.68±25.66	379.04±25.03	370.00±0
	DO (day)	0.996	100.70±22.41	100.83±23.29	90±0
	EPP (day)	0.648	95.66±22.06	96.25±23.37	90±0
			CC (n=69)	CT (n=8)	TT (n=2)
g.336 C>T	S/C	0.981	1.39±0.49	1.06±0.18	1.00± 0
	CI (day)	0.993	381.07±25.19	365.75±23.30	370.00±0
	DO (day)	0.993	102.28±25.19	87.50±19.82	90±0
	EPP (day)	0.576	96.96±22.51	86.25±19.23	90±0
			CC (n=69)	CT (n=10)	
g.351 C>T	S/C	0.435	1.36±0.48	1.32±0.42	
	CI (day)	0.502	379.80±25.82	375.40±19.20	
	DO (day)	0.391	100.87±23.39	97.70±12.99	
	EPP (day)	0.759	96.07±23.34	93.99±9.49	
			TT (n=49)	TG (n=23)	GG (n=7)
g.372 T>G	S/C	0.347	1.39±0.53	1.36±0.53	1.07±0.19
	CI (day)	0.902	381.76±28.26	376.22±20.71	371.57±4.16
	DO (day)	0.588	102.88±25.00	98.09±18.75	91.43±3.78
	EPP (day)	0.832	98.57±25.25	91.30±16.87	90±0
			GG (n=62)	AG (n=15)	AA (n=2)
g.482 G>A	S/C	<0.001	1.37±0.49 ^{a}	1.29±0.43 ^{b}	1.45±0.07 ^{c}
	CI (day)	<0.001	382.75±25.86 ^{a}	364.80±17.31 ^{b}	385.00±21.21 ^{c}
	DO (day)	<0.001	100.03±22.30 ^{a}	100.40±23.38 ^{b}	114.50±20.51 ^{c}
	EPP (day)	0.316	95.39±22.11 ^{a}	106.00±25.01 ^{b}	90.00±0 ^{c}
			CC (n=62)	CT (n=15)	TT (n=2)
g.589 C>T	S/C	0.993	1.39±0.49	1.23±0.42	1.25±0.35
	CI (day)	0.79	379.65±23.43	378.07±32.96	375.50±7.78
	DO (day)	0.375	100.27±20.97	102.00±20.08	95.00±7.07
	EPP (day)	0.186	94.84±20.47	100.00±29.28	90.00±0

S/C= service/conception; CI= calving interval CI; DO= days open; and EPP= estrus post partus; ED= estrus duration. Different superscript letters between Mean ± SEM indicate a significant difference (P<0.05)

The research of Nestor at al. (2020) stated that STAT5A polymorphism 19:42407732 was not associated with the reproductive parameters evaluated in this study of a Holstein cow herd in Mexico. Genotype AG of COQ9 polymorphism 18:25527339 was associated with lower SPC and is suggested as a molecular marker to improve Holstein cow reproductive performance in dairy herds in Mexico. A Chi-square test by Basant et al. (2022) showed that the Egyptian water buffalo population was in Hardy-Weinberg equilibrium. STAT5A gene might be a potential molecular marker for effective animal selection and breeding programs.

Sudhakar (2021) said that Significant associations were not observed in support of *STAT5A* as a marker for milk production traits in either Ongole or crossbred cattle of indicine admixture, and no reason could be found to consider this locus as a universal marker for milk production traits in indicine cattle and buffaloes. Considering the monomorphic nature of the gene in buffaloes and their higher milk fat content than bovine milk much remains to be explored regarding the underlying differences across the bovine and the bubaline species. Tina et al. (2022) stated that it is evident from this investigation that STAT5A polymorphism could be a promising indirect marker to improve milk production in crossbred cattle of Kerala.

CONCLUSION

There are 9 SNPs identified in this study, which are g.100 C>G; g.117 C>T; g.232 C>T; g.234 C>T; g.336 C>T; g.351 C>T; g.372 T>G; g.482 G>A and g.589 C>T). Most SNPs (eight of the nine) were not significantly different in the Chi-square (X2) results, meaning the population was in HWE conditions. There is an association of the STAT5A gene with the reproductive parameters S/C, CI, DO, and EPP at SNP g.482 G>A so that it can be used as a marker in the implementation of the selection of superior Ongole Grade Cow of Rembang.

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Comparison of Two Methods for Sperm Plasma Membrane Integrity Assessment in Frozen Murrah Buffalo Semen

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ABSTRAK

Rahayu JD, Purwantara B, Said S, Arifiantini RI. 2025. Perbandingan dua metode untuk penilaian integritas membran plasma sperma dalam semen beku kerbau murrah. JITV 30(1):19-27. DOI:http://dx.doi.org/jitv.v30i1.3488.

Integritas membran plasma sperma dalam semen beku sangat penting dalam menilai kualitas semen dan secara langsung terkait dengan kesuburan. Integritas membran plasma sperma dapat diuji menggunakan beberapa metode, termasuk Uji Hypo-Osmotik Swelling (HOS) Tes dan pewarnaan Carboxyfluorescein Diacetate-Propidium Iodide Staining (CFDA-PI). Penelitian ini membandingkan integritas membran plasma semen kerbau Murrah beku menggunakan HOS-Tes dan CFDA-PI. Semen diperoleh dari empat kerbau jantan, masing-masing mewakili kelompok produksi yang berbeda. Semen beku dicairkan secara, dan kualitasnya dinilai. Integritas membran plasma setiap sampel dievaluasi menggunakan metode pewarnaan Uji HOS dan CFDA-PI. Uji-T dua sampel digunakan untuk menganalisis data dan menunjukkan bahwa terdapat perbedaan yang signifikan secara statistik dalam integritas membran plasma sperma antara metode HOS-Tes (64,83%) dan pewarnaan CFDA-PI (58,97%) (P<0,05). Penilaian HOS-Tes optimal untuk efektivitas biaya, kecepatan, dan kenyamanan. Uji ini dapat diterapkan di laboratorium dan lapangan dengan kemudahan yang sama. Pewarnaan CFDA-PI sebaliknya, merupakan standar yang baik untuk akurasi dan presisi. Hal ini memberikan dasar yang komprehensif untuk menentukan kualitas semen beku.

Kata Kunci: CFDA-PI, HOS-tes, Kerbau Murrah, Membran Plasma Sperma

ABSTRACT

Rahayu JD, Purwantara B, Said S, Arifiantini RI. 2025. Comparison of two methods for sperm plasma membrane integrity assessment in frozen murrah buffalo semen. JITV. JITV 30(1):19-27. DOI:http://dx.doi.org/jitv.v30i1.3488.

The integrity of the sperm plasma membrane in frozen semen is crucial in assessing semen quality and is directly linked to fertility. The plasma membrane integrity of sperm can be tested using several methods, including the Hypo-Osmotic Swelling Test (HOS-Test) and Carboxyfluorescein Diacetate-Propidium Iodide Staining (CFDA-PI). This study compares the integrity of the plasma membrane of frozen Murrah buffalo semen using the HOS-Test and CFDA-PI. The semen was obtained from four buffalo bulls, each representing a different production batch. The straw was thawed individually, and quality was assessed. The plasma membrane integrity of each sample was evaluated using the HOS-Test and CFDA-PI staining methods. A two-sample t-test was used to analyze the data and demonstrate that there is a statistically significant difference in sperm plasma membrane integrity between the HOS-Test (64.83%) and CFDA-PI staining (58.97%) methods (P<0.05). The HOS-Test assessment is optimal for cost-effectiveness, speed, and convenience. It can be implemented in laboratory and field settings with equal ease. In contrast, CFDA-PI staining is a good standard for accuracy and precision. It provides a comprehensive foundation for determining the quality of frozen semen.

Key Words: CFDA-PI, HOS-Test, Murrah Buffalo, Sperm Plasma Membrane

INTRODUCTION

The buffalo (*Bubalus bubalis*) is one of the large ruminants with considerable potential for development in Indonesia. One of the breeds of buffalo found in Indonesia is the Murrah buffalo. The Murrah buffalo is a dairy breed that produces meat and livestock (Rajoriya et al. 2016). Buffalo breeding can be conducted through either natural mating or artificial insemination. The utilization of artificial insemination in buffaloes has the potential to enhance the genetic quality of the progeny, as it involves the use of frozen semen from selected superior bulls. In artificial insemination programs, frozen semen plays an important role in improving the genetic quality of the sperm. After freezing, a frozen semen evaluation must be carried out to ensure that the semen used for insemination is of good quality. The frozen semen used in the AI program must meet the minimum post-thaw motility (PTM) standard of 40% (SNI 2021). In addition, the semen must have optimal sperm motility, viability, intact plasma membrane and acrosome integrity, normal morphology, and high deoxyribonucleic acid (DNA) content to facilitate oocyte fertilization (Chelucci et al. 2015).

All parts of the sperm are covered by a plasma membrane, which acts as a protection against environmental changes and as a transport system for components from the inside of the cell to the outside or vice versa, maintaining the integrity of the biochemistry and structure of the sperm (Trimble & Grinstein 2015). Hence, the integrity of the plasma membrane determines sperm quality. Sperm with good plasma membrane integrity can survive, undergo complex changes in the female reproductive organs, and fertilize eggs. Damage to the plasma membrane and acrosome reduces the fertility potential of sperm (Morrell et al. 2018). Therefore, assessing active and functional plasma membrane integrity is essential for successful fertilization.

The assessment of sperm plasma membrane integrity can be performed on both fresh and frozen sperm. The most commonly employed methodology for evaluating the integrity of the sperm plasma membrane is the hypo-osmotic swelling test (HOS-Test). The fundamental premise of this methodology is the capacity of the sperm plasma membrane to undergo osmotic expansion within a hypo-osmotic milieu. The capacity to identify sperm damage is advancing with technological and scientific advancement (Moore & Hasler 2017). Fluorescent agents were initially used to assess mammalian sperm plasma membrane integrity, as delineated by Harrison & Vickers (1990). One of the dyes employed is carboxyfluorescein diacetate and propidium iodide (CFDA-PI). CFDA-PI dye is widely used in sperm quality assessment to evaluate sperm viability and membrane integrity. This dye is very effective in identifying live and dead sperm. It provides information on the integrity of the sperm membrane, which is very important in determining the ability of the sperm to fertilize an egg.

The study of Murrah buffalo plasma membrane integrity using HOS-Test and CFDA-PI staining has yet to be previously reported in the scientific literature. The HOS test can provide a good assessment of the ability of the sperm plasma membrane to function physiologically. In contrast, CFDA-PI staining provides a more comprehensive assessment of sperm viability and membrane integrity, with more quantitative and objective results. Therefore, the objective of this study was to compare the integrity of the plasma membrane of frozen Murrah buffalo sperm using HOS-Test and CFDA-PI staining. The two methods (HOST and CFDA-PI) were chosen to represent the conventional and state-of-the-art methods. The results are expected to provide more accurate integrity testing information for plasma membranes.

MATERIALS AND METHODS

All procedures in this study were approved by The Ethical Committee of the National Research and Innovation Agency (BRIN) under No: 093/KE.02/SK/05/2023.

Research time and place

The study was conducted between November and December 2023 at the Stem Cell Laboratory, located within the Genomics Building of the National Research and Innovation Agency, Bogor. It employed frozen semen samples from the Lembang Artificial Insemination Centre (AIC). The methodology for producing frozen semen adheres to the standards set forth by the Indonesian National Standard SNI 4869-2:2021.

Research procedure

Frozen semen was derived from four buffalo bulls aged between 10 and 12 years, with six batch codes selected at random, each consisting of five straws. Six batch codes were necessary to ensure the data collected from the study could be transmitted accurately and consistently. Consequently, 120 straws were utilized. The frozen semen is initially thawed using a water bath at 37°C for 30 seconds, after which it is transferred to a micro-tube and stored at 37°C for observation.

Sperm motility evaluation

The sperm motility was observed using a computer-assisted sperm analysis (CASA) system. A volume of 5 µl of semen was deposited onto an object glass and covered with a cover glass. The sperm were analyzed in four fields with a cell count of 300–400 cells using the software program Sperm Vision (Minitüb, Tiefenbach, Germany) on a warming table at 37°C.

Sperm viability evaluation

The viability of sperm was evaluated using eosin-nigrosin staining by the methodology proposed by Kumar et al. (2017). In this staining technique, live sperm remain unstained and appear transparent, whereas dead sperm are stained red. The viability of the sperm was determined by enumerating the live and dead cells across a minimum of 10 fields of view, with a minimum of 200 cells evaluated in each sample.

Acrosome status evaluation

The sperm's acrosome status was determined by staining with Fluorescein Isothiocyanate Peanut Agglutinin (FITC-PNA). The staining process was conducted in a dark room to prevent external light sources from affecting the results. A minimum of 200 cells were observed under a fluorescence microscope.

Sperm morphology evaluation

The sperm morphology was observed using the carbolfuchsin-eosin dye (William's stain), following the established methodology. The sperm morphology was observed under a microscope with 400x magnification, and a minimum of 200 normal and abnormal sperm were counted.

Hypo Osmotic Swelling Test (HOS-Test)

The HOS-Test method for assessing sperm membrane integrity is based on the methodology proposed by Agarwal et al. (2016). The HOS solution was maintained at 37°C in a water bath. A 30 µl sperm sample was added to 300 µl of HOS solution, homogenized, and incubated at 37°C. Subsequently, following a 30-minute incubation period, the integrity of the sperm membrane in frozen semen was assessed by adding 5 µl of the solution mixture to a microscope slide, which was then covered with a cover slip and observed under a microscope at 400x magnification. The sperm exhibiting intact plasma membranes exhibited a curved tail reaction. In contrast, a straight tail indicated damage to the plasma membranes. The total number of sperm was at least 500, and the number of reacted and unreacted sperm was enumerated.

Carboxyfluorescein Diacetate-Propidium Iodide (CFDA-PI)

The evaluation of sperm plasma membrane integrity with the CFDA-PI dye (Sigma Aldrich, Germany) is based on the methodology outlined by Harrison & Vickers (1990), with minor modifications. The CFDA dye stock solution was prepared by dissolving the dye in 4 mg/mL dimethyl sulfoxide (DMSO). The PI dye stock solution was prepared by dissolving the dye in 0.5 mg/mL PBS (both solutions were prepared in a dark room and stored at -20°C). A 20 µL aliquot of frozen-thawed buffalo semen was combined with 40 µL of CFDA dye and 5 µL of PI dye and incubated for 10 minutes at 37°C in the dark. Subsequently, 5 µL of the sample was transferred to a microscope slide and covered with a cover slip. The sperm were analyzed by fluorescence microscopy (Axiophot Zeiss) using excitation and emission filters at 490/515 nm and 400x magnification. The criteria were based on those set forth by Kumar et al. (2017), whereby sperm with intact plasma membranes exhibited bright green fluorescence, while those with damaged membranes displayed bright red fluorescence. The sperm exhibited green and red fluorescence, indicative of moribund sperm. A minimum of 500 sperm were observed and enumerated.

Statistical analysis

The data were tabulated and presented as mean and standard deviation. The frozen semen quality was subjected to an ANOVA (analysis of variance) analysis at the 95% significance level. The data obtained from the HOS-Test and CFDA-PI were subjected to statistical analysis using the T-test for independent samples with a confidence level of 95%. The correlation between the quality of frozen semen and the integrity of the plasma membrane of Murrah buffalo sperm was subjected to statistical analysis using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

The motility, viability, acrosome integrity, and sperm abnormalities of the Murrah buffalo frozen semen exhibited favorable quality (Table 1). No statistically significant differences were observed in motility, acrosome integrity, or sperm abnormalities of frozen semen among the buffalo bulls ($P>0.05$). The sperm motility values ranged from 57.20±2.01 to 58.78±0.92%. The data in Table 1 illustrate the quality of frozen semen from various Murrah buffalo bulls. According to the Indonesian National Standard guidelines, sperm motility in frozen semen should exceed 40%, with a maximum abnormal sperm count of 20%. The results demonstrate that sperm motility and abnormalities in this study comply with the established quality standards for frozen buffalo semen. Sperm motility is crucial for evaluating sperm fertility (Yaghoobi et al. 2022).

Abnormalities in the shape or structure of sperm, such as an abnormal head, damaged tail, or bent tail, can inhibit sperm function during fertilization. Therefore, sperm morphology evaluation is an important aspect of sperm quality analysis. The prevalence of sperm abnormalities ranged from 6.20±0.73% to 7.10±1.10%. However, the sperm abnormalities in this study were still categorized as relatively minor; thus, the samples still met the standards for insemination. The presence of a considerable number of sperm abnormalities has been demonstrated to have a detrimental impact on fertility (Matabane et al. 2017). Most abnormal sperm shapes in this study were secondary abnormalities, including circular, bent, asymmetrical, or broken tails. These abnormal conditions can inhibit the ability of sperm to move normally toward the egg. Primary abnormalities

Table 1. The mean quality of frozen Murrah buffalo semen from each bull

Name and Bull Code	Sperm Quality ± Standard Deviation (%)			
	Motilities	Viabilities	Acrosome Integrity	Abnormalities
Alex (131118)	57.20±2.01[a]	64.02±2.28 [a]	94.04±1.43[a]	7.10±1.10 [a]
Big Lake (131220)	57.97±1.46[a]	64.87±1.05 [ab]	95.37±0.68 [a]	6.66±0.77 [a]
Caesar (131321)	58.25±1.08 [a]	65.85±1.22 [b]	95.16±1.02 [a]	6.56±1.16 [a]
Millenio (131219)	58.78±0.92 [a]	65.94±0.58 [b]	95.32±0.96 [a]	6.20±0.73 [a]

[a b] Different superscripts in the same column mean a significant difference (P<0.05). A total of 120 samples were obtained from six production batches, with each batch comprising five straws

include macrocephaly, microcephaly, and pear-shaped heads; abnormalities in the spermatogenesis process may cause this. Sperm with head abnormalities are often unable to penetrate the egg membrane effectively.

The highest sperm viability was observed in buffaloes designated Caesar and Millenium, with values of 65.85±1.22 and 65.94±0.58%, respectively. The lowest viability was observed in the buffalo designated as Alex, with a value of 64.02±2.28%. Significant differences (P<0.05) were observed in sperm viability between individual Murrah buffaloes. Differences in sperm viability can be influenced by various internal factors, such as age, reproductive health, and genetics, as well as external factors, such as incorrect techniques in storing frozen semen or improper thawing, which damage sperm and reduce their viability. Nevertheless, sperm viability remains satisfactory. Viability assessment is essential in sperm analysis (Fischer et al. 2020), as it can elucidate damage to the plasma membrane, particularly in the head region, resulting in reduced sperm viability and motility (Dolnik et al. 2019).

The values for acrosome integrity ranged from 94.04±1.43 to 95.37±0.68%. No significant differences (P>0.05) were observed in sperm acrosome integrity between individual Murrah buffaloes. Acrosome integrity was rated excellent, with no significant changes in the acrosomal membrane structure. Ito and Toshimori (2016) elucidate that the sperm head is enveloped by four distinct types of membranes: the plasma membrane, the outer acrosomal membrane, the inner acrosomal membrane, and the nuclear envelope. Olexikova et al. (2019) observed that damage to the acrosome could result in the release of acrosomal enzymes, thereby reducing the sperm's fertilization capacity. As Reis et al. (2016) highlight, the integrity of the acrosome and the maintenance of its enzymes are paramount for the acrosome reaction process.

Figure 1 illustrates the results of the plasma membrane integrity test for each method. The HOS test method identifies two categories of reactions: intact membranes and defective membranes. The CFDA-PI staining method reveals three distinct categories of reactions: intact membrane, damaged membrane, and moribund. The findings of this study indicate that the HOS-Test method exhibited an average of 64.83% intact plasma membranes and 35.17% damaged plasma membranes. The CFDA-PI staining method yielded an average of 58.97% intact plasma membranes, 28.55% damaged plasma membranes, and 12.48% moribund. Table 2 demonstrates that the p-value (P<0.05) indicates statistically significant differences between the mean values of the HOS-Test and CFDA groups.

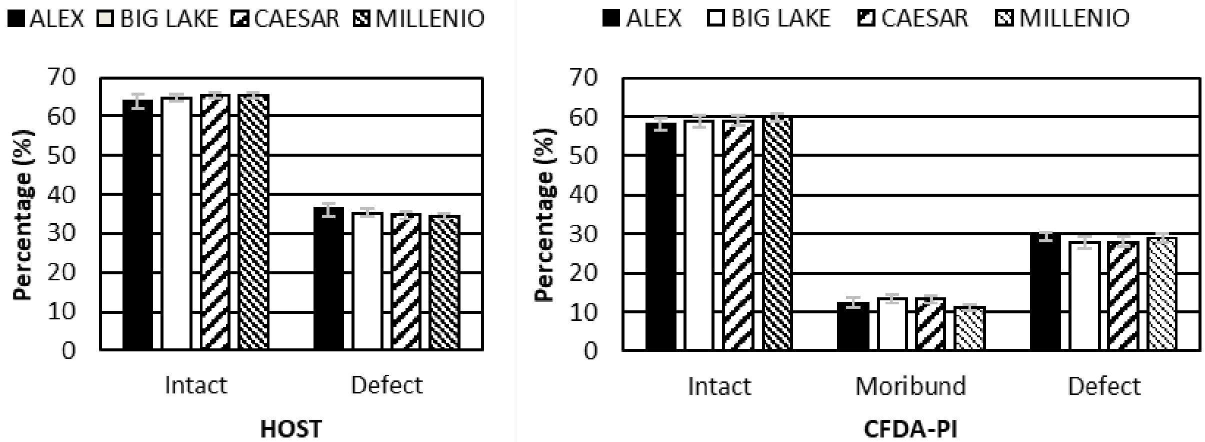

Figure 1. Comparative diagram of the average results of the plasma membrane integrity test of Murrah buffalo semen with HOS-Test and CFDA-PI. HOST: Hypo-Osmotic Swelling Test, CFDA-PI: Carboxyfluorescein diacetate-propidium iodide

Table 2. A comparative analysis of sperm plasma membrane integrity was conducted using the HOS-Test and CFDA-PI dye

Method	Mean	SD	Levene's Test for Equality of Variances		T-test for Equality of Means
			T (T-Test)	P-value	Sig. (2-tailed)
HOS-Test	64.83	1.26	1.44	0.23	0.00
CFDA-PI	58.97	1.40			0.00

HOS-Test: Hypoosmotic swelling test, CFDA-PI: carboxyfluorescence diacetate-propidium iodide. Sig. (2-tailed) indicates a significant difference (P<0.05). A total of 120 samples were obtained from six production batches, with each batch comprising five straws

The results of the sperm membrane integrity analysis, conducted using the HOS-Test method and CFDA staining, demonstrated statistically significant differences (P<0.05). It was postulated that the disparate responses of the two methods were responsible for the considerable discrepancies observed in the percentage of membrane integrity. Figure 1 illustrates that the percentage value of plasma membrane integrity of frozen Murrah buffalo semen, as determined by the HOS-Test method, is higher than that obtained by CFDA-PI staining. The results of the CFDA-PI assessment were categorized into three groups, which led to a lower value for the intact plasma membrane in this evaluation compared to the HOS-Test. In CFDA-PI staining, the condition of a deteriorating plasma membrane can be classified as intact in the HOS-Test. CFDA-PI is highly effective at identifying damage to the sperm plasma membrane. Sperm that are either damaged or dead with compromised plasma membranes were precisely identified using PI, as PI can only enter sperm that have lost membrane integrity. Sperm with intact plasma membranes but internal damage, such as DNA damage, may still show green fluorescence from CFDA, even when nearing death, highlighting this method's sensitivity to internal sperm damage.

The statistical test results indicated a statistically significant positive correlation between sperm motility and the integrity of the sperm plasma membrane, as determined by both the HOS test (r= 0.96) and CFDA-PI staining (r= 0.97). The viability of the sperm was found to be positively correlated with the integrity of the plasma membrane, as determined by CFDA-PI staining (r= 0.99). A significant negative correlation was observed between sperm abnormality and plasma membrane integrity values, as determined by HOS-Test (r= -0.98).

The integrity of the plasma membrane is vital for optimizing sperm function and survival (Bezzera et al. 2018). Damage to the sperm plasma membrane can disrupt cellular metabolism and reduce fertility due to the release of essential enzymes from the acrosome (Olexikova et al. 2019). The HOS-Test method yielded two distinct sperm responses following exposure to the HOS solution: sperm with curled tails and sperm with straight tails (Figure 2). The swelling of the sperm tail in the HOS solution indicates water transport across the membrane, which in turn demonstrates the cell's capacity to maintain equilibrium between its external and internal fluids (Ramu & Jeyendran 2013). Sperm cells in HOS solution undergo biochemical swelling, increasing their volume to achieve equilibrium between the intracellular fluid space and the extracellular environment (Rashedi et al. 2016). The fluid influx into the cell causes a volume change, causing the tail to fold (Figure 2.a).

The HOS test is a straightforward, cost-effective, and user-friendly method for evaluating the integrity of the plasma membrane in diverse species (Zeidan et al. 2018). As Nordhoff (2015) notes, a limitation of this approach is the restricted incubation period that must be allowed to obtain the optimal HOS test response. The assessment of samples using the HOS-Test method is a time-consuming process that requires careful execution. Even if the initial assessment indicates normal cellular function, it is essential to conduct additional tests to ascertain the absence of damage or evidence of infertility (Ramu & Jeyendran 2013).

The sperm examined in this study exhibited three distinct response criteria following exposure to the CFDA-PI dye. These were observed as green cells (intact membrane), red cells (dead or damaged membrane), and green-red cells (moribund). Similarly, Golher et al. (2018) reported comparable findings when evaluating the integrity of sheep sperm crossed with CFDA-PI, which exhibited three distinct response types: intact membrane, moribund, and dead. The dye CFDA can penetrate intact membranes and bind to esterases, thereby enabling the identification of living cells (Dolnik et al. 2019).

The CFDA dye can penetrate the plasma membrane and is hydrolyzed by intracellular esterases, producing fluorescent compounds that emit green fluorescence. In contrast, PI dye can only penetrate dead cells, resulting in the emission of red fluorescence. The study by Kumar et al. (2017) also demonstrated that motile sperm stained with CFDA fluorochrome exhibited widespread cytoplasmic staining while remaining unstained with PI. Given that CFDA is an enzyme-based staining method (enzyme substrate conversion to fluorescent product), and given the issue of time dependence. Figure 3(b) illustrates PI-positive cells, damaged sperm, and

Table 3. Correlation between the quality parameters of frozen semen from Murrah buffaloes

Variable (sperm)		Motilities	Viabilities	Acrosome	Abnormalities	Membrane Integrity	
						HOST	CFDA-PI
Motilities		1	0.94	0.85	-0.99**	0.96*	0.97*
Viabilities		0.94	1	0.78	-0.92	0.84	0.99**
Acrosome		0.85	0.78	1	-0.84	0.78	0.85
Abnormalities		-0.99**	-0.92	-0.84	1	-0.98*	-0.94
Membrane Integrity	HOST	0.96*	0.84	0.78	-0.98*	1	0.88
	CFDA-PI	0.97*	0.99**	0.85	-0.94	0.88	1

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level. HOST= Hypo-Osmotic Swelling Test, CFDA-PI= Carboxyfluorescein diacetate-propidium iodide. A total of 120 samples were obtained from six production batches, with each batch comprising five straws

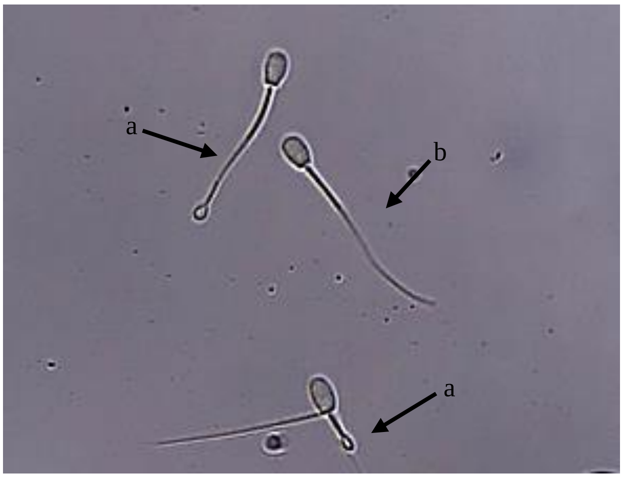

Figure 2. Membrane integrity assessment of Murrah buffalo sperm using the HOS test. Intact plasma membrane (a); defective (b)

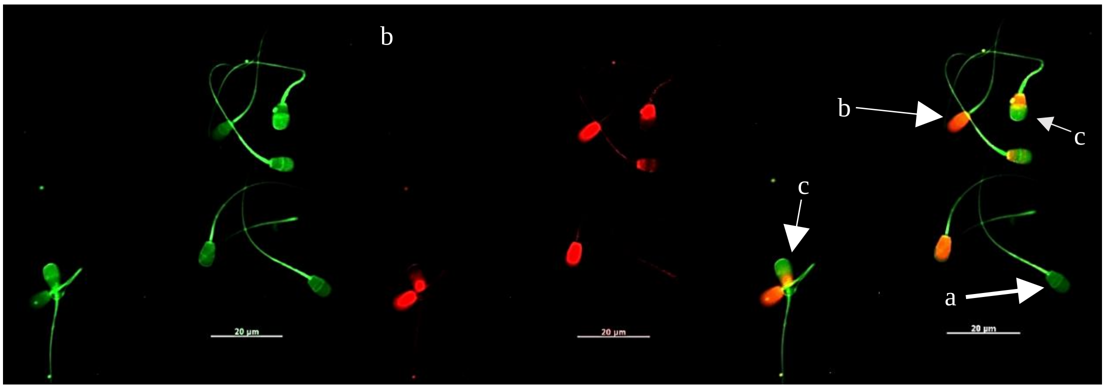

Figure 3. Membrane integrity assessment of Murrah buffalo sperm using CFDA-PI staining. intact plasma membrane (a); defective (b) moribund (c)

fluorescent red. This figure suggests that the PI dye has penetrated and stained cells with damaged plasma membranes.

Sperm exhibiting a dual coloration comprising green and red (Figure 3) are designated as moribund sperm. This phenomenon occurs due to the persistence of esterase enzyme activity within the plasma membranes of sperm cells despite the absence of metabolic activity (Kumar et al. 2017). This residual esterase activity is the underlying cause of both green and red categories among sperm cells. Davey & Guyot (2020) define moribund cells as metabolically inactive despite appearing microscopically intact. Consequently, sperm that have recently undergone apoptosis may still exhibit green coloration due to the continued activity of esterase enzymes, which can result in their misidentification as cells with intact membranes. As Singh et al. (2016) have observed, moribund sperm display compromised membrane integrity and undergo a transition from a viable state to a moribund one before eventually dying. The plasma membrane of dead sperm loses its ability to resist propidium iodide (PI), resulting in the cells appearing red.

Fluorescent probes can be employed to evaluate the structural integrity and functionality of sperm by binding to and staining specific cellular components (Dolnik et al. 2019). The principal disadvantages of CFDA-PI staining include the high cost of the requisite equipment, the complexity of the fluorescent staining process, and the time required for the subsequent evaluation. Bezerra et al. (2023) demonstrated that cells tagged with CFDA emit fluorescence briefly and then rapidly lose contrast, presenting a significant visualization challenge. Despite these limitations, fluorescence staining remains an advancing technology, and its application in assessing sperm plasma membrane integrity is expected to grow.

The integrity of the plasma membrane serves to protect the sperm's interior physically, regulate the movement of substances and ions that are crucial for metabolic processes, and maintain electrolyte balance. The loss of plasma membrane integrity and reduced sperm motility are pivotal indicators employed to distinguish between dead and living cells. Damage to the plasma membrane can result in metabolic disorders that impair both sperm motility and viability (Gaczarzewicz et al. 2015). In general, higher plasma membrane integrity is associated with improved sperm motility. A breach in the central membrane can impede mitochondrial functionality, reducing adenosine triphosphate (ATP) production and declining sperm motility (Barbagallo et al. 2020).

The results of this study indicate a positive correlation between the integrity of the sperm plasma membrane and the viability of the sperm. The integrity of the sperm plasma membrane is a critical factor influencing sperm viability, particularly in the head region (Dolnik et al. 2019). Prior research has indicated that the assessment of sperm viability may serve as an indicator of plasma membrane structure integrity (Sukmawati et al. 2014). Moreover, a negative correlation has been observed between sperm abnormalities and plasma membrane integrity, indicating that higher sperm abnormalities are associated with lower plasma membrane integrity. As Garcia-Vazquez et al. (2016) have observed, sperm morphology analysis may prove a valuable means of detecting changes related to sperm membrane integrity. Matabane et al. (2017) underscored the importance of evaluating sperm morphology and plasma membrane integrity for predicting sperm fertility.

The integrity of the plasma membrane can be evaluated using either the HOS-Test or the CFDA-PI test, according to the specific requirements and perspectives of the researcher. The HOS test is a more cost-effective and uncomplicated procedure, utilizing a straightforward instrument that can be performed in any setting. In contrast, the CFDA-PI test is a more complex procedure that requires the input of a specialist and the use of costly equipment, such as fluorescence microscopes, which are typically only available in specialized laboratories. However, this issue can be addressed by providing better training or collaborating with other laboratories. CFDA-PI is more sensitive in detecting damaged or moribund sperm than the HOS Test.

Furthermore, the HOS-Test may yield positive results for moribund sperm, which could lead to erroneous conclusions. Conversely, the CFDA-PI test is more precise, offering three distinct categories of results and exhibiting a robust positive correlation with sperm motility and viability. The discrepancy in accuracy between the two methods is approximately six percent.

CONCLUSION

This study's findings indicate notable differences between the HOS-Test and CFDA assessments of frozen Murrah buffalo semen, as evidenced by the statistical analysis. Both methods are appropriate for detecting plasma membrane integrity; however, selecting the most appropriate method can be adjusted according to the specific requirements of the situation. The HOS-Test assessment is a rapid, cost-effective, and practical method employed in laboratory and field settings. CFDA-PI staining may be employed to conduct a more accurate and precise assessment of sperm plasma membrane integrity, thereby providing a more detailed and accurate basis for determining the quality of frozen semen.

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Alternative Quantitative Digital Analysis of Agarose Gel PCR Products for Detection of Molecular Markers in Livestock

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ABSTRAK

Suyatno, Hafid A, Saputra F, Prabowo TA. 2025. Alternatif analisis digital kuantitatif produk PCR gel agarosa untuk deteksi penanda molekuler pada ternak. JITV 30(1):28-34. DOI:http://dx.doi.org/10.14334/jitv.v30i1.3448.

Metode Polymerase Chain Reaction (PCR) konvensional sudah menjadi prasyarat dalam penelitian biologi molekuler. Reaksi PCR mudah disusun dan hanya membutuhkan sebagian kecil dari urutan nukleotida target yang kompleks, sehingga PCR menjadi metode yang mudah dan akurat untuk digunakan dalam analisis biokimia dan molekuler. PCR secara umum dibedakan menjadi dua yaitu PCR kualitatif dan real-time PCR kuantitatif (RT-qPCR). Metode RT-qPCR lebih presisi namun memiliki kelemahan yaitu biayanya jauh lebih mahal dan membutuhkan peralatan lebih rumit dibanding PCR konvesional. Produk PCR divisualisasikan menggunakan elektroforesis gel agarose yang menghasikan pita. Seiring dengan perkembangan teknologi digital, pita yang dihasilkan dapat dianalisis dengan menggunakan sowftware digital yang biasa digunakan untuk menganalisis foto seperti ImageJ dari NIH. Hasil uji coba menggunakan perangkat lunak ImageJ untuk menganalisis CD44 dengan gen housekeeping β-Actin menunjukkan bahwa ekspresi gen dapat diekspresikan secara kuantitatif. Ekspresi kuantitatif CD44 dan β-Actin diperoleh dengan membandingkan persentase plot puncak CD44 dan β-Actin. Ekspresi CD44 lebih tinggi daripada β-Actin setelah dianalisis menggunakan perangkat lunak ImageJ. Hasil ini juga konsisten dengan hasil RT-qPCR yang membutuhkan peralatan dan reagen PCR yang lebih kompleks. Metode analisis hasil PCR semi kuantitatif menggunakan perangkat lunak ImageJ dapat menjadi alternatif bagi laboratorium peternakan dan veteriner yang memiliki keterbatasan anggaran penelitian dan peralatan.

Kata Kunci: Kuantifikasi Ekspresi Gen, ImageJ Software, Biologi Molekuler, RT-PCR, Laboratorium Veteriner

ABSTRACT

Suyatno, Hafid A, Saputra F, Prabowo TA. 2025. Alternative quantitative digital analysis of agarose gel PCR products for detection of molecular markers in livestock. JITV 30(1):28-34. DOI:http://dx.doi.org/10.14334/jitv.v30i1.3448.

The conventional polymerase chain reaction (PCR) method has become a prerequisite in molecular biology research. The PCR reaction is easy to prepare and only requires a small portion of the complex target nucleotide sequence, making PCR an easy and accurate method to use in biochemical and molecular analysis. PCR is generally divided into two categories: qualitative PCR and quantitative real-time PCR (RT-qPCR). The RT-qPCR method is more precise but has the disadvantage that it is much more expensive and requires more complicated equipment than conventional PCR. PCR products were visualized using agarose gel electrophoresis, which produced bands. Along with the development of digital technology, the resulting bands can be analyzed using digital software commonly used to analyze photos, such as ImageJ from the NIH. The trial results using ImageJ software to analyze CD44 compared to the housekeeping gene β-Actin demonstrated that gene expression can be quantified. Quantitative CD44 and β-Actin expression measurements were obtained by comparing the percentage of their respective peak plots. Analysis showed that CD44 expression was higher than β-Actin when evaluated with ImageJ software. These findings align with RT-qPCR results, which require more advanced PCR equipment and reagents. The semi-quantitative PCR analysis method using ImageJ offers a practical alternative for livestock and veterinary laboratories with limited budgets and resources.

Key Words: Gene Expression Quantification, ImageJ Software, Molecular Biology, RT-PCR, Veterinary Laboratories

INTRODUCTION

Genetic material consists of DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid), which are substances that control all body activities. The role of DNA and RNA is what encourages researchers to develop methods of detecting genetic material for various purposes. In 1983, a biochemist named Kary Mullis discovered the Polymerase Chain Reaction (PCR) technique, which became the basis of modern PCR that is developing today (Bartlett et al. 2003). PCR is a technique of multiplying a small number of specific nucleotide sequences in the DNA of a complex organism in vitro so that it is ready for analysis (Kubista et al. 2006). The PCR reaction is easy to set up. It only requires a small portion of the target nucleotide sequence, making PCR an easy, cheap, and accurate method for biochemical and molecular analysis (Green

& Sambrook 2019). The PCR method has been widely used in forensic analysis (Morling 2009; Gibson-Daw et al. 2018), food technology (De Medici et al. 2015; Chapela et al. 2015), medical diagnostics (Zauli 2020; Ai et al. 2020), livestock and veterinary diseases (Hewajuli et al. 2014; Hamond et al. 2014; Knapp et al. 2014; Kishimoto et al. 2017) and various research interests in the field of molecular biology. PCR has also become popular since it was used as a standard test for diagnosing SARS-CoV-2, the cause of COVID-19, which was designated by WHO as a pandemic (Waller et al. 2020; Zhu et al. 2020).

PCR is generally divided into two categories: qualitative PCR and quantitative real-time PCR (RT-qPCR). In principle, qualitative and quantitative PCR are the same process; the only difference is the interpretation of the results. In qualitative PCR, only positive or negative results, as indicated by bands on the agarose gel electrophoresis, result when read on a UV transilluminator. RT-qPCR shows positive or negative results and can also show how much DNA or genes are present in the sample (Garibyan and Avashia, 2013). Various quantitative parameters make the RT-qPCR method more precise (Kralik & Ricchi 2017). However, RT-qPCR has the disadvantage that it is much more expensive than conventional PCR and requires more complicated equipment than conventional PCR.

The results of the analysis of PCR products using agarose gel electrophoresis when read in a UV-transilluminator are in the form of a band image of the target gene. The strength and weakness of the resulting band indicate the target gene's expression. Therefore, along with the development of digital technology, there is potential to analyze the bands produced using digital software commonly used to analyze photos, such as ImageJ (Rueden et al. 2017). However, there are still very few references to using this software, especially in Indonesia's veterinary and livestock fields. This article will discuss and demonstrate the use of ImageJ software for gene expression analysis of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) results. This method can be an alternative for laboratories in developing countries with limited research budgets, especially livestock and veterinary laboratories.

MATERIALS AND METHODS

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

According to the manufacturer's instructions, RNA was isolated from ovine testicular cells using Agilent RNA Isolation Kits (Agilent, USA). All reagent preparations are set on ice. Reverse transcriptase reactions were performed by adding ReverTra Ace (Toyobo, Japan) to the RNA solution. The mixture was incubated for 10 minutes at 30°C, 60 at 42°C, and 5 at 99°. Standard PCR reactions will be performed at 1000 ng cDNA per 20 µL PCR reaction mixture. PCR products will be separated and visualized on a 2% (b/v) agarose gel containing ethidium bromide. The visualization results of PCR products in agarose gel are then analyzed quantitatively using ImageJ software (NIH).

Visualization of PCR products in agarose gel using ImageJ Software (NIH)

Image-J software to analyze PCR results begins with downloading ImageJ software from NIH that is compatible with the computer operating system. Extract the zip file and find the ImageJ image icon in the folder. Import the agarose gel image to be analyzed by opening the file menu and selecting the file in the saved folder; the image must be converted into an 8-bit image first. The next step is to reduce the background or noise in the agarose gel image, the yellow arrow points to the background or noise that needs to be removed before quantification. How to reduce background or noise by clicking on the rectangle tool and selecting the background or noise to be removed, then clicking analyze and set measurements, then selecting a mean gray value, all and all other options can be deselected, and then clicking okay. Again, click the analyze and measure dialog box, showing the mean gray value of the background or noise. Select the yellow rectangle box from the agarose gel image, click process math, subtract, enter the mean gray value of the background or noise, and click OK. The next step is to invert the image so that the gel band in the image appears black on a white background. Select the rectangle tool again, then draw a rectangle on the gel band. Click analyze gel, select the first lane dialog box, and confirm if the bands are arranged horizontally. Click yes to continue if the gel band is in the horizontal position. Now, the rectangle box on the gel is marked as one. Again, click analyze gel and plot lanes. Next, select the line tool and draw a straight line connecting the bottom ends of the plotted lanes. This step displays the area occupied by the bands on the gel image. Once done, click inside the plot. Again, click on analyze gel and label peaks; this step displays the percentage intensity of the gel, then copy the entire result and paste it into an Excel file. Next, repeat the previous procedure for β-Actin gene housekeeping in the same way to calculate the intensity of the percentage area. After the results are obtained, copy and paste the entire data into the Excel file again. To measure the normalized intensity of the target gene gel band against the housekeeping gene, use the formula of the percentage intensity of the area obtained by the target gene divided by the percentage of the housekeeping gene, and then the resulting value can be plotted on a graph so that the densitometric analysis of the agarose gel band has been completed.

Quantitative Real-Time PCR (RT-qPCR)

Complementary DNA (cDNA) from the previous RT-PCR reaction was then used in quantitative PCR (RT-qPCR) to determine CD44 gene expression levels on the Rotor-Gene Q (Qiagen, USA), utilizing the SsoFast EvaGreen Supermix (Biorad, USA). The sequence of the β-Actin is as follows: Forward: 5'-TCCCTGGAGAAGAGCTACGA -' 3 and Reverse: 5'-ACATCTGCTGGAAGGTGGAC -3'. The sequence of the CD44 is as follows: Forward: 5'-CGGATACCAGAGACTACGGC -'3 and Reverse: 5'-CCGCATAGGACCTGAGGTTG -3'. The RT-PCR master mix was made with a total volume of 20 µL with the following composition: SsoFast EvaGreen Supermixes (10 µL), 10 µM forward primer (0.4 µL), 10 µM reverse primer (0.4 µL), cDNA template (2 µL) and Nuclease Free Water (7.2 µL). A two-step amplification program was used with pre-denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 5 seconds) and annealing (58°C for 15 seconds). The amplification of both the target and reference genes was performed in triplicate. The relative quantification of target gene mRNA was determined using the comparative CT method based on the difference in cycle threshold (ΔCT) values between the target and reference genes.

RESULTS AND DISCUSSION

Conventional polymerase chain reaction (PCR) should be a prerequisite in molecular biology research. However, the data obtained can only be interpreted as "positive" (detectable) or "negative (not detectable)". Conventional PCR is an endpoint assay where the amplified PCR product (amplicons) can only be detected at the end of electrophoresis using ethidium bromide (EtBr) and other nucleic acid dyes. Quantitative PCR (qPCR) is a more sensitive assay. However, qPCR is still not a standard test tool in most veterinary laboratories and, most importantly, in less developed countries where access to real-time PCR technology is limited or non-existent.

PCR visualization on an agarose gel

Figure 1 a) shows the visualization of one of the bands on the agarose gel with background/noise in the green box. This background/noise can be removed by performing the "subtract" step described in the materials and methods. Meanwhile, Figures 1.b) and 1.c) show the results of the subtract and invert image process using ImageJ software from the expression of the CD44 gene

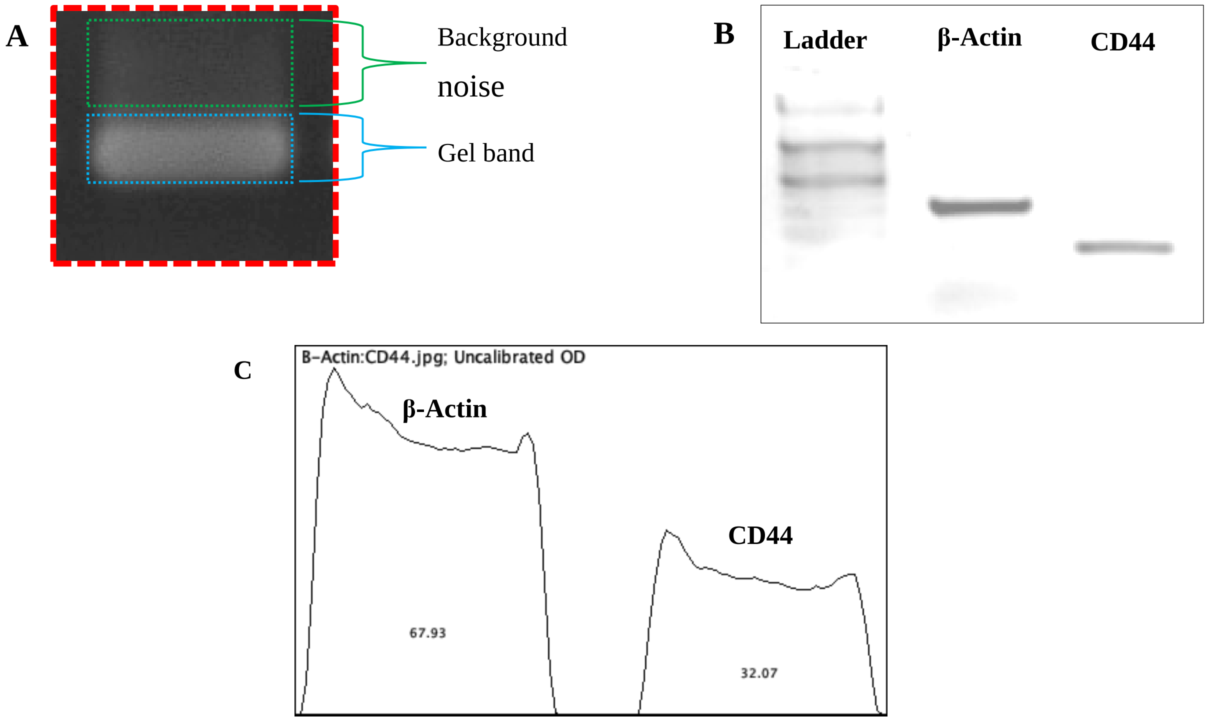

Figure 1. Visualization of RT-PCR results on agarose gel: a) gel band image and the resulting background/noise; b) the result of the subtract and invert image process using ImageJ software from gene expression of CD44 as the target gene and the β-Actin gene as the housekeeping gene; c) plot peak of optical density with Image-J software to determine the strength and weakness of gene expression from RT-PCR results of agarose gel electrophoresis

as the target gene and the β-Actin gene as the housekeeping gene. Visually, the expression of the CD44 gene in Figure 1.b shows that sample 2 has more thickness than the other samples.

In general, the PCR stage starts with the denaturation of DNA templates by heat, annealing, and extension, which repeatedly occurs in an enzymatic reaction with a DNA polymerase catalyst (Green & Sambrook 2018). The results of this PCR process need to be analyzed further to be interpreted. PCR products can generally be detected by two methods, including: 1) staining PCR products with chemical dyes such as ethidium bromide or 2) labeling PCR primers or nucleotides with fluorescent dyes (fluorophores) before PCR amplification (Garibyan & Avashia 2013). After staining, agarose gel electrophoresis is the simplest and easiest method to visualize and analyze PCR products. Electrophoresis separates charged molecules in an electric field (Fatchiyah et al. 2011). With this method, PCR products are distinguished based on their size, which is then compared with the visualization of the control (ladder).

Conventional PCR tests are now available in most veterinary pathology laboratories. However, the data obtained can only be interpreted as "positive" or "negative (not detected)." With the development of digital technology, there is potential to analyze the bands generated using digital software commonly used to analyze photographs, such as ImageJ (Rueden et al. 2017). Some other image processing tools include Matlab (Fan & Quake 2007; Zhu et al. 2014; Dimov et al. 2014) and LabView (Zhong et al. 2011).

Densitometric analysis of agarose gel electrophoresis results using ImageJ

ImageJ is a pioneer software for scientific data analysis in the form of images developed by the US National Health Institute (NIH) since 1987 (Schneider et al. 2012). ImageJ has been widely used for scientific data analysis, such as PCR (Lee & Back 2017; Chung et al. 2019), immunocytochemical staining (Das et al. 2014; Lee et al. 2015), immunoblotting (Jiao et al. 2014; Gallo-Oller et al. 2018), and colony counting in cell culture (Guzmán et al. 2014; Choudhry 2016). ImageJ provides an alternative low-cost PCR solution, especially for developing countries with limited RT-qPCR equipment. In this paper, the use of ImageJ software to analyze electrophoresis images has been demonstrated. The target gene analyzed was CD44 with the housekeeping gene β-Actin. The CD44 gene encodes a cell-surface glycoprotein pivotal in numerous biological processes, including cell adhesion, migration, proliferation, and signaling (Laohavisudhi et al. 2022). CD44 plays crucial roles in immune responses, wound healing, and cancer biology, often overexpressed in tumors and contributing to cancer stem cell maintenance, metastasis, and drug resistance(Laohavisudhi et al. 2022; Shen et al. 2022). The dysregulation of CD44 is linked to various diseases, including rheumatoid arthritis, cancer, and infectious diseases. CD44 serves as a valuable biomarker for cancer prognosis and a potential therapeutic target in oncology and autoimmune conditions. The activation of the CD44 receptor with its major ligand hyaluronan has been shown to promote breast cancer metastasis to the liver (Ahmad et al. 2023). The β-Actin gene encodes β-Actin, a key cytoskeletal protein that maintains cell structure, motility, and intracellular transport. In mammals, β-Actin is highly conserved and ubiquitously expressed in almost all cell types, playing a critical role in processes such as cell division, signaling, and adhesion. Due to its stable and consistent expression under normal conditions, β-Actin is widely used as a housekeeping gene in molecular biology experiments such as quantitative PCR and Western blot (Bustin 2000).

The rapid development of diseases affecting the veterinary community, both animals and humans, requires accurate and rapid diagnostic methods. RT-qPCR has become a standard method of diagnosis in veterinary laboratories (Toohey-Kurth et al. 2020). The method described in this study is not intended to replace the qPCR method but to be an alternative to quantitative assessment of low-cost PCR products, especially for veterinary laboratories or livestock laboratories that have limited facilities and budgets. This method can also be used as an early indicator of possible trends that may exist across the evaluated specimens to make quick empirical decisions on the choice of specimens for further PCR testing. With the growing number of emerging pathogens, some of which are zoonotic, this method can also be used as an initial identification for faster and more precise follow-up testing decisions on possible new disease outbreaks that will develop either in animals or, if possible, zoonotic.

Table 1. Relative expression of CD44 to B-Actin as determined by optical density (OD) Image-J software

No	Genes	Area (OD)	Percent
1	B-Actin	34252.85	67.93
2	CD44	16171.18	32.07
Relative expression			0.47

Table 2. Expression level of CD44 as determined by RT-qPCR

No.	Sample	β-Actin	CD44	ΔCt CD44
1.	Sample T (control)			
	R1	18.23	28.71	10.48
	R2	18.04	28.59	10.55
	R3	20.27	28.37	8.10
	Average ΔCt CD44 T (control)			9.71
2.	Sample P1			
	R1	15.37	23.56	8.19
	R2	15.57	20.94	5.37
	R3	16.65	21.89	5.24
	Average ΔCt CD44 P1			6.27
	Fold Difference			10.88

Quantitative analysis of CD44 gene expression using RT-qPCR

Quantitative Real-Time PCR (qRT-PCR) is a powerful technique that allows researchers to quantify gene expression levels in biological samples accurately. This study focuses on the analysis of the CD44 gene, with the β-Actin gene serving as the housekeeping gene for normalization. The analysis begins by measuring each sample's Ct (cycle threshold) values for CD44 and β-Actin. The difference between these Ct values, known as ΔCt, is then calculated to determine the relative expression of CD44 within each sample (Garcia and Ma 2005). To assess changes in CD44 expression between samples or conditions by comparing the ΔCt value of the target sample to that of a control sample, resulting in the ΔΔCt value. The final step involves calculating the relative fold change in expression using the formula 2^(-ΔΔCt) (Garcia & Ma 2005; Kishore et al. 2013; Arya et al. 2017).

The Cycle Threshold (Ct) value indicates the cycles at which the fluorescence signal amplification exceeds a certain threshold to be considered significant (Kubista et al. 2006; Hahn & Lapaire 2013; Kralik and Ricchi 2017). In real-time PCR (qPCR), the Ct value determines the initial target DNA or RNA amount in a sample. A low Ct indicates that the target DNA or RNA is present in large quantities at the beginning, so the signal is detected more quickly, while a high Ct indicates a low amount of target because it requires more cycles to reach the threshold of detection (Kubista et al. 2006; Forootan et al. 2017). The data presented in Table 2 shows that the Ct value of β-Actin is consistently lower than that of CD44 across all samples; this indicates that β-Actin expression is higher than CD44, aligning with the results obtained through ImageJ analysis. Further analysis using ΔCt and ΔΔCt reveals that the relative fold change in CD44 expression between the control and sample P1 is 10.88.

CONCLUSION

The trial results using ImageJ software to analyze CD44 compared to the housekeeping gene β-Actin demonstrated that gene expression can be quantified. Quantitative CD44 and β-Actin expression measurements were obtained by comparing the percentage of their respective peak plots. Analysis showed that CD44 expression was higher than β-Actin when evaluated with ImageJ software. These findings align with RT-qPCR results, which require more advanced PCR equipment and reagents. The semi-quantitative PCR analysis method using ImageJ offers a practical alternative for livestock and veterinary laboratories with limited budgets and resources.

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CSN1S1 Gene Polymorphism of Indonesian Local PE, Saanen, and Sapera Goats

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ABSTRAK

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CSN1S1 (alpha-s1 casein) gen merupakan salah satu dari empat gen utama pengontrol produksi kasein susu. Variasi genetik pada gen ini diketahui mempengaruhi kualitas protein susu kambing perah. Studi ini mempelajari polimorfisme genetik dari gen CSN1S1 menggunakan metoda *direct sequencing* pada kambing lokal Peranakan Etawah (PE), eksotis Saanen, dan Sapera sebagai kambing persilangan keduanya. Sampel darah dikumpulkan dari induk betina pada kambing Sapera 66 ekor, kambing Saanen 15 ekor, dan kambing PE 14 ekor dari Balai Penelitian Ternak di Bogor, Jawa Barat. Program MEGA11 digunakan untuk menganalisis data sekuen gen CSN1S1 pada fragmen DNA ekson 12 dan parsial intron 12. Paket program Popgen 3.2 dipakai untuk menganalisis frekuensi alel dan genotipe, serta nilai keseimbangan Hardy-Weinberg (H-W), dan tingkat heterosigositas (Ho). Teridenditikasi ada empat SNP: g.10243 G>A, g.10250 A>G, g.10277 G>A, dan g.10283 T>G. Berdasarkan frekuensi alel dan genotipe, nilai keseimbangan H-W dan tingkat Ho menunjukkan bahwa gen CSN1S1 pada keempat SNP tersebut bersifat polimorfik untuk ketiga genotipe kambing. SNP teridentifikasi dapat dipertimbangkan sebagai kandidat penanda seleksi kualitas protein susu. Penelitian ini memberikan wawasan tentang keragaman genetik gen CSN1S1, sebagai dasar seleksi molekuler untuk meningkatkan kualitas protein susu pada PE lokal dan persilanganny.

Kata Kunci: Gen CSN1S1, Kambing Perah , SNP

ABSTRACT

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CSN1S1, or alpha-s1 casein, gene is one of milk's four significant casein-controlling genes. Genetic variations in this gene have been proposed to affect milk protein quality in dairy goats. This study investigated the genetic polymorphism of the CSN1S1 gene using a direct sequencing method in local Peranakan Etawah (PE), exotic Saanen, and their cross or Sapera goats. Blood samples were collected from 66 Sapera, 15 Saanen, and 14 PE does at the Indonesian Research Institute for Animal Production (IRIAP) in Bogor, West Java Province. The MEGA11 program was used to analyze the sequence data of the CSN1S1 gene in DNA fragment exon 12 and partial intron 12. A Popgen 3.2 packet program was applied to analyze allele and genotype frequencies, Hardy-Weiberg (H-W) equilibrium, and the degree of heterozygosity (Ho) values. Four SNPs were identified: g.10243 G>A, g.10250 A>G, g.10277 G>A, and g.10283 T>G. Based on the allele and genotype frequencies, H-W equilibrium, and Ho values, the CSN1S1 gene at the four loci was polymorphic in all three goat genotypes. These SNPs may be considered candidate selection markers for milk protein quality. This study provides insights into the genetic diversity of CSN1S1, which is crucial for molecular selection to improve milk protein quality in local PE and its crosses.

Key Words: CSN1S1 Gene, Dairy Goat, SNP

INTRODUCTION

The Indonesian community currently consumes approximately 4.3 million tons of milk; however, only 22% of this amount comes from domestic production, primarily cow milk. Although cow milk production is significantly higher, dairy goat milk presents a viable development opportunity owing to lower production costs and consumer benefits (Hammam et al. 2022). As the market trend for goat milk consumption grows, production has significantly increased worldwide. Compared to cow milk, goat milk has more important nutrients, including protein, fat, calcium, phosphorus, iron, vitamin A, and several B vitamins. However, it contains less lactose, which is advantageous for those intolerant to lactose (Nayik et al. 2022). The chemical structure, protein structure, and amino acid composition of goat milk decrease the probability of allergies. Furthermore, it is easier to digest, especially in patients with gastrointestinal disorders, because of its smaller fat globules and higher levels of short- and medium-chain fatty acids (Stergiadis et al. 2019).

Genetic and breeding practices substantially influence animal milk production's quantitative and qualitative aspects. Higher milk production can be achieved by selecting specific breeds or individuals within breeds, leading to a significant increase in the milk yield over time (Brito et al. 2021). Certain breeds or individuals may also produce milk with high protein, fat, or specific minerals and vitamins (Zaalberg et al. 2019; Rahmatalla et al. 2021). Casein is the main protein in milk and accounts for as much as 80-85% of the total protein content (Chauhan et al. 2021; Rahmatalla et al. 2022). It is essential to absorb calcium microelements in newborn digestion (Rezaei et al. 2016). Casein proteins constitute a significant portion of the total protein content of milk. These are essential for forming micelles, which are crucial for transporting calcium, phosphate, and other minerals (Chauhan et al. 2021; Hammam et al. 2022; Nayik et al. 2022). From an industrial perspective, high-casein milk could yield a greater quantity of cheese curd (Wang et al. 2019). CSN1S1 (alpha-s1 casein) gene is one of the four genes responsible for milk's casein content. CSN1S1 gene plays an important role in casein secretion (Hassanin et al. 2022). Research has shown low beta-casein and kappa-casein transportation in low-expressed alpha-s1 casein in goat milk. Approximately 40% of cow milk contains alpha-S1 caseins, whereas goat milk has varying levels of casein expression owing to highly polymorphic casein genes. The CSN1S1 gene is composed of 19 exons and 18 introns (Hassanin et al. 2022; Rahmatalla et al. 2022). Base mutations (SNPs) and their interactions within the same or various genes might cause variable genetic responses in different breeds of the same animal (Dettori et al. 2024). Some previous studies have looked at casein gene genetic variants and how they affect milk components in *goat* breeds. High genetic variability in the CSN1S1 gene and the significant effects of its SNPs on the concentration of milk components have been discovered by previous studies on the CSN1S1 gene in goats (Anggraeni et al. 2021; Nayik et al. 2022; Khaldi et al. 2023).

The Peranakan Etawah (PE) goat is the only Indonesian local goat that can be used for meat and milk production. Breeding programs to improve the genetics of milk production and quality of this goat have not been extensively implemented; consequently, milk production and quality remain suboptimal (Anggraeni et al. 2020). In contrast, the Saanen dairy goat breed is the most-reared dairy goats worldwide because of their high milk yield (Devendra & Hannein 2019). However, these dairy goats are native to subtropical areas and are not adaptive in tropical regions. The development of Sapera, an improved dairy goat breed, resulted from a breeding program conducted by researchers at the IRIAP. This innovative cross-mating, comprising an equal genetic contribution from Saanen and PE goats, emerged from early scientific investigations (Anggraeni et al. 2020). This breeding program aims to produce high milk and protein yields. A preliminary study on genetic polymorphism of the CSN1S1 gene at the g.12164G>A locus showed high genetic variation with the frequency of the G allele against the A allele higher in PE goats (0.578) and Saanen goats (0.625) but lower in Sapera goats (0.333) (Anggraeni et al. 2021).

Thus, this study aimed to increase our understanding of the genetic polymorphisms of CSN1S1 using sequencing techniques in local PE, Saanen, and Sapera goats. Understanding the genetic diversity of CSN1S1 is crucial for developing effective breeding programs that aim to enhance the milk protein quality of local PE dairy goats and their crosses.

MATERIALS AND METHODS

Location and period of research

The Dairy Goat Research Station, a division of the Indonesian Research Institute for Animal Production (IRIAP), Ciawi, Bogor, West Java, Indonesia, conducted research on animal observations and the collection of milk and blood from dairy goats in 2021 and 2022. The IRIAP is in Banjarwaru Village within the Ciawi Subdistrict of Bogor Regency, West Java, Indonesia. IRIAP is located at an altitude of over 350 m asl with an average temperature of around 25.2ºC (22.8-33.2 ºC) and an average relative humidity (RH) of 87 5 % (73-94 %).

Molecular analysis of the CSN1S1 gene was performed at the Laboratory of Animal Molecular Genetics, part of the Department of Animal Production and Technology, at the Faculty of Animal Science of Bogor Agricultural University.

Feeding management

All animals were maintained optimally and fed regarding physiological status. Green fodders (leaves and grass) were fed approximately 10% of body weight (ad libitum), around 3-4 kg/hd/d. Meanwhile, concentrate feed was given as a source of protein and energy with a protein content of between 16-18% with TDN 70-80%, about 2% of body weight or 0.2-1 kg/hd/d. Concentrate is given to the does around 0.8-1 kg/hd/d.

Research sample

Ninety-five blood samples were analyzed at the Animal Molecular Genetics Laboratory of the Faculty of Animal Science of Bogor Agricultural University. Blood samples obtained were from all the does of the three goat genotypes, including the local PE breed (n= 14 hd.), exotic Saanen breed (n= 15 hd.), and cross Sapera genotype (n= 66 hd.), whose blood composition is 50% PE and 50% Saanen.

These goats were in various stages of lactation, ranging from 1 to 8 months, with periods of lactation from 1 to 4 and a kidding period from 2021 to 2022. All animals were maintained at the dairy goat experimental station of the IRIAP, Ciawi sub-district, Bogor, West Java, Indonesia.

Fresh blood (approximately 3 ml per sample) was collected from each animal's jugular vein. The samples were transferred to a 10-ml EDTA-containing tube and stored under refrigeration for preservation and subsequent analysis.

Molecular analysis

DNA extraction was performed at 10-50 µg/mL. PCR was performed on an AB system machine. Primers were created manually and optimized using Primer Stat software. The primers used for the amplification of exon 12 and a partial segment of intron 12 were designed with the forward primer (F) sequence for 5'-CTCATCCTCTGTCCTCTTCT -3' and the reverse primer (R) sequence for 5'-CTGTGCTTTCACAAGGAGGC -3' (Figure 1).

The PCR process was performed in a 26 µL reaction mixture containing 2 µL (10-50 µg/mL) DNA extract, 0.3 µL (10 µM) forward primer, 0.3 µL (10 µM) reverse primer, 10.4 µL distilled water, 12.5 µL My Taq Hs red mix produced by BioScience (USA), and 0.5 µL MgCl2. The temperature profile was 1 minutes for initial denaturation at 95°C, 35 cycles of 15 s at 95°C, 30 s at 60°C, and 10 s at 72°C, with a 5 min final extension at 72°C. Sanger sequencing was used to analyze the DNA sequences of exon 12 and partial intron 12 of CSN1S1. Direct sequencing using Sanger sequencing was conducted at the Macrogen Laboratory in South Korea to analyze the sequence of the targeted amplicon.

Data analysis

Hardy-Weinberg (H-W) equilibrium and the degree of heterozygosity were assessed using the equation following the study by Anggraeni et al. (2021) to calculate allele and genotype frequencies.

Ethical approval

The methodology employed in this study was approved by the ICARD Experimental Animal Welfare Commission of the Indonesian Agricultural Research and Development Agency (approval number ICARD/IRIAP/Rm/11/2021).

RESULTS AND DISCUSSION

CSN1S1 gene sequence

In our study, utilizing AJ504710.2 as the reference base, four SNPs were detected in the CSN1S1 fragment target in all goats. The SNPs were identified for g.10243 G>A, g.10250 A>G, g.10277 G>A, and g.10283 T>G (Figure 2, Figure 3). The numerical designations represent the specific positions within the CSN1S1 gene sequence. For instance, SNP g.10243 G>A indicates a substitution from Guanine (G) to Adenine (A) at the 10243rd base pair from the initiation of the CSN1S1 gene. Two of these SNPs, g.10243 G>A and g.10250 A>G, were identified in the exon 12 region of the CSN1S1 gene. The exon region of a gene is responsible for encoding proteins; consequently, mutations in this region may affect the structure and function of the protein encoded by the CSN1S1 gene (Lim et al. 2018).

The CSN1S1 gene is involved in the production of casein, which affects milk quality (Rahmatalla et al. 2021; Rahmatalla et al. 2022). Hence, these SNPs might have significant implications for milk protein. The CSN1S1 gene is known to be involved in the production of casein, which affects milk quality (Hassanin et al. 2022). Consequently, these SNPs may have significant implications for milk protein composition. The presence of two SNPs in the gene's coding region suggests a potential impact on the structure of the casein protein.

These findings open up new possibilities for studying the genetics of milk production and quality in goats, particularly the potential for breeding goats for higher-quality milk based on their CSN1S1 gene sequence. This investigation identified a missense mutation in caprine subjects' exon 12 region of the CSN1S1 gene. Specifically, the single nucleotide polymorphism (SNP) g.10243 G>A resulted in an amino acid substitution from Arginine (amino acid code: R) to Lysine (amino acid code: K). This alteration could affect the milk protein quality and functionality. Amino acids play a critical role in protein structure and function.

Arginine and Lysine are essential amino acids with distinct properties (Meuzelaar et al. 2016). Arginine contains a guanidin group, which can form multiple hydrogen bonds, whereas Lysine features an amino group in its side chain (Rahmatalla et al. 2021). These two amino acids share similarities in their overall charge. The variations in their side chains may influence protein folding, stability, and function (Meuzelaar et al. 2016). Missense mutations in the CSN1S1 gene have been demonstrated to influence dairy goats' milk protein composition, yield, and quality (Widodo et al. 2023). A mutation involving the substitution of Arginine with histidine has been observed to correlate with a diminished presence of αs1-casein in milk, subsequently influencing coagulation characteristics and cheese production efficiency (Hammam et al. 2022). The findings indicate that alterations in amino acid sequences can impact milk quality, although the specific mutation observed in the referenced study differs from the one examined in our research. Nevertheless, the specific impact of the Arg-to-Lys mutation in the CSN1S1 gene on milk protein quality and function remains insufficiently examined.

Figure 1. Visualization of forward and reverse primers. The reference base sequence is AJ504710.2

Figure 2. Visualization of mutation points occurring in the target amplicon. SP denotes the Sapera goat, PE denotes the PE goat, and SA denotes the Saanen goat. The reference base was obtained from AJ504710.2

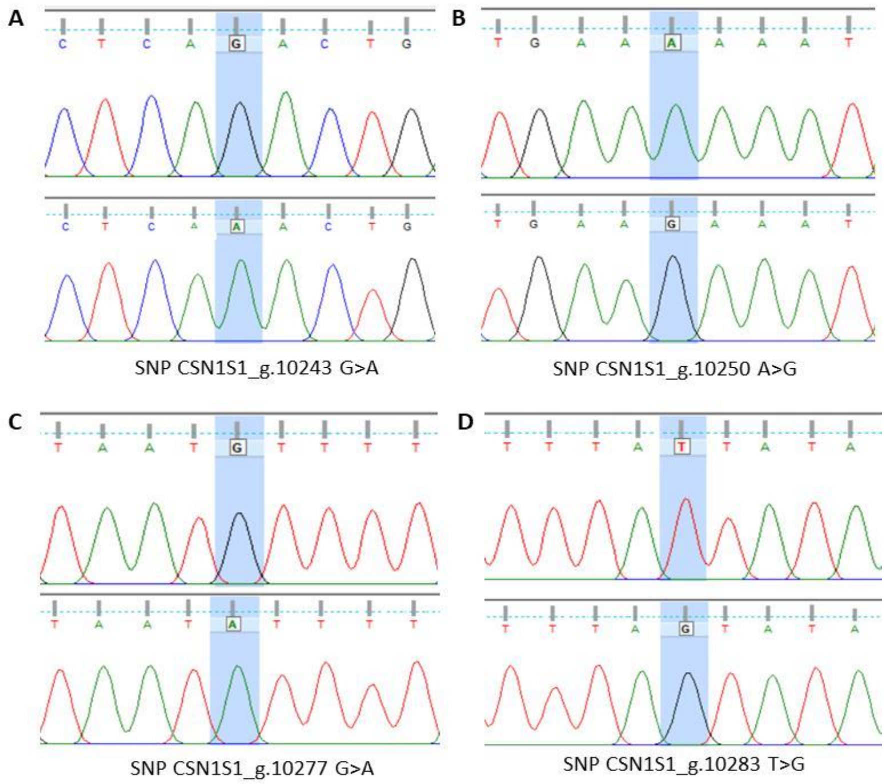

Figure 3. The sequence chromatograms of the SNPs from the CSN1S1 gene exon 12 and partial intron 12 (obtained from Finch TV images). The upper and lower panels depict non-mutant and mutant genotypes, respectively. (A) SNP CSN1S1_g.10243 G>A (B) SNP CSN1S1_g.10250 A>G (C) SNP CSN1S1 g_10277 G>A (D) SNP CSN1S1_g.10283 T>G

Genotype and allele frequencies

Table 1 presents the genotyping results of the SNPs present in the targeted amplicon from Sapera, Saanen, and PE goats. Across all four SNP locations (CSN1S1_g.10243 G>A, CSN1S1_g.10250 A>G, CSN1S1_g.10277 G>A, and CSN1S1_g.10283 T>G), a predominantly consistent genotype is observed in all three genotypes.

Moreover, the allele frequencies exhibit minimal variation across the breeds for each SNP. The findings, however, differed from the previous study by Anggraeni et al. (2021) concerning the genetic polymorphism of the CSN1S1 gene at the g.12164G>A locus.

The previous research found a significant genetic variation, with the G allele frequency surpassing that of the A allele in PE goats (0.578) and Saanen goats (0.625). The differences may be attributed to variations in single nucleotide polymorphisms (SNPs) within the same caprine population. A subsequent investigation conducted by (Rahmatalla et al. 2021) on eight native and exotic goat breeds identified a novel single nucleotide polymorphism (SNP) of SCN1S1 in exon 12 at locus g.7213522443A/G. This polymorphism exhibited high genetic variation in six breeds, with the A/G allele resulting in a genotype frequency ranging from approximately 0.50 to 0.75.

Heterozygosity and Hardy-Weinberg Equilibrium

This investigation assessed genetic diversity among the Saanen, PE, and Sapera goats, utilizing heterozygosity as a primary measure. Heterozygosity values approaching 0 indicate limited genetic diversity, whereas values nearing 1 suggest substantial genetic diversity (Sahoo et al. 2023). The observed heterozygosity (Ho) and expected heterozygosity (He) values derived from this investigation are presented in Table 2. Interestingly, none of the populations studied were in Hardy-Weinberg equilibrium (X^2 test > X^2 table). The preliminary study on the CSN1S1 gene at g. 12164G>A locus reported the SNP in H-W equilibrium for Sapera, PE, and Saanen goats (Anggraeni et al. 2021). The observation above suggests that, in the absence of external factors, genetic variation would remain constant across successive generations. Any deviation from this equilibrium state could be attributed to phenomena such as genetic drift, inbreeding, or non-random mating practices within these goat populations, which can significantly alter gene frequencies.

Table 1. Genotype and allele frequencies of the four single nucleotide polymorphisms (SNPs) in Sapera, Saanen, and PE goat populations

Dairy goat type	N	Genotype			Allele	
CSN1S1_g.10243 G>A		AA	AG	GG	A	G
SAPERA	66	0.196	0.000	0.803	0.197	0.803
SAANEN	15	0.067	0.000	0.933	0.067	0.933
PE	14	0.071	0.000	0.929	0.071	0.929
CSN1S1_g.10250		AA	AG	GG	A	G
SAPERA	66	0.818	0.000	0.812	0.818	0.182
SAANEN	15	0.933	0.000	0.067	0.933	0.067
PE	14	0.929	0.000	0.071	0.929	0.071
CSN1S1_g.10277 G>A		AA	AG	GG	A	G
SAPERA	66	0.182	0.000	0.818	0.182	0.818
SAANEN	15	0.067	0.000	0.933	0.067	0.933
PE	14	0.071	0.000	0.929	0.071	0.929
CSN1S1_g.10283 T>G		GG	GT	TT	G	T
SAPERA	66	0.197	0.000	0.803	0.197	0.803
SAANEN	15	0.067	0.000	0.933	0.067	0.933
PE	14	0.071	0.000	0.929	0.071	0.929

Table 2. Observed heterozygosity (Ho), expected heterozygosity (He), and chi-square (χ^2) test values

Dairy goat type	N	Ho value	He value	X^2 test
CSN1S1_g.10243 G>A				
Sapera	66	0.00	0.32	68.24
Saanen	15	0.00	0.13	29.04
PE	14	0.00	0.14	27.04
CSN1S1_g.10250 A>G				
Sapera	66	0.00	0.30	68.46
Saanen	15	0.00	0.13	29.04
PE	14	0.00	0.14	27.04
CSN1S1_g.10277 G>A				
Sapera	66	0.00	0.30	68.46
Saanen	15	0.00	0.13	29.04
PE	14	0.00	0.14	27.04
CSN1S1_g.10283 T>G				
Sapera	66	0.00	0.32	68.24
Saanen	15	0.00	0.13	29.04
PE	14	0.00	0.14	27.04

Table 2 presents the observed heterozygosity (Ho), expected heterozygosity (He), and X^2 test values for each genotype at the identified SNPs. A comparison of the actual genotype frequencies with those expected under Hardy-Weinberg equilibrium revealed significant deviations for all SNPs. This observation strongly indicates the influence of genetic forces, potentially including non-random mating, genetic drift, or selection, resulting in an observed departure from equilibrium. Elucidating the nature of these deviations is crucial for future research, particularly regarding their potential implications in population genetics and the development of effective breeding strategies (Baneh et al. 2020; Sahoo et al. 2023).

While these findings provide valuable insights into the genetic diversity of these goat breeds, it is imperative to acknowledge the limitations of our study. The sample size was relatively small, and there exists potential for selection bias. To address potential issues with statistical analysis, we implemented rigorous statistical tests appropriate for smaller sample sizes. However, these limitations do not diminish the significance of our findings but rather elucidate areas for further investigation.

Subsequent investigations employing larger sample sizes and more comprehensive analyses are necessary to corroborate and extend our findings. Such research would not only validate our results but also provide a more detailed understanding of the genetic diversity of these caprine populations. This knowledge is crucial for designing effective breeding strategies and improving the quality of *caprine* milk, a valuable resource in the dairy industry.

CONCLUSION

Genetic polymorphism study in the CSN1S1 gene, specifically at exon 12 and partial intron 12 regions, across local, exotic, and cross-bred goats identified four single nucleotide polymorphisms (SNPs): g.10243 G>A, g.10250 A>G, g.10277 G>A, and g.10283 T>G. Notably, the g.10243 G>A SNP led to an amino acid change from Arginine to Lysine. This alteration in exon 12 of the CSN1S1 gene could potentially impact the quality of milk and protein. These discoveries offer a valuable foundation for upcoming studies for improving dairy goat milk quality through genetic selection. The outcomes further underscore the importance of understanding the genetic diversity and composition of dairy goat populations, which can be instrumental in developing effective breeding programs.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this published article, neither the funding nor the content

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The Effects of Canola Oil, Vitamin E, and Selenium Supplementation in the Ration on Blood Metabolites Profile and Liquid Semen Quality in Fat-Tailed Rams

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ABSTRAK

Sutarno AP, Khotijah K, Arifiantini RI, Pamungkas FA, Nurlatifah A. 2025. Pengaruh suplementasi minyak kanola, vitamin E, dan selenium dalam pakan pada profil metabolit darah dan kualitas semen cair domba Ekor Gemuk jantan. JITV 30(1):42-51. DOI:http://dx.doi.org/jitv,v30.i1.3497.

Tujuan penelitian ini adalah mengevaluasi pengaruh pemberian minyak kanola, vitamin E, dan selenium dalam pakan pada profil metabolit darah dan kualitas semen cair pada domba ekor gemuk jantan. Ternak yang digunakan sebanyak 10 ekor domba ekor gemuk berumur 10 – 14 bulan dengan bobot awal 23.84±3.91 kg. Rancangan yang digunakan ialah rancangan acak kelompok (RAK) dan rancangan acak kelompok faktorial (RAKF) 2 x 2 dengan 2 perlakuan pakan (R0: ransum kontrol, R1: R0 + minyak kanola + vitamin E + selenium dan 2 jenis pengencer. Peubah yang diukur meliputi konsumsi nutrien, pertambahan bobot badan harian, efisiensi penggunaan pakan, metabolit darah, biokimia plasma semen, dan kualitas semen cair. Hasil penelitian menunjukkan bahwa pemberian minyak kanola dan antioksidan tidak mempengaruhi konsumsi bahan kering, pertambahan bobot badan harian, dan metabolit darah (P>0,05). Akan tetapi, terdapat peningkatan asupan nutrien berupa lemak kasar, asam lemak, efisiensi penggunaan pakan, kadar kolesterol total, dan kolesterol LDL darah (P<0,05). Pemberian minyak kanola dan antioksidan secara deskriptif juga menunjukkan peningkatan kadar trigliserida plasma semen dan LDL, sementara secara bersamaan mengurangi kadar glukosa dan kolesterol plasma semen. Pemberian minyak kanola dan antioksidan tidak memengaruhi viabilitas sperma (P>0,05), tetapi menunjukkan peningkatan motilitas sperma semen cair pada hari ke-3 (P<0,05). Sebagai simpulan, pemberian suplementasi minyak kanola, vitamin E, dan selenium dalam ransum domba jantan ekor gemuk mengakibatkan peningkatan kadar kolesterol total dan LDL dalam darah sekaligus mengurangi konsentrasi kolesterol dalam plasma semen. Disamping itu, suplementasi minyak kanola dan antioksidan meningkatkan asupan nutrien, efisiensi pakan, dan memperpanjang masa simpan semen cair.

Kata Kunci: Antioksidan, Domba Ekor Gemuk Jantan, Minyak Kanola, Lemak, Semen Cair

ABSTRACT

Sutarno AP, Khotijah K, Arifiantini RI, Pamungkas FA, Nurlatifah A. 2025. The effects of canola oil, vitamin E, and selenium supplementations in the ration on blood metabolites profile and liquid semen quality of Fat-tailed rams. JITV 30(1):42-51. DOI:http://dx.doi.org/jitv,v30.i1.3497.

This research aimed to assess the impact of dietary supplementation with canola oil, vitamin E, and selenium on blood metabolite profile and the quality of liquid semen in fat-tailed rams. The livestock consisted of 10 fat-tailed rams aged 10 to 14 months, with an average initial body weight of 23.84±3.91 kg. Randomized block design (RBD) and factorial randomized block design (FRBD) 2 x 2 were used for evaluating performance and liquid semen variables, with two feed treatments (R0: control diet, R1: R0 + canola oil + vitamin E + selenium). The variables measured included nutrient intake, daily body weight gain, feed efficiency, blood metabolites, seminal plasma biochemistry, and liquid semen quality. The results showed that canola oil and antioxidants did not influence dry matter intake, daily body weight gain, and blood metabolites (P>0.05). However, there was an increase in the nutrient intake of crude fat, fatty acids, feed efficiency, total cholesterol levels, and blood LDL cholesterol (P<0.05). In the ration, Canola oil and antioxidant supplementations were descriptively increased seminal plasma triglycerides and LDL. Additionally, supplementations of canola oil and antioxidants in the feed reduced seminal plasma glucose and cholesterol levels. The administration of canola oil and antioxidants did not affect sperm viability (P>0.05) but increased liquid semen motility on day 3 (P<0.05). In conclusion, supplementing canola oil, vitamin E, and selenium in the diet of fat-tailed rams increased total cholesterol and LDL levels in the blood while reducing cholesterol concentration in seminal plasma. Additionally, these dietary interventions improve nutrient intake and feed efficiency and extend the shelf life of liquid semen.

Key Words: Antioxidants, Canola oil, Fat, Fat-tailed Rams, Liquid Semen

INTRODUCTION

The lamb meat production and the sheep population in West Java have increased and decreased by 5.1% and 15.6%, respectively (BPS 2024). This condition raises concerns about the challenges in increasing meat production to meet the continuously rising market demand. The issue can be addressed by enhancing the population of fat-tailed rams as breeding stock. Fat-tailed rams are known as local meat sheep with good growth potential in this context. Optimizing the function of fat-tailed rams as breeding stock is achieved by managing nutrients and meeting feed requirements to improve reproduction. Feed plays a crucial role in the reproductive performance of sheep, particularly in providing sufficient energy sources.

Energy deficiency in males negatively impacts reproductive performance (De Souza et al., 2019). Alternative energy sources for livestock can be achieved through fat supplementation since the nutrient provides 2.24 times more energy than carbohydrates (Pramono et al. 2018). Fat plays an important role as an energy reserve and supports hormone synthesis, plasma membrane composition, and sperm motility function. The nutrient is included in the energy metabolism processes of sperm and seminal plasma (Van Tran et al. 2017; Alagawany et al. 2019; Fitriyah & Isyaturriyadhah 2021). Therefore, fat-source feed such as oil should be added to livestock rations.

According to Younis Talpur et al. (2009), canola oil contains 56.89% and 10.66% monounsaturated and polyunsaturated fatty acids. Oleic acid supplementation is reported to function as an energy source to enhance sperm motility (Zhu et al. 2020), maintain membrane fluidity, and improve sperm motility (Ferramosca et al. 2017). Linoleic acid supplementation enhances motility and acrosome integrity (Ezazi et al. 2019) and increases semen volume, concentration, total motility, viability, and plasma membrane integrity of sperm (Masoudi & Dadashpour Davachi 2021).

Feed is optimized by adding antioxidants such as vitamin E and the mineral selenium (Se) to reduce oxidative stress. Vitamins and minerals are crucial in livestock's growth and reproductive health (Zubair et al. 2015). Semen quality is a determining factor in optimizing the reproductive function of fat-tailed sheep for superior breeding stock. Sound quality is expected to increase the population of rams through artificial insemination (AI) or natural mating. AI can use liquid semen as an alternative because the process is fast and only requires the addition of diluent materials to fresh semen and storing at a temperature of 4-5°C (Zakiya et al. 2020; Rokana et al. 2022). In ruminant livestock, feeding high levels of fatty acids raises concerns about affecting consumption, which will affect semen quality because the addition of fatty acids in the diet causes a change in the fatty acid composition of the sperm membrane (Jafaroghli et al. 2014; Nassan et al. 2018). Therefore, this research aimed to assess the impact of dietary supplementation with canola oil, vitamin E, and selenium on blood metabolite profile and the quality of liquid semen in fat-tailed rams.

MATERIALS AND METHODS

Animals and diets

The Animal Maintenance has approved this research and Use Ethics Commission of the National Research and Innovation Agency (Number: 204/K.E.02. /SK/11/2023). The livestock used in this experiment consisted of 10 fat-tailed rams aged 10 to 14 months, with an average initial body weight of 23.84±3.91 kg divided into 2 treatments and 5 replications. The animals were maintained at the Meat and Work Animal Nutrition Laboratory, Pen Facility B, Faculty of Animal Science, IPB University, in individual pens equipped with feeding and drinking water facilities. Additionally, the experimental diet consisted of elephant grass *(Pennisetum purpureum)* and concentrate. The diet was provided at 3% of body weight with a forage-to-concentrate ratio 30:70 based on dry matter (DM). The formulation was isoenergetic and isoprotein, following the needs of male sheep, according to (NRC 2007). The experimental diets used were R0: control diet, R1: R0 + canola oil + vitamin E + selenium. Table 1 shows the nutrient composition of the concentrate used.

Nutrient consumption

Feed and nutrient intake measurements were conducted daily during the maintenance phase. The nutrients calculated included DM intake, crude protein (CP), crude fiber (CF), crude fat (CF), nitrogen-free extract (NFE), total digestible nutrients (TDN) intake, and fatty acid intake. The calculations for measuring feed intake and nutrient consumption can be performed using the formulas DM intake = feed intake (g) X % DM of feed, CP intake = DM intake (g) X % CP of feed, CF intake = DM intake (g) X % CF of feed, NFE intake = DM intake (g) X % NFE of feed, TDN intake = DM intake (g) X % TDN of feed, and fatty acid intake = Crude fat intake (g) x % fatty acid content.

Measurements of average daily weight gain

Body weight measurements in rams were conducted before and after the treatment. Meanwhile, weighing was performed using a digital scale with a capacity of 100 kg. The calculation of average daily gain (ADG) was obtained using the formula ADG= [(final weight – initial weight) / duration of maintenance (days)].

Table 1. The composition of ingredients concentrate, dosage of canola oil, vitamin E, selenium, and nutrient content (Fatty acids) of the treatment concentrate

Feed materials	Treatment	
	R0 (%)	R1 (%)
Canola oil	-	6.00
Soybean meal	20.71	20.71
Pollard	20.00	20.00
Rice bran	22.86	22.86
Cassava pulp	24.29	24.29
Molasses	10.00	10.00
Premix	0.71	0.71
$CaCO_3$	0.71	0.71
Salt	0.71	0.71
Vitamin E	-	500 IU
Selenium	-	0.5 ppm
Composition [(1)]	Treatment	
	R0 (%)	R1 (%)
Oleic acid	21.69	37.71
Linoleic acid	2.13	14.02
Linolenic acid	0.00	0.96

R0= control diet, R1= R0+ canola oil + vitamin E + selenium, * (1)= Integrated Laboratory of IPB University

Feed efficiency calculation

Feed efficiency was calculated based on the ADG and divided by feed intake before multiplying by 100%.

Blood metabolite analysis

Blood metabolite analysis was conducted using plasma blood samples. Plasma was obtained from blood centrifuged at 3000 rpm for 15 minutes. Subsequently, the obtained plasma was placed into tubes and analyzed using the enzymatic method. Total protein, glucose, triglycerides, cholesterol, HDL, and LDL were obtained using the Bio maxima, Greiner Glucose GOD-PAP, Greiner Triglycerides, Greiner Cholesterol, and Greiner HDL Cholesterol kits. The variables were analyzed using a 5010V+ photometer at the Bogor Center for Primate Studies Laboratory (PSSP).

Semen collection

Semen was collected towards the end of feeding treatment at 07:00 WIB using an electro-ejaculator. Preputial shaving and washing were performed with 3% sodium bicarbonate while drying was carried out before collection. The electrode of the electroejaculator (30 cm in length and 2 cm in diameter) was inserted into the rectum approximately 15 cm. Subsequently, low-voltage electrical stimulation was applied repeatedly with a voltage of 3-6 volts for 10 seconds, and the operator performed the electrical stimulation. The collected semen was gathered in tubes for evaluation.

Fresh semen plasma biochemical analysis

The plasma semen obtained from semen collection was separated by centrifuging fresh ram semen (3000 rpm, 20 minutes) and stored at -20°C until further analysis. The stored semen was thawed at room temperature and centrifuged at 10,000 rpm for 20 minutes. Subsequently, plasma biochemistry was analyzed using biological equipment, and the enzymatic colorimetric assay method was used with an ST-type spectrophotometer. The reagents were analyzed using a Chemistry Analyzer 220 V at the IRATco Laboratory Service, Dramaga, Bogor, Indonesia.

Liquid semen evaluation

Preparing liquid semen starts with creating two types of extenders: Tris egg yolk and Citrate fructose egg yolk extender. The preparation of the egg yolk tris extender followed the method of Kulaksiz et al. (2012)

by dissolving 3.63 g, 1.99 g, and 0.5 g of tris(hydroxymethyl)aminomethane, citric acid, and fructose in 100 ml of distilled water. Meanwhile, the preparation of the fructose citrate extender was based on the method of Arifiantini & Purwantara (2010) by dissolving 1.25 g, 2.32 g, and 100 ml of fructose, sodium citrate dihydrate, and distilled water, respectively. Semen suitable for processing was fresh with motility greater than 70%.

The preparation of liquid semen starts with calculating the concentration of spermatozoa and determining the amount of extender needed, with a target concentration of 100 million/ml. Each fresh semen sample suitable for processing into liquid is divided into two glass test tubes. Each tube is supplemented with egg yolk tris or fructose citrate extender. In addition, the liquid semen is covered with plastic wrap and placed in a glass beaker without a water jacket. The sample is stored in a refrigerator at a temperature of 3-5ºC, analyzed periodically for 5 days, and evaluated microscopically for viability, motility, and intact plasma membrane.

Determining viability percentage is conducted by counting the number of live sperm and dividing it by the total sperm count, then multiplying the result by 100. Motility observation is performed using a microscope with a magnification of 400 times across five different fields of view, and the assessment is given on a scale of 0-100%. The percentage of intact plasma membrane can be calculated by dividing the number of spermatozoa that reacted by the total number of spermatozoa counted and multiplying the result by 100%.

Statistical analysis

Randomized block design (RBD) and factorial randomized block design (FRBD) 2 x 2 were used for performance and liquid semen variables, with two feed treatments (R0: control diet, R1: control diet + canola oil + vitamin E + selenium). The extenders used were egg yolk tris and fructose citrate based on body weight and sperm concentration. Data were analyzed using an Independent Sample T-Test, while the results of the plasma semen biochemistry were subjected to descriptive analysis. Data analysis was performed using IBM SPSS Statistics 25 software.

RESULTS AND DISCUSSION

Nutrient intake

Nutrient intake in rams treated with canola oil and antioxidants did not have a significant effect ($P > 0.05$) on DM, CP, CF, nitrogen-free extract (NFE), and TDN. However, the intake had a significant effect ($P < 0.05$) on crude fat and fatty acids (Table 2).

The similar nutrient content in the diets resulted in no significant differences in DM intake among treatments, indicating that feed sources like canola oil and antioxidants do not alter the aroma or texture. Fat-tailed rams fed ration supplemented with canola oil had higher crude fat and fatty acid intakes than the control group, likely due to the higher fat content. Maia et al. (2012) reported that adding canola, sunflower, or castor oil to the diets of Dorper x Santa Ines sheep did not affect DM intake but increased fat intake. Increased fat and fatty acid intake may improve semen quality, benefiting rams used for breeding. Díaz et al. (2016) highlighted the role of fatty acids in male reproductive function, particularly in improving membrane fluidity, motility, and viability of the sperm.

Average daily gain and feed efficiency

Average daily weight gain in fat-tailed rams treated with canola oil showed no significant effect ($P>0.05$). However, the variable improved feed efficiency ($P<0.05$), as reported in Table 3.

The variation in average daily weight gain between the control and treatment groups can be attributed to the absence of significant differences in nutrient intake. According to Riemas et al. (2021), supplementing unsaturated fatty acids in fat-tailed rams did not affect average daily weight gain. This result was because DM intake was similar, resulting in an equivalent nutrient supply to the animals. The addition of canola oil led to a significantly higher improvement in feed efficiency than the control. Therefore, rams consuming a diet of fatty acids from canola oil reported relatively better feed and energy efficiency. According to Parakkasi (1999) and Abrori et al. (2022), higher feed efficiency values showed better use in enhancing sheep growth.

Blood metabolites profile

Blood metabolites in fat-tailed rams treated with canola oil and antioxidants showed a significantly higher cholesterol level ($P < 0.05$) compared to the control. However, there was no significant effect ($P > 0.05$) on total protein, glucose, triglycerides, and high-density lipoprotein (HDL) cholesterol (Table 4).

Total blood protein levels of fat-tailed rams remained within the normal range (Table 4). This condition is attributed to the lack of significant treatment effect on CP intake, resulting in similar absorption and metabolism. Siska and Anggrayni (2021) stated that the levels of total blood protein are influenced by the amount of dietary protein consumed. Blood glucose levels in the experimental rams treated with canola oil did not differ significantly from the control. This condition is due to insufficient treatment effect on feed intake. Similar results were observed by Jafaroghli et al. (2014), where

Table 2. Average nutrient intake in fat-tailed rams

Variables	Treatment		
	R0 (g $head^{-1}day^{-1}$)	R1 (g $head^{-1}day^{-1}$)	P-value
Total dry matter	787.74±117.98	708.34±71.58	0.23^{ns}
Crude protein	134.05±20.19	116.88±11.75	0.13^{ns}
Crude fiber	141.69±21.18	120.30±12.31	0.08^{ns}
Crude fat	27.61±4.14	41.44±4.15	0.00^{s}
Nitrogen free extract	396.44±59.61	350.45±35.36	0.17^{ns}
Total digestible nutrients	528.73±79.51	494.75±49.87	0.44^{ns}
Oleic acid	4.00±0.60	12.77±1.26	0.00^{s}
Linoleic acid	1.14±0.17	5.29±0.52	0.00^{s}
Linolenic acid	0.00±0.00	0.32±0.03	0.00^{s}

R0= control diet, R1= R0+ canola oil + vitamin E + selenium ^{ns}= Non significant (P>0.05), ^{S}= Significan*t* (P<0.05)

Table 3. Average daily gain and feed efficiency in fat-tailed rams

Variables	Treatment		
	R0	R1	P-value
Average daily weight (g $head^{-1}day^{-1}$)	71.61±10.90	84.51±11.09	0.10^{ns}
Feed efficiency (%)	9.71±0.71	12.00±1.87	0.02^{s}

R0= control diet, R1= R0+ canola oil + vitamin E + selenium ^{ns}= Non significant (P>0.05), ^{S}= Significan*t* (P<0.05)

Table 4. Blood metabolites profile of experimental fat-tailed rams

Variables	Treatment		P-value	Normal range
	R1	R0		
Total protein (g dl^{-1})	4.58±0.30	4.60±0.37	0.85^{ns}	4.50-7.20[1]
Glucose (mg dl^{-1})	32.68±8.50	21.48±19.39	0.27^{ns}	26.18-72.08[2]
Triglycerides (mg dl^{-1})	30.82±7.05	37.62±7.62	0.18^{ns}	27.19-60.09[2]
Total cholesterol (mg dl^{-1})	65.60±9.20	82.25±11.55	0.04^{s}	36,80-91,50[3]
HDL cholesterol (mg dl^{-1})	34.40±5.59	39.75±2.87	0.12^{ns}	26,00-53,33[3]
LDL cholesterol (mg dl^{-1})	25.03±2.66	35.15±7.64	0.02^{s}	15,60-39.25[3]

R0= control diet, R1= R0+ canola oil + vitamin E + selenium, ^{ns}= *Non significant* (P>0.05), ^{S}= Significant (P<0.05), HDL= High density lipoprotein, LDL= Low density lipoprotein,[1] Mitruka 1981, [2] Eshratkhah et al. 2008, [3]Sarmin et al. 2021

Moghani rams fed fish oil and vitamin C did not show any significant effect on blood glucose levels, and the same results by Khotijah et al. (2020) on sheep-fed vegetable oil ration did not have a significant effect either. Blood triglyceride levels in the experimental rams remained within the normal range (Table 4). This result is attributed to the nutrient content of feed meeting energy requirements and leading to differences in blood triglyceride levels. Triglycerides are broken down when insufficient dietary energy (Suharti et al. 2017; Ramadhina et al. 2019; Nurmalia et al. 2020). Adding canola oil and antioxidants increased sheep's blood and LDL cholesterol levels within the normal range (Table 4). This result is due to the significantly different intake of unsaturated fatty acids. According to Śpitalniak-Bajerska et al. (2020), increased cholesterol levels occur due to higher intake of unsaturated fatty acids. Susilowati et al. (2015) stated that the compound enhanced sperm membrane integrity. The elevated LDL levels correspond with the increased cholesterol concentration.

Plasma seminal biochemistry

Based on the descriptive analysis results, Table 5 presents the plasma seminal biochemistry of fat-tailed rams fed the control diet and the canola oil treatment. Plasma seminal glucose concentration in the present experiment was lower than those reported by Almadaly et al. (2021) in Ossimi rams, and the reported levels ranged from 47.04 to 65.82 mg dl^{-1}. Meanwhile, the triglyceride concentration in the present experiment was lower than that of Hafez (2009), who reported values ranging from 229.71 to 242.76 mg dl^{-1} in Rahmani rams. The cholesterol levels in the plasma seminal of fat-tailed rams remained within the normal range compared to those reported by Gündoğan and Gündoğan (2006). The results showed that cholesterol levels in the plasma seminal of Akkaraman and Awassi rams were 111.00 mg dl^{-1}. The HDL and LDL cholesterol levels were higher compared to Motalebipour et al. (2022), that HDL and LDL levels in Afshari rams are between 14.50-19.00 mg dl^{-1} and 15.50-21.50 mg dl^{-1}, respectively. The total cholesterol and LDL concentration in seminal plasma was lower than in the control, while the opposite was observed in blood plasma (Table 5 and Table 4), suggesting that the lipids in seminal plasma may not originate solely from the blood, but that other factors may also be influencing this. According to the research by Lu et al. (2016), lipid levels in seminal plasma may not come directly from the blood. However, there is a possibility that they also originate from epithelial cells in the male reproductive system. Additionally, age affects lipid levels in male seminal plasma. As age increases, lipid levels tend to rise.

Quality of liquid semen

The quality of liquid seminal from the experimental fat-tailed rams fed with the control diet, canola oil, and two extender treatments showed no interaction in motility, viability, and plasma membrane integrity ($P > 0.05$), as reported in Tables 6, 7, and 8. There are no established quality standards for liquid semen in sheep in Indonesia. Therefore, the variable refers to the minimum quality requirements for frozen semen in goats and sheep (BSN 2023).

Sperm motility indicates sperm fertility in males Santoso et al. (2021). Sperm motility in the experimental fat-tailed rams fed with oil-based diets using extenders on day 3 showed a significant difference, maintaining

Table 5. Plasma seminal biochemistry in experimental fat-tailed rams

Variables	Treatment	
	R0	R1
Glucose (mg dl^{-1})	45.59	40.45
Triglycerides (mg dl^{-1})	152.89	171.36
Total cholesterol (mg dl^{-1})	127.21	113.00
HDL cholesterol (mg dl^{-1})	43.21	65.43
LDL cholesterol (mg dl^{-1})	48.25	38.43

R0= control diet, R1= R0+ canola oil + vitamin E + selenium, HDL= High density lipoprotein, LDL= Low density lipoprotein

Table 6. The percentage of sperm motility in fat-tailed rams fed with different diets varied between the tris egg yolk extender and the citrate fructose egg yolk

Storage time (Day)	Treatment	Extender	
		Tris egg yolk	Citrate fructose egg yolk
0	R0	77.40±11.10	77.40±11.10
	R1	84.00±6.55	84.00±6.55
1	R0	67.00±10.36	55.00±7.07
	R1	70.00±8.66	68.33±12.58
2	R0	47.00±7.58	47.50±3.53
	R1	51.67±2.88	55.00±10.00
3	R0*	31.00±18.50	40.00±7.07
	R1*	46.66±2.88	50.00±10.00
4	R0	9.00±8.98	20.00±16.35
	R1	38.33±5.77	36.67±12.58

R0= control diet, R1= R0+ canola oil + vitamin E + selenium; *= indicates a significant effect ($P<0.05$)

sperm motility above 40% (P<0.05). Furthermore, the sperm motility of experimental sheep on the control diet was lower than that of those fed rations with canola oil treatment in both extenders. This result is due to the diet's oleic, linoleic, and linolenic acids that sustain motility. Unsaturated fatty acids can maintain sperm motility (Ferramosca et al. 2017; Kogan et al. 2021). Although no effect was observed from the two extenders, the extenders provide nutritional contributions in the form of energy sources, specifically carbohydrates, to maintain sperm activity during storage. According to Arifiantini & Purwantara (2010), carbohydrates influence sperm motility in the extenders, and sperm motility ceases when carbohydrates are absent.

Sperm viability in liquid semen treated with canola oil showed no significant difference (P > 0.05) compared to the control (Table 7). Even though there was no significant difference, viability was maintained up to day 4 in the canola oil treatment (R1). This result was influenced by the high fat intake, affecting the increase in blood cholesterol and plasma seminal levels within the normal range. According to Motalebipour et al. (2022),

Table 7. The percentage of sperm viability in the experimental fat-tailed rams fed with different diets varied between the tris egg yolk extender and the citrate fructose egg yolk

Storage time (Day)	Treatment	Extender	
		Tris egg yolk	Citrate fructose egg yolk
0	R0	86.51±4.90	86.51±4.90
	R1	90.60±3.14	90.60±3.14
1	R0	80.59±4.06	80.44±3.57
	R1	87.60±4.02	85.99±2.10
2	R0	70.61±6.25	72.86±5.57
	R1	79.94±3.58	76.77±5.65
3	R0	50.96±12.85	53.03±17.31
	R1	67.88±15.51	67.92±6.50
4	R0	11.70±11.68	37.23±19.68
	R1	58.69±7.68	64.73±6.37

R0= control diet, R1= R0+ canola oil + vitamin E + selenium; *= indicates a significant effect (P<0.05)

Table 8. The percentage of intact plasma membrane integrity in the experimental fat-tailed ram sperm fed with different diets varied between the egg yolk tris extender and the citrate egg yolk extender

Storage time (Day)	Treatment	Extender	
		Tris egg yolk	Citrate fructose egg yolk
0	R0*	82.94±5.75	82.94±5.75
	R1*	70.61±16.16	70.61±16.16
1	R0*	74.36±3.43	73.74±4.96
	R1*	65.43±12.53	64.66±7.77
2	R0	64.87±9.28	63.81±8.12
	R1	59.47±11.31	57.40±12.48
3	R0	46.70±12.19	42.72±25.38
	R1	55.22±11.97	51.02±12.47
4	R0	11.84±11.83	31.53±16.82
	R1	45.81±0.96	45.11±14.46

R0= control diet, R1= R0+ canola oil + vitamin E + selenium; *= indicates a significant effect (P<0.05) sperm viability is influenced by the increased fat levels in the blood and semen to improve plasma membrane integrity and viability.

The plasma membrane integrity of sperm in the experimental fat-tailed rams fed with the control diet was significantly higher on days 0, 1, and 2 ($P<0.05$). On day 3, the treatment diet showed higher results than the control, as reported in Table 8. The maintenance of intact plasma membranes is influenced by the high levels of blood cholesterol, vitamin E, and selenium in the diet to reduce ROS during storage. High cholesterol concentrations in the blood also affect sperm membranes from cold shock (Motalebipour et al. 2022). Prolonged storage increases ROS levels and impacts energy metabolism, motility, viability, and plasma membrane integrity (Abdel-Khalek et al. 2016; Monova & Ducha 2019; Naz et al. 2022).

Maintaining motility, viability, and plasma membrane integrity was supported despite no interaction between the diet and extenders. This fact is evidenced by the motility, viability, and plasma membrane integrity of sperm used for AI up to day 3. The egg yolk tris and fructose citrate egg yolk were reported by Arifiantini and Purwantara (2010) and Rather et al. (2017) to be equally effective in preserving liquid semen in Holstein Friesian cattle and ram; this is because tris and citrate egg yolk contain phospholipids and lecithin to protect sperm from cold shock.

CONCLUSION

In conclusion, the supplementation of canola oil, vitamin E, and selenium in the diet of fat-tailed rams increased total cholesterol and LDL levels in the blood while reducing cholesterol concentration in seminal plasma. Additionally, these dietary interventions improved nutrient intake, feed efficiency, and the shelf life of liquid semen.

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Adapted Local Feed Supplement for African Dwarf Sheep in the Rainy Season

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ABSTRAK

Dokui F, Houndonougbo FM, Babatoundé S, Mouteïrou AAA, Chrysostome CAAM.. 2025. Suplemen pakan lokal yang diadaptasi untuk domba kerdil Afrika di musim hujan. JITV 30(1):52-58. DOI:http://dx.doi.org/jitv.v30i1.3298.

Tujuan dari penelitian ini adalah untuk membuat dan mengidentifikasi suplemen pakan terbaik untuk domba kerdil Afrika selama musim hujan. Untuk itu, dua jenis suplemen pakan (batu jilat multi-nutrisi dan pakan konsentrat) dibuat berdasarkan bahan pakan lokal. Empat perlakuan diet ditetapkan, dan masing-masing diuji pada sekelompok 6 domba yang ditempatkan di kandang individu. Kelompok pertama tidak diberi suplemen, kelompok kedua diberi suplemen pakan konsentrat, kelompok ketiga diberi suplemen pakan konsentrat dan batu jilat, dan kelompok terakhir diberi suplemen batu jilat saja. Hewan-hewan tersebut berada di padang rumput alami selama 5,30 jam setiap hari. Percobaan ini dilakukan selama 84 hari. Asupan pakan dicatat setiap hari dan berat badan diukur setiap dua minggu. Asupan pakan, rasio konversi pakan, pertambahan bobot badan harian rata-rata, biaya pakan, dan efisiensi pakan ekonomis dihitung. Domba-domba tersebut memakan pakan konsentrat dalam jumlah yang lebih besar daripada batu jilat. Performa pertumbuhan domba yang disuplementasi dengan konsentrat (5,51 kg) lebih baik daripada domba yang tidak disuplementasi (3,52 kg); domba yang disuplementasi dengan batu jilatan memiliki rasio konversi pakan terbaik dan keuntungan ekonomis terbaik (28,49 unit uang yang diperoleh dari 1 unit yang diinvestasikan dalam pakan). Batu jilat yang terbuat dari bahan pakan lokal lebih cocok daripada pakan konsentrat untuk domba kerdil Afrika selama musim hujan. Tidak menarik untuk menggabungkan pakan konsentrat dan batu jilat sebagai suplemen pakan untuk domba kerdil Afrika di musim hujan.

Kata Kunci: Domba Kerdil Afrika, Keuntungan Ekonomi, Suplemen Pakan, Kinerja Pertumbuhan, Musim Hujan

ABSTRACT

Dokui F, Houndonougbo FM, Babatoundé S, Mouteïrou AAA, Chrysostome CAAM.. 2025. Adapted local feed supplement for African dwarf sheep in the rainy season. JITV 30(1):52-58. DOI:http://dx.doi.org/jitv.v30i1.3298.

This study aimed to manufacture and identify the best feed supplements for African dwarf sheep during the rainy season. Two feed supplements (multi-nutritional lick stone and concentrate feed) were made based on local feedstuffs. Four different dietary treatments were set for four groups; each group contained 6 sheep but in individual pens. The first group was not supplemented, the second was supplemented with concentrate feed, the third was supplemented with both concentrate feed and lick stone, and the last was supplemented with lick stone alone. The animals were in a natural pasture for 5.30 hours each day. The experiment was carried out over an 84-day period. The feed intake was registered daily, and the body weight was measured biweekly. Feed intake, feed conversion ratio, average daily gain, feed cost, and economical feed efficiency were calculated. The sheep ate more significant amounts of concentrate feed than of the lick stone. The growth performance of the sheep supplemented with the concentrate (5.51 kg) was better than that of the non-supplemented sheep (3.52 kg); those supplemented with lick stone had the best feed conversion ratio and the best economic return (28.49 Unit of money gained for 1 unit invested in the feed). Lick stone based on local feedstuffs is more suitable than concentrate feed for African dwarf sheep during the rainy season. It is of no interest to mix feed concentrate and lick stones together as feed supplements for African dwarf sheep in the rainy season.

Keywords: African Dwarf Sheep, Economic Return, Feed Supplements, Growth Performance, Rainy Season

INTRODUCTION

A great part of ruminants' feed consists of fodder. Hence, the problem of feeding small ruminants is increasing due to urban fringe development, which negatively affects small ruminant production through the widespread use of weedicides and destruction of natural pastures (Shinde & Mahanta 2020; Khan et al. 2021; Abdulai 2022). Some years ago, feeding problems were not noticed during the rainy season, but the situation has changed due to population growth. According to the United Nations (United Nations 2019), the world population is projected to grow from 7.7 billion in 2019 to 10.9 billion in 2100. The impact of this growth will be most significant in lower-income regions such as sub-Saharan Africa (Bajagai et al. 2016; United Nations 2019). Feeding this population will require an increase of more than 60% in food and an important contribution by livestock, which represent more than 40% of the global value of agricultural production (Alexandratos & Bruinsma 2012). The already substantial trend toward destroying natural resources, especially pastureland, will increase further if an adequate solution is not found.

To deal with this situation, the wise management of natural resources is essential (Dokui et al. 2023). Especially in small ruminant feeding and nutrition, managing the industrial and agricultural by-products that are widely considered waste to produce adequate feed supplements could be a great solution. It could help to maintain and increase productivity as well as contribute to a reduction of greenhouse gas emissions (Haque 2018). Apart from the conflicts between breeders and farmers noticed in many countries because of bad land management, the destruction of natural pasture is the source of climate change due to the imbalance between animals and crops. According to Teague et al. (2016), ruminants reduce overall GHG emissions, facilitate essential ecosystem services, increase soil carbon (C) sequestration, and reduce environmental damage. Increasing the dietary level of concentrate in ruminant feeding reduces methane production (because of less use of fiber sources) and increases livestock productivity (Haque 2018). In sub-Saharan countries such as Benin, where ruminants represent more than 60% of all protein (FAOSTAT 2019), wise management of the agricultural and industrial by-products to provide an efficient feed supplement for ruminants seems to be a suitable solution (Montcho et al. 2016; Dokui et al. 2023). But when it comes to ruminants, there are numerous types of feed supplements.

This study aimed to test two feed supplements for their impact on the growth and economic performance of African dwarf sheep and determine which option is most appropriate for them during the rainy season in Benin.

MATERIALS AND METHODS

Study area

The study was conducted in the rainy season on a farm in the village of Houngbo in the municipality of Toffo, southern Benin, which is characterized by a subequatorial climate with two dry and two rainy seasons. The annual rainfall is 1100 mm during the rainy season and 800 mm during the dry season. The average temperature varies between 27 and 31ºC. Apart from some forest galleries, the flora consists mainly of herbaceous and shrubby savannah. On the various plantations, the oil palm (*Elaeis guinensis*), which holds a part of the natural grassland, is especially observed.

Experimental design and feeding

The experiment was carried out over 84 days, preceded by 14 days of adaptation. Twenty-four weaned dwarf sheep were weighed and placed in 4 groups of 6 animals each. Animals were housed in individual pens, 1 m x 1 m, with a feeding and water trough. The average weight of the animals at the beginning of the trial was 11.04±1.98 kg, 11.05±1.82 kg, 11.09±1.83 kg, and 11.06±197 kg, respectively, for the four groups. Hence, the average weight of the sheep in each group was similar at the beginning of the study.

The animals foraged daily from 08:30 h to 12:00 h and from 16:00 h to 18:00 h in the natural grassland. The first group was the control group, which received no supplements. The second group was supplemented with concentrate (pelleted feed) based on local feedstuffs, the third group received this concentrate feed and multi-nutritional lick stone based on local feedstuffs, and the last group was supplemented with multi-nutritional lick stone only. The natural pasture was abundant and composed of *Panicum maximum* C1, *local Panicum maximum, and Tridax proc*ess; once the sheep returned from the pasture, they were led to their individual box. Water was offered to all animals at all times without limitation (ad libitum). The ingredients used to make the concentrate feed were oyster shells, common salt, orange peel, rice bran, wheat bran, palm kernel meal, and cassava meal. The nutritional composition of the supplements made is shown in Table 1.

Chemical composition of the feed supplements

The chemical composition of the feed indicates its nutritional value. Hence, to know the chemical composition of the concentrate and lick stone used in this study as the supplements, the AOAC (Horwitz and Latimer 2005) procedure was used to determine their composition in terms of dry matter (DM) (#930.15), phosphorus (P) (#946.06), calcium (Ca) (#978.05), magnesium (Mg) (#2006.03), organic matter (OM) (#942.05), ash (#942.05), and total nitrogen (TN) (#990.03) values (Table 1).

Data and statistical analysis

The data collected on each sheep included daily feed supplement intake and body weight. These data were used to calculate average feed intake, feed conversion ratio, average daily gain, feed cost, and economic feed efficiency. Parameters were compared using analysis of variance when the data followed a normal distribution and the Kruskal Wallis test if the normality assumption was not met. The Student-Newman-Keuls (SNK) post-hoc test was performed in cases of significance ($P<0.05$); all these analyses were performed in R software version 4.2.1 (R Core Team 2022). The standard deviations of the means were calculated and added to them, and the differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSION

Chemical composition of the feed supplement

Table 1 reveals that the concentrate feed contains greater amounts of organic matter and phosphorus than the multi-nutritional lick stone. The multi-nutritional lick stone has shown a good balance in organic matter, ash, total nitrogen, calcium, and magnesium. The dry matter of both supplements reveals that they were more concentrated in nutrients, in contrast to fodder. The feed supplements used were multi-nutritional lick stones and concentrate feed based on local feedstuffs. An analysis of the chemical composition revealed that the lick stone had a good balance of nutrients except for phosphorus. This difference might be explained by the lack of dicalcium phosphate or feedstuffs such as cassava peel and palm kernel meal in the lick stone, in contrast to the concentrate. Because the aim was to make the lick stones based essentially on local feedstuffs, adding commercial ingredients like dicalcium phosphate was avoided. This imbalance was not observed in the concentrate feed because of the ingredients of dicalcium phosphate, cassava peels, palm kernel meal, and orange peels. Cassava peels have been proven to be a source of phosphorus (Khalil 2022). As the ratio of phosphorus to calcium should be 1.0:1.7 because the two minerals provide bone with the necessary strength for the main activities of the sheep, such as grazing and walking (Ternouth & Coates 1997; Karn 2001), it is compulsory to find local feedstuffs rich in phosphorus that can correct this imbalance. The concentrate feed had a good nutrient balance but was less rich than the lick stone except for phosphorus and organic matter. The concentrate used lacks sources of minerals and protein such as charred bone meal, quicklime, cement, and urea, and the lesser use of oyster shells and common salt, which are a good source of minerals. The high incorporation of ingredients such as cassava peels, rice bran, and orange peel raised the organic matter composition of the concentrate feed relative to the lick stone. Compared to the feed supplements described by the studies of Babatoundé et al. (2016) and Montcho et al. (2016) and the nutritional requirements set by Noziere et al. (2018) and NRC (2007), the feed supplements used in the present study have good potential to fill the nutritional gaps of African dwarf sheep.

Feed supplement intake

Table 2 reveals the amount of the average daily feed supplement taken by the sheep of each group. The multi-nutritional lick stones were taken in lesser amounts in the presence of the concentrate feed than when served alone. Combining concentrate feed and lick stone raised the overall feed intake relative to the groups that received only one of the two supplements.

For animals, feed is essential for production and the different functions of the organism. Its quality and quantity are the primary determinants of livestock's growth performance and economic return. Throughout the study, the feed intake of sheep given the multi-nutritional lick stone was lower than that of sheep given concentrate feed because the lick stone is more compact and was made to be licked instead of eaten like concentrate feed. Even when some sheep tried to eat chunks of lick stone, its solidity did not allow them to take in a great quantity. Apart from that, the composition of the lick stone was such that even a small quantity was more concentrated in nutrients needed by the sheep. Also, the group of sheep that had both lick stone and concentrate feed increased their ingestion of concentrate feed slightly; this confirms the result of Zhao et al. (2022), who reported that the lick stones could improve the feed intake because of their ability to improve the digestibility of the diet and the passage of roughage through the gastrointestinal tract. The 18.44±4.61 g/d of lick stone intake revealed by this study is in the range of 13.3 g/d to 500 g/d reported by Zhao et al (2022). The lick stone was taken in somewhat greater amounts when served alone than combined with concentrate feed. This finding confirms the ability of animals to adjust their feed ingestion according to their nutritional need (Clauss et al. 2007; Forbes 2007). The lick stone intake (18.44±4.61 g/d) of the group fed only with lick stone was lower than the 75 g to 110 g/d reported by Yahya et al. (2022). It might be due to the average initial body weight (35 kg) of Yankasa rams used by those authors instead of dwarf sheep used in this study, which had 11.06 kg as the initial average body weight. The quantity of concentrate feed intake was close to that reported by Montcho et al. (2016), who used a multi-nutritional block for African dwarf sheep. African dwarf sheep seem to appreciate the concentrated feed in the rainy season and the multi-nutritional block in the dry season (Dokui et al. 2022).

Table 1. Chemical composition of feed supplements (% dry matter basis, unless otherwise stated)

Item	DM	OM	Ash	TN	P (g/kg)	Ca (g/kg)	Mg (g/kg)
LS	87.81	31.91	68.09	40.62	0.007	96.05	75.81
CF	86.58	78.27	21.73	17.5	8.99	54.031	14.11

LS= Multi-nutritional lick stone, CF= Concentrate feed, DM= Dry matter, OM= Organic matter, TN= Total nitrogen

Table 2. Feed supplement intake (g)

Item	CF	CF + LS			LS	P-value
		CF	LS	CF + LS		
FI_1	174.42±46.25^b	192.47±34.07^{bc}	14.33±2.79^a	218.82±17.81^c	21.26±3.62^a	<0.001
FI_2	249.79±30.87^b	267.94±19.31^b	21.87±6.53^a	274.85±16.98^b	17.55±4.36^a	<0.001
FI_3	236.16±23.68^b	237.12±54.60^b	10.20±1.57^a	254.57±46.83^b	16.51±9.34^a	<0.001
FI	220.12±31.89^b	232.51±25.93^b	15.47±2.79^a	249.41±25.93^b	18.44±4.61^a	<0.001

FI_1= Feed intake in the first month, FI_2= Feed intake in the second month, FI_3= Feed intake in the third month, FI= feed intake over the 84-day period, CF= concentrate feed, LS= multi-nutritional lick stone based on local feedstuffs, P= probability

Feed conversion ratio

Table 3 shows the amount of each feed supplement needed to produce a unit of body weight. It shows that more concentrated feed than the lick stone was needed to produce one unit of body weight. Combining concentrate feed and lick stone increased the feed supplement needed to produce one unit of body weight.

The feed conversion ratio permitted us to measure each feed supplement's potential to increase the sheep's body weight. The multi-nutritional lick stone showed better potential to improve the body weight gain of African dwarf sheep than the concentrate feed. The chemical analysis confirms the good balance of nutrients in the lick stone. The fact that the sheep fed with concentrate feed in combination with lick stone have the best feed intake but not the best feed conversion ratio proves that the concentrate feed was less convertible to body weight than the lick stone. The feed conversion ratio (6.55 to 13.63) of the concentrate reported by a previous study (Amuda and Okunlola 2020) was better than the 56.80±12.59 of the concentrate revealed by this study; this is due to the significant utilization of feedstuffs such as rice bran and cassava peels, which can limit the digestibility of the concentrate and, thus, the availability of the nutrients (NRC 2007; Noziere et al. 2018). The feed conversion ratio (9.38±0.94) of the multi-nutritional lick stone based on local feedstuffs was better than (13.2 to 38.8) reported by Hatungimana & Ndolisha (2015); this might be justified by the great use of ingredients like *Penisetum purpureum* and *Leucaena leucocephala* which are not mostly used in the lick stone.

Average daily gain

The average daily gain observed was due to the effect of forage intake and the feed supplement for those supplemented. However, it was solely due to forage intake for the non-supplemented group. The impact of supplementation needs to be evaluated.

Table 4 shows how the body weight of the sheep changed during the trial period. The table shows that the sheep fed with concentrate feed grew faster than the non-supplemented sheep, especially after the first month. The combination of concentrate feed and multi-nutritional lick stone did not significantly affect the growth of African dwarf sheep during the rainy season. Overall, the non-supplemented gained 50.54%, 30.04%, and 7.23% less weight than those supplemented with concentrate feed, combination concentrate feed-lick stone and lick stone alone.

Their average daily weight gain measures animals' growth speed. For the African dwarf sheep used in this study, the average daily gain enables us to determine the effect of each treatment on the growth performance of the sheep. The concentrate feed exhibited the potential to increase daily body weight growth faster than no supplementation. This finding is backed up by the fact that the sheep's intake of concentrated feed was greater than their intake of lick stones. The concentrate feed's composition in terms of fiber sources such as rice bran, cassava peel, and orange peels makes it a feed better suited for ruminants. Gallo et al. (2019) confirmed that a minimum of 15% nitrogen detergent fiber (NDF) is required in the feed of ruminants to improve their ruminal activity and growth performance. The concentrate feed seems more fit for this purpose than the lick stone, a source of minerals more than fiber. The average daily gain across all groups (44.96 g/day to 63.21 g/day) revealed by this study is less than 90.48 g/day reported by Amuda and Okunlola (2020). As the body weight gain is greatly determined by breed and the initial body weight, the initial average body weight of the sheep in this study (11.06 kg) was well under the 15 kg weight of the sheep used by those authors, which may explain that difference. The average daily gain across all groups (44.96 g/day to 63.21 g/day) revealed by this study was close to the gains (28.6 g/day to 57.1 g/day) reported by Aye & Adegun (2010).

Feed supplement cost and Economic feed efficiency

Table 5 reveals that both the concentrate feed alone and the concentrate feed in combination with the lick stone required a greater cost than the lick stone served alone to produce a kilogram of body weight. The lick

Table 3. Feed conversion ratio (g of feed/g of body weight)

Item	CF	CF+LS	LS	*p-value*
FCR_1	41.33±17.47[b]	110.72±25.00[c]	5.76±1.27[a]	<0.001
FCR_2	76.49±17.63[b]	104.73±18.51[c]	13.74±1.44[a]	<0.001
FCR_3	52.58±9.52[b]	90.81±9.68[c]	8.65±1.27[a]	<0.001
FCR	56.80±12.59[b]	102.09±15.00[c]	9.38±0.94[a]	<0.001

FCR_1= Feed conversion ratio for the first month, FCR_2= Feed conversion ratio for the second month, FCR_3= Feed conversion ratio for the third month, FCR= Feed conversion ratio over the 84-day period, CF= Concentrate feed, LS= Multi-nutritional lick stone base on local feedstuffs

Table 4. Average daily gain (g/day)

Item	NS	CF	CF+LS	LS	*p-value*
ADG_1	57.96 ± 10.56	63.91 ± 14.45	70.29 ± 23.78	58.33 ± 9.88	0.499
ADG_2	24.79 ± 5.80[a]	53.33 ± 19.83[b]	40.15 ± 9.09[ab]	27.22 ± 10.08[ab]	0.002
ADG_3	43.04 ± 8.65[a]	72.38 ± 12.25[b]	53.14 ± 12.25[ab]	49.34 ± 20.46[ab]	0.024
ADG	41.93 ± 6.68[a]	63.21 ± 12.67[b]	54.53 ± 8.37[ab]	44.96 ± 9.88[ab]	0.004
BWG (kg)	3.52 ± 0.56[a]	5.31 ± 1.06[b]	4.58 ± 0.70[ab]	3.78 ± 0.83[ab]	0.004

ADG_1= Average daily gain in the first month, ADG_2= Average daily gain in the second month, ADG_3= Average daily gain in the third month, ADG= Average daily gain over the 84-day period, BWG= Body weight gain over the 84-day period, CF= Concentrate feed, LS= Multi-nutritional lick stone base on local feedstuffs

Table 5. Feed supplement cost (money invested in feed supplement/kg of body weight gain)

Item	CF	CF + LS	LS	*p*-value
FC_1	200.34 ± 84.67[ab]	274.81 ± 181.38[b]	64.47 ± 16.32[a]	0.020
FC_2	391.11 ± 66.51[b]	615.08 ± 211.53[c]	108.05 ± 21.83[a]	<0.001
FC_3	294.87 ± 65.93[b]	550.14 ± 138.89[c]	49.55 ± 9.13[a]	<0.001
FC	295.44 ± 62.63[b]	480.01 ± 122.43[c]	74.02 ± 10.37[a]	<0.001

FC_1= Feed cost during the first month, FC_2= Feed cost during the second month, FC_3= Feed cost during the third month, FC= Feed cost over the 84-day period

stone required less cost to produce a unit of body weight than the other options.

Overall, the price of one kg of all feed supplements was under the average one kilogram of body weight. All the feed supplements used in this study can provide the breeders with a good economic return; this confirms the fact that the local feedstuffs have nutritional potential and should be explored to reduce the feed cost of the dwarf sheep to improve the economic return for breeders (Dokui et al. 2022). However, the lick stone was more affordable, whereas the combination with concentrate was less affordable for the breeders. Besides the feed conversion ratio influencing the feed cost, the lick stone had the best feed conversion ratio.

Table 6 shows the amount of money gained for one unit of money invested in feeding. The analysis of those values in each group revealed that the lick stone allowed the best economic return. The combination of concentrate feed and lick stone didn't significantly affect the economic return of African dwarf sheep during the rainy season relative to providing no supplement, whereas the lick stone alone did have a significant effect.

Adopting a new feed that is not economically viable is impossible. To prevent this, in animal feed and nutrition, the economic return of every new feed developed is evaluated using economic feed efficiency (EFE); this allows us to determine the amount of money gained or wasted for one unit of money invested in the feed. It enables researchers to know if the economic return has resulted from the efficacy of the feed as measured by the body weight gained. This study has revealed that the lick stone alone provided the best economic return compared to the concentrate feed, the concentrate combined with the lick stone, or no supplement. The fact that the EFE of the lick stone was the best confirmed that the nutrients in the lick stone were more convertible, as shown by the feed conversion ratio. It leads the lick stone to be more advantageous for the sheep, leading to this result. It is a waste of money to give combined concentrate feed and lick stone for African dwarf sheep during the rainy season because that is not economically sustainable. The fact that the lick stone allowed the best economic return might be explained by the fact that during the rainy season, in

Table 6. Economic feed efficiency (money gained / money invested in feed supplement)

Item	CF	CF+LS	LS	*p*-value
EFE_1	8.94±4.17[a]	7.62±4.17[a]	26.01±6.52[b]	<0.001
EFE_2	4.87±2.05[a]	2.95±0.87[a]	23.32±2.78[b]	<0.001
EFE_3	6.83±2.38[a]	4.34±0.61[a]	36.14±7.14[b]	<0.001
EFE	6.88±2.40[a]	4.97±1.14[a]	28.49±2.53[b]	<0.001

EFE_1= Economic feed efficiency in the first month, EFE_2= Economic feed efficiency in the second month, ECE_3= Economic feed efficiency in the third month, EFE= Economic feed efficiency over the 84-day period, CF= Concentrate feed, LS= Multi-nutritional lick stone base on local feedstuffs, *p*= probability

contraction to the dry season, fodder is available but has a deficiency in protein and minerals like calcium and magnesium (Azando et al. 2022; Olomonchi et al. 2022). The EFE (4.97±1.14) revealed by this study was better than (-0.28 to 0.14) reported by the study by Inweh et al. (2021) for West African dwarf goats. That might be justified by the chemical composition of the feed in this study, which is better balanced than theirs. The fact that the growth performance of goats is sometimes lower than sheep's might also contribute to this difference between the EFEs (Bosso et al. 2007).

CONCLUSION

The aim of this study was to make different feed supplements and determine which one was better for the growth and economic performance of African dwarf sheep during the rainy season. The results have proved that the multi-nutritional lick stone based on local feedstuffs can increase feed intake. The lick stone was better converted to the body weight than the concentrate feed. Concentrate feed allowed for better growth performance than non-supplemented sheep, but it is not economically viable. The multi-nutritional lick stone based on local feedstuffs has allowed the best economic return. It is a waste of money to combine feed concentrate and lick stone for the African dwarf sheep in the rainy season because that decreases the economic return. The multi-nutritional lick stone based on local feedstuffs is economically viable for African dwarf sheep during the rainy season in Benin. As the climate conditions of the West African countries are close to each other, this study shall help the remaining countries to choose wisely the type of feed supplement they should use during the rainy season.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Physiological Responses of Saanen Does: A Comparative Study of Traditional Wooden and Aluminium Galvanized Iron Housing System in a Tropical Climate

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ABSTRAK

Omar F, Kari A, Awang R, Komilus CF. 2025. Respon fisiologis kambing Saanen betina: studi perbandingan sistem kandang kayu tradisional dan kandang aluminium besi galvanis di iklim tropis. JITV 30(1):59-67. DOI:http://dx.doi.org.10.14334/jitv.v30i1.3462.

Respon fisiologi merupakan indikator kunci untuk menilai kesejahteraan hewan ternak dan berfungi sebagai referensi penting dalam mendeteksi kondisi abnormal yang disebabkan oleh faktor lingkungan yang tidak sesuai. Penelitian ini bertujuan untuk mengevalusi respon fisiologi kambing Saanen betina berusia 9 sehingga 12 bulan dengan berat badan 23.54±1.15 kg, ditempatkan secara acak pada kandang kayu traditional (T_0) dan kandang aluminium besi galvanis (T_1). Setelah masa adaptasi selama 14 hari, variabel termoregulator seperti suhu rektal (RT), detak jantung (HR) dan laju pernapasan (RR) diukur setiap minggu selama 90 hari. Kadar kortisol serum juga diukur pada minggu ke-0, ke-4, ke-8 dan ke-12. Selain itu, parameter iklim micro seperti suhu lingkungan (AT) dan kelembapan relatif (RH) dicatat setiap minggu di setiap sistem kandang untuk menghitung indeks suhu-kelembapan (THI). Jenis kandang tidak berpengaruh signifikan (P>0.05) terhadap parameter iklim, namum variasi diamati sepanjang hari. AT dan THI dicatat tinggi pada sore hari, sedangkan Rh dicatat tinggi pada pagi hari pada kedua jenis rumah. Khususnya, RR dan HR secara signifikan (P<0.05) dipengaruhi oleh sistem kandang tetapi RT tetap tidak terpengaruh. Namun, kadar kortisol serum tidak terpengaruh oleh jenis kandang. Temuan ini menunjukkan bahwa kedua sistem kandang mungkin menyebabkan stres pada hewan, namun T_1 memberikan kondisi pembuangan panas yang lebih baik dan memiliki biaya pemeliharaan yang lebih rendah. Dengan demikian, T_1 menjadi alternatif yang lebih sesuai daripada T_0 di iklim tropis, yang berpotensi meningkatkan kesejahteraan dan kenyamana kambing Saanen.

Kata Kunci: Aluminium, Detak Jantung, Respons Fisiologis, Suhu, Kayu

ABSTRACT

Omar F, Kari A, Awang R, Komilus CF. 2025. Physiological responses of Saanen does: A comparative study of traditional wooden and aluminium galvanized iron housing system in a tropical climate. JITV 30(1):59-67. DOI:http://dx.doi.org.10.14334/jitv.v30i1.3462.

Physiological responses are key indicators that serve as valuable references for detecting abnormal conditions caused by unsuitable environmental factors. This study aimed to evaluate the physiological responses of Saanen in two different housing systems. Twelve female Saanen does, ages 9 to 12 months with an average weight of 23.54±1.15 kg, were randomly assigned to either a traditional wooden house (T0) or an aluminum galvanized iron house (T1). After 14 14-day adaptation period, thermoregulator variables, including rectal temperature (RT), heart rate (HR), and respiratory rate (RR), were measured every week over 90 days. Serum cortisol levels were also measured at weeks 0, 4, 8, and 12. Additionally, microclimate parameters, ambient temperature (AT), and relative humidity (RH) were recorded in each housing system weekly to calculate the temperature-humidity index (THI). Housing types did not significantly (P>0.05) affect the environmental parameters, but variations were observed throughout the day. High AT and THI were recorded in the afternoon, while RH was higher in both houses in the morning. Notably, RR and HR were significantly (P<0.05) affected by the housing system. However, RT remained unaffected. Serum cortisol levels, however, were unaffected by housing type. These findings suggest that both housing systems may induce animal stress, but T1 provided better heat dissipation and lower maintenance costs. Thus, T1 presents a more suitable alternative to T0 in tropical climates, potentially improving the welfare and comfort of Saanen does.

Key Words: Aluminum, Heart Rate, Physiological Responses, Temperature, Wood

INTRODUCTION

Saanen goats are widely recognized as one of the leading dairy goat breeds, prized for their high milk production and adaptability to diverse environmental conditions (Gökdai et al. 2020). However, maintaining optimal physiological health and welfare in tropical climates is challenging due to environmental stressors such as high temperature and humidity. Housing design is critical in mitigating these stressors influencing the livestock's physiological responses, behaviors, and overall well-being (Nunes et al. 2014). Inadequate housing can lead to numerous issues in dairy goats. For instance, Simões and Gutiérrez (2017) highlight that inadequate nutrient intake and environmental conditions can lead to metabolic diseases affecting overall health and production in dairy goats. Additionally, under heat stress, goats exhibit increased respiratory rates and altered metabolic processes, which can hinder hormone production essential for lactation, such as prolactin (Fonseca et al. 2016; Henn et al. 2021)

In Malaysia, smallholder farmers have traditionally relied on wooden housing systems due to their availability, low initial cost, and ease of construction. However, wood structures are prone to moisture absorption, insect infestation, and rapid decomposition in tropical environments, which can compromise the health and welfare of animals (Awang et al. 2020). Over time, these issues can lead to higher maintenance costs and reduced durability, raising concerns about the long-term sustainability of this housing option.

In contrast, aluminum galvanized iron houses are emerging as a modern alternative with numerous advantages, including outstanding durability, insect resistance, and lower maintenance requirements (Awang et al. 2020). These systems also have the potential to provide better ventilation and heat dissipation, which are crucial in hot, humid climates (Awang et al. 2020). Despite these benefits, the feasibility of this housing system on the physiological parameters of livestock, particularly in tropical climates, remains largely unexplored. The knowledge gap is particularly significant given the importance of physiological responses as animal welfare indicators.

The physiological responses can be one of the simple indicators of access to animal welfare in livestock. According to the International Physiology Journal (2023), physiological responses are the measurements of living organisms' characteristics that describe the functions and processes occurring within the body. These include heart rate, blood pressure, body temperature, respiratory rate, oxygen saturation, muscle strength, metabolic rate, and hormonal levels. Febretrisiana et al. (2022) stated that physiological responses can be used as references to assess abnormal livestock conditions caused by unsuitable livestock environments. Monitoring these physiological parameters is essential in assessing the effectiveness of housing designs in providing a comfortable and stress-free environment for livestock. By ensuring these physiological responses remain within normal ranges, the farmer can safeguard the well-being of their animals, ultimately leading to better health outcomes and higher productivity.

Heat stress is one of the most significant challenges in livestock management, particularly in tropical climates. This condition occurs when environmental factors such as high temperature, humidity, solar radiation, and wind speed exceed an animal's ability to regulate its body temperature (Sejian et al. 2018). Heat stress can elevate body temperature, respiratory rate, and heart rate, reducing feed intake and overall performance. These effects depend on various factors, including the animal's age, breed, reproductive status, and environmental conditions, such as air temperature fluctuations and altitude (Macías-Cruz et al. 2016; Abecia et al. 2017). Consequently, proper housing and environmental management are vital in mitigating heat stress and supporting optimal physiological function in livestock.

Given the importance of housing systems in moderating these physiological responses, this study aims to fill the gap in knowledge regarding the impact of traditional wooden versus aluminum galvanized iron housing on the physiological well-being of Saanen in a tropical climate. By systematically evaluating the physiological responses of Saanen does under both housing conditions, this research seeks to provide critical insights into the suitability of these housing systems in tropical livestock farming. The findings from this study have the potential to inform better housing designs that improve animal welfare, promote sustainable farming practices, and offer cost-effective solutions for smallholder farmers.

MATERIALS AND METHODS

Ethics approval

The research protocol for this study was approved by the UniSZA Animal and Plant Research Ethics Committee (UAPREC) under approval number UAPREC/008/003. Ethical considerations were made to ensure that the animals were handled with care and that all procedures adhered to the animal welfare guidelines.

Study design

The experiment was conducted over 90 days (12 weeks) at Ladang Universiti Sultan Zainal Abidin (UniSZA), Pasir Akar. The farm is situated at 102.471009 longitude and 5.643865 latitude. The average annual ambient temperature ranged from 25.7°C to 30.2°C, with an average humidity of 80.0%. This area received 181.4 mm of annual rainfall. The primary objective of this study is to evaluate the physiological responses of Saanen houses in two housing systems: traditional wooden and aluminum galvanized iron houses. The first housing system, T1, was a traditional wooden structure with slatted wooden flooring and wooden walls with an asbestos roof, utilizing the existing facilities at the farm. The second housing system, T2, was a modern design composed of aluminum and galvanized iron, featuring an aluminum mesh floor and walls made of aluminum galvanized iron with a zinc roof. Each pen's dimensions adhered to the standard space allowance of 0.75–1.0 square meters per goat, as recommended for goats aged 9 to 12 months (Acharya et al. 2017). Each pen was equipped with water drinkers and feed troughs to provide the animals with a consistent food and water supply.

Experimental animals

Twelve healthy, non-lactating, young adult Saanen goats, aged 9 to 12 months and averaging 23.54±1.15 kg, were randomly selected from the UniSZA farm flock. The goats were divided into two groups of six, one housed in traditional wooden pens (T_0) and the other in aluminum galvanized iron pens (T_1). Each goat was tagged for individual identification. All goats had a daily diet consisting of 40% commercial concentrate in the morning and 60% *Brachiaria humidicola* in the morning and afternoon. Table 1 shows the proximate analysis of feed used during the experiment. Water was provided ad libitum to both groups. Prior to data collection, the goats underwent a two-week acclimation period in their assigned housing to allow for adjustment to the new environmental conditions.

Microclimate variables

The microclimatic conditions within the two housing systems were asses by measuring the ambient temperature (AT) and relative humidity (RH) were measured using a digital thermohydrometer (SNDWAY SW572, China) with a measurement range of -20°C to 60°C (±0.3°C) for temperature and 0% to 100% (±3%) for relative humidity. Measurements were taken at three-time points daily: 8:30 a.m., 1:00 p.m., and 4:30 p.m., on the same days when physiological data were collected. The recorded AT and RH values were used to calculate the temperature-humidity index (THI), a commonly used indicator of heat stress, to provide a more comprehensive assessment of thermal comfort in each housing system. THI was calculated using the equation provided by Habeeb et al. (2018):

$$THI = (1.8 \times AT + 32) - ((0.55 - 0.0055 \times RH) \times (1.8 \times AT - 26))$$

This index provided a more comprehensive understanding of the environmental comfort levels within the wooden and aluminum galvanized iron housing systems.

Thermoregulatory variables

After the two-week adaptation period, thermoregulatory variables, including respiratory rate (RR), heart rate (HR), and rectal temperature (RT), were recorded at the same three-time points daily (8:30 a.m., 1:00 p.m., and 4:30 p.m.). The goats were restrained using a low-stress technique, positioned in the corner of the pen with their chin gently held to prevent movement. This procedure took approximately 1 to 3 minutes per goat.

Rectal temperature (RT) was measured by inserting a flexible-tip digital thermometer into the goat's rectum for 30 seconds. Heart rate (HR) was measured using a stethoscope placed on the left thoracic region over the aortic arch. Heartbeats were counted for 15 seconds and multiplied by four to obtain beats per minute (de Oliveira et al. 2018; Ferreira et al. 2020) . Respiratory rate (RR) was measured by counting the number of abdominal movements (breaths) for 15 seconds and multiplying by four to get breaths per minute. These measurements provided valuable insights into the goats' thermoregulatory responses to the varying environmental conditions within each housing system.

Blood sampling and analysis

Blood samples were collected from all goats via jugular venipuncture using a 21G needle at four-time points: week 0, week 4, week 8, and week 12. Blood was drawn into plain tubes immediately before morning feeding at 8.30 a.m. and then allowed to sit at room

Table 1. Proximate analysis of feeds used during the experiment

Parameters (% Dry Matter basis)	*Brachiaria humidicola*	Concentrate feed
Dry matter	50.4	87.0
Crude protein	3.4	14.0
Crude fat	<0.1	4.0
Crude fibre	15.2	20.0

temperature for 4 hours. The blood was centrifuged at 600xg for 5 minutes at room temperature using a tabletop centrifuge, after which the serum was separated and stored at -20°C for later analysis. Serum cortisol concentrations were determined using a commercial ELISA kit (Elabscience Biotechnology, China). The standard diluents were prepared and diluted according to the manufacturer's instructions before being added to the standard wells. In the test sample wells, 50 µL of serum sample was added, followed by 50 µL of biotinylated detection antibody working solution. The plate was sealed and incubated for 45 minutes at 37°C. After incubation, the liquid was discarded, and each well was filled with washing buffer, mixed gently, and shaken for 1–2 minutes before discarding the liquid. This washing step was repeated three times, and the wells were tapped onto absorbent paper to remove excess liquid. Following this, 100 µL of HRP conjugate working solution was added to each well, and the plate was sealed and incubated for 15 minutes at 37°C. The washing procedure was repeated five times, as described earlier. Next, 50 µL of substrate reagent was added to each well, and the plate was sealed and incubated for 10 minutes at 37°C, protected from light to prevent degradation of the chromogens. After incubation, 50 µL of stop solution was added to each well. Finally, the optical density (OD) values were measured at 450 nm using a microplate reader, and cortisol concentrations were determined by plotting a standard curve based on the standard concentrations and their corresponding OD values.

Statistical analysis

Statistical analysis was performed using SPSS version 27. Descriptive statistics, including means and standard deviations, were calculated for all microclimatic (AT, RH, and THI) and physiological responses (RT, HR RR) variables. A T-test was used to compare the microclimate variable and thermoregulatory responses between two housing systems within each of the times of day (morning, afternoon, and evening). A one-way ANOVA was conducted to evaluate differences in microclimatic and physiological response variables across three daily time points (morning, afternoon, and evening) within each housing system. Significant was set at $P < 0.05$. The correlation between THI with RT, RR, and HR was analyzed.

RESULTS AND DISCUSSION

Microclimate variables

Table 2 presents the microclimate variable observation across different housing systems at various times of the day in Saanen. The microclimate analysis showed that RH in the morning was significantly higher in aluminum galvanized iron housing (78.3 ± 1.41%) compared to traditional wooden housing (72.1±2.00%) with P value = 0.02. This difference could be attributed to the material properties of aluminum galvanized iron, which retains less moisture

Table 2. The microclimate variable across different housing systems at different times of day in Saanen does

Microclimate parameters	Times of day (N=3)	Housing system (N=2) Traditional wooden	Aluminium galvanized iron	Pooled mean	P value
RH (%)	Morning	$72.1±2.00^{a}$	$78.3±1.41^{a}$	$75.2±1.32^{a}$	0.02*
	Afternoon	$58.7±2.00^{b}$	$60.0±1.56^{b}$	$59.3±1.24^{b}$	0.6
	Evening	$72.3±4.61^{a}$	$72.1±4.72^{a}$	$72.3±3.22^{a}$	0.95
	Overall	67.8±2.08	70.1±2.14	69.0±1.49	0.44
AT (°C)	Morning	$30.6±0.71^{a}$	$28.9±0.55^{a}$	$28.9±0.55^{a}$	0.06
	Afternoon	$35.2±0.48^{b}$	$34.2±0.27^{b}$	$34.1±0.29^{b}$	0.06
	Evening	$31.6±1.12^{a}$	$31.5±1.17^{c}$	$31.6±0.79^{a}$	0.98
	Overall	32.5±3.32	31.5±3.29	32.0±0.41	0.24
THI	Morning	$82.5±0.77^{a}$	$80.8±0.6^{a}$	$81.7±0.53^{a}$	0.11
	Afternoon	$86.9±0.41^{b}$	$85.7±0.66^{b}$	$86.3±0.26^{b}$	0.02*
	Evening	$83.7±0.95^{a}$	$83.5±1.04^{b}$	$83.6±0.69^{c}$	0.91
	Overall	84.4±3.05	83.3±3.09	83.8±0.38	0.18

Means with different superscripts in the column differed significantly (P<0.05) than traditional wooden housing, leading to increased indoor humidity due to reduced ventilation or air exchange (Salonvaara et al. 2004). However, the lack of significant differences during the afternoon and evening indicates that both housing systems maintain similar humidity levels.

Regarding AT, there were no significant differences (P>0.05) between the housing systems on any day. However, traditional wooden housing consistently recorded slightly higher temperatures with mean AT in traditional wooden housing (32.5±3.32°C) and aluminum galvanized iron housing (31.5±3.29°C), but these differences were minimal; this suggests that both houses provided similar environments throughout this experiment.

While THI was significantly lower in aluminum galvanized iron housing during the afternoon (85.7±0.66) compared to traditional wooden housing (86.9±0.41) with a P value= 0.02, this reduction in THI during the afternoon could be attributed to aluminum's reflective and conductive properties, which reduce heat (Pradhan et al. 2021). No significant differences in THI were noted during the morning and evening in both housing types (P>0.05), suggesting that overall, both housing systems maintained similar THI levels.

The current result suggests stressful conditions were present in both housing systems in Saanen. However, aluminum galvanized iron provides more suitable conditions, allowing Saanen to experience sensible and latent heat loss. According to Sejian et al. (2021), the optimal AT range for goats is between 6°C to 27°C with RH 60% to 80%. In a recent study, the average AT in both housing systems exceeded this range (28.9±0.55°C to 35.2±0.48°C), while RH was within the normal range except in traditional wooden housing during the afternoon (58.7±2.00%). It was also observed that in both houses, the highest AT and lowest RH recorded during this study were in the afternoon. These findings align with Borges and Rocha (2018) and Singh et al. (2017), who also reported peak AT and reduced RH in the afternoon.

The combined values of RH and AT are critical for assessing heat stress and thermal comfort in livestock (Habeeb et al. 2018; Sejian et al. 2021). According to Aleena et al. (2018), THI values of 70 or less are considered comfortable for goats, values above 75 indicate heat stress, and those exceeding 85 suggest severe heat stress. This study's THI values ranged from 80.8±0.6 to 86.9±0.41, indicating that the goats experienced low to severe heat stress. However, Silanikove & Koluman (2015) and Srivastava et al. (2021) proposed that THI values above 90 represent severe to extreme heat stress. The discrepancy between these thresholds could be attributed to differences in the study locations, climate, or goat breeds.

Notably, goats in both housing systems experienced severe heat stress during the afternoon due to high AT and low RH. Such stressful conditions can prompt goats to alter their behaviors and physiological responses as adaptive mechanisms to cope with the environment. Due to their morphological and physiological characteristics, goats are generally more resilient to heat stress than cattle and sheep (Sejian et al. 2021).

Thermoregulatory variables

The objective of the current study was to determine the physiological responses of the Saanen in different types of housing: traditional wooden and aluminum galvanized iron houses. Table 3 shows the variation of the RT, RR, and HR in traditional wooden and aluminum galvanized iron houses at various times of the day. RT of Saanen does in both housing systems showed no significant differences (P > 0.05) at any time of the day. The overall RT was similar between the traditional wooden (39.4±0.02°C) and aluminum galvanized iron housing (39.5±0.02°C). In contrast, significant differences were observed between housing systems (P<0.05). Does in the wooden system exhibited higher (61.9±1.68 breaths/min) than those in the aluminum galvanized iron system (53.9±1.54 breaths/min). In both housing systems, RR peaked during the afternoon compared to morning and evening values. HR also differed significantly (P<0.05) between housing systems, with higher HR observed in the aluminum galvanized iron housing (101.1±1.30 beats/min) compared to the traditional wooden housing (94.8±1.38 beats/min). However, no significant differences were observed in HR at specific times of the day (P>0.05). These patterns are consistent with previous studies by Borges and Rocha (2018), Beyleto et al. (2022), and Singh et al. (2023), which reported an afternoon peak in RR compared to other times.

Rectal temperature is a critical indicator of livestock's core body temperature and heat stress (Rejeb et al. 2016). The lack of significant differences in RT between the two housing systems suggests that both environments provided similar thermal regulation for Saanen. The lower morning temperature suggests that both housings effectively maintained a stable thermal environment during more incredible hours, keeping the goats in a thermoneutral state where their body temperature was well-regulated and within a healthy range. However, the significant rise in body temperature during the afternoon in both houses can be attributed to the elevated THI. By evening, as THI decreased, the goat's body temperature also lowered, yet it remained within the 37°C–41°C range, as Okoruwa (2014) reported.

RR and HR are vital indicators of both the environmental conditions and the animal’s health status. Under normal, unstressed conditions, goats' respiratory and heart rates typically range between 12 breaths/min to 20 breaths/min (Gupta & Mondal, 2021) and 16 beats/min to 34 beats/min (Samimi et al. 2018), respectively. However, this rate can increase significantly due to various factors such as heat stress, physical activity, and illness. In the present study, RR was significantly influenced by both housing systems at different times of the day. It reflects the goats' effect on maintaining thermal balance in varying environmental conditions. For instance, studies by Ribeiro et al. (2016) and (Silanikove 2000a) reported that goats typically increase their RR in response to high AT to dissipate excess body heat. The observed respiratory rates in the current study align with these physiological responses, particularly during the afternoon when environmental temperature peaks and heat stress are most pronounced, consistent with Borges & Rocha (2018) and Santos et al. (2021).

According to (Silanikove 2000a; Silanikove, 2000b), the severity of heat stress can be qualified according to respiratory rate (breaths/min) (low: 40–60, medium: 60–80, high: 80–120, and severe: >200). The recent study indicates that the goats in the traditional wooden house experienced medium heat stress while aluminum galvanized iron experienced low heat stress; this could be due to the house's material, which caused the high RR in a traditional wooden house. While wood offers some insulation, it also retains heat and limits air circulation (Michaud et al. 2020), creating a warmer environment for the goats. As a result, goats in traditional wooden houses showed higher RR as they tried to cool down. In contrast, aluminium galvanized iron house reflects heat better and promotes airflow. Increased air movement help lower the effective temperature experience by goats (Jacobson 2013). This environment allows them to maintain a lower respiratory rate, reducing heat stress. Thus, an elevated RR in goats is a clear indicator of thermal discomfort due to heat stress.

According to Heath & Olusanya (1985), HR is the normal beat rate of the arteries as blood is pushed through them to heart. HR can be altered due to animals' biological activities or an external factor such as temperature. Interestingly, HR increases during heat stress to dissipate heat to its surroundings by enhancing blood flow to the body's surface (Shilja et al. 2016). The goat's physiological adaptation HR was 106.7±15.27 beats/min (de Souza et al. 2014). In a recent study, the HR was still below the range of physiological adaptation. Interestingly, the HR rate remains consistent in the morning and afternoon but increases slightly in the evening. This result is inconsistent with previous study by Singh et al. (2017) and Borges & Rocha (2018), where the HR peaks in the afternoon in their study. This evening's HR increase in this study, despite the cooling external temperatures, could be due to the cumulative effect of the day's heat stress, indicating the goats were still recovering from the afternoon's high temperatures, leading to a sustained elevation in HR as their bodies continue to cool down.

Table 3. Thermoregulatory variables of Saanen does (N=12) in different housing system at various times of day

Thermoregulatory variables	Times of day (N=3)	Housing system (N=2)			
		Traditional wooden	Aluminium galvanized iron	Pooled mean	P value
RT (°C)	Morning	$39.3±0.03^{a}$	$39.4±0.04^{a}$	$39.4±0.03^{a}$	0.384
	Afternoon	$39.5±0.03^{ab}$	$39.5±0.03^{ab}$	$39.5±0.02^{ab}$	0.41
	Evening	$39.4±0.03^{b}$	$39.5±0.04^{b}$	$39.5±0.03^{b}$	0.19
	Overall	39.4±0.02	39.5±0.02	39.4±0.02	0.09
RR (Breath/min)	Morning	$49.7±2.23^{a}$	$43.6±1.82^{a}$	$46.6±1.46^{a}$	0.04
	Afternoon	$72.7±2.66^{b}$	$63.6±2.97^{b}$	$59.5±2.04^{b}$	0.02
	Evening	$64.3±3.02^{b}$	$55.2±2.67^{c}$	$31.6±2.04^{c}$	0.03
	Overall	61.9±1.68	53.9±1.54	57.7±1.16	<0.001
HR (Beats/min)	Morning	$96.5±2.55^{a}$	$100.3±2.42^{a}$	$98.5±1.76^{a}$	0.28
	Afternoon	$94.5±2.34^{a}$	$100.1±1.98^{a}$	$97.4±1.54^{a}$	0.07
	Evening	$93.2±2.29^{a}$	$102.9±2.28^{a}$	$98.3±1.67^{a}$	0.003
	Overall	94.8±1.38	101.1±1.30	98.1±0.96	<0.001

Means with different superscripts in the column differed significantly (P<0.05)

Table 4. Relationship between temperature humidity index and rectal temperature, respiratory rate, and Saanen's heart rate in both houses

Correlated parameter	Type of houses	
	Traditional wooden	Aluminium galvanized iron
Temperature humidity index and mean rectal temperature	0.483**	0.386**
Temperature humidity index and mean respiratory rate	0.489**	0.438**
Temperature humidity index and male heart rate	-0.007	0.172*

**Correlation significant at the level P<0.01, *Correlation significant at level P<0.05

Table 5. Cortisol concentration of Saanen is in different types of houses.

	Type of houses	Weeks				Overall mean	P-value
		0	4	8	12		
Cortisol (ng/ml)	Traditional wooden	87.82±1.70	90.17±0.16	90.32±0.47	89.18±0.60	89.37±0.49	0.375
	Aluminium galvanized iron	89.49±0.82	87.80±0.82	87.35±0.91	89.02±0.61	88.42±0.47	

Table 4 shows the relationship between THI and RT, RR, and HR of Saanen in both types of houses. The results show that both housing systems exhibit a medium correlation with RT and RR; this suggesting as THI increases, the RR and RT also increase, indicating a thermoregulatory response to heat stress. Similar findings have been reported by Srivastava et al. (2021), who demonstrate that THI directly influences the RT and RR in small ruminants. Polsky and von Keyserlingk (2017) stated that elevated RR could be a significant stress indicator in evaluating thermally stressed animals as it represents a key mechanism for dissipating excess body heat through evaporating cooling (Shilja et al. 2016) While for the HR, in the aluminum galvanized iron house, have small positive correlation with THI indicating the HR increases slightly with heat stress, possibly as a compensatory mechanism to maintain thermoregulatory and blood flow, as describe by (Gupta et al. 2013). In contrast, in traditional wooden houses, HR did not show a correlation with THI, which may suggest a less pronounced effect of thermal load in this environment. Interestingly, this finding contradicts Srivastava et al. (2021), who observed a highly positive correlation between HR and THI in their study.

Cortisol concentration

Cortisol is the primary stress-related hormone used to measure goat stress (Silanikove, 2000a). When the goats are exposed to stress, their hypothalamic-pituitary-adrenal (HPA) axis is activated, which causes an increase in blood cortisol concentration. Cortisol secretion induces physiological adaptations that equip animals to abolish stress-induced effects (Adenkola & Ayo, 2010).

Table 5 shows the concentration of Saanen's cortisol in different types. The mean concentration observed in Saanen was higher than reported (30 to 46 ng/ml) by Singh et al. (2023). This elevated concentration may reflect the additional thermal load associated with Malaysia's tropical climate, characterized by high ambient temperatures and relative humidity. Similarly, Idris et al. (2024) reported that exposure to higher THI levels led to increased fecal cortisol concentrations in beef cattle, emphasizing the impact of thermal stress.

Interestingly, the type of houses did not influence the serum concentration of cortisol between the two types of houses where the Saanen experienced the same level of stress. However, as Hydbring-Sandberg et al. (2022) noted, cortisol alone may not provide a comprehensive measure of stress. Evaluating stress responses requires complementary indicators, such as thermoregulatory variables or additional hormonal markers, to ensure a holistic understanding. Further studies are needed to examine the interactions between housing systems, environmental conditions, and stress physiology. This approach could provide insights into optimizing goat welfare and productivity in hot climates while minimizing stress-related effects.

CONCLUSION

In conclusion, this study suggests that both housing types present stressful conditions for the goats, particularly in the afternoon. However, the aluminum galvanized iron house provided more suitable and comfortable conditions, aiding both sensible and latent heat loss. Goats housed in this system showed improved thermal comfort and welfare, suggesting its potential to enhance productivity and growth performance in tropical climates like Malaysia. This housing system is a viable alternative to traditional wooden housing. Future research should focus on the long-term feasibility and performance of aluminum galvanized housing to further validate its effectiveness over time.

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   The author is recommended to use the Mendeley Program (http://www.mendeley.com) and the citation style of Taylor & Francis - Council of Science Editors (author-da e). Mendeley's program aims to avoid mistakes in citations and reference writing. Cited references are preferably 80% of the primary article in the last ten years. They should not be from unpublished papers, except thesis and dissertation, such as practical guidance and research reports. The download is allowed if it is from an electronic magazine, genome database, or patent.

Citation in the references:

The literature in the reference is written alphabetically based on the author's name. The same author is written sequentially, starting from earlier order.

Example of reference writing

Primary paper:

Barbato O, De Felice E, Todini L, Menchetti L, Malfatti A, Scocco P. 2021. Effects of feed supplementation on nesfatin-1, insulin, glucagon, leptin, T3, cortisol, and BCS in milking ewes grazing on semi-natural pastures. Animals. 11:682. DOI:10.3390/ani11030682.

Book:

a. Alshelmani M, Abdalla E, Kaka U, Basit M. 2021. Advances in poultry nutrition research. In: Kumar Patra A, editor. Adv Poult Nutr Res. London (UK): IntechOpen; p. 19–32. DOI: 10.5772/intechopen.91547.

b. Reece W. 2015. Respiration in mammals. New Jersey (USA): Willey-Blackwell.

c. Van Soest P. 2018. Nutritional ecology of the ruminant. 2nd ed. New York (USA): Cornell University Press.

Proceeding:

Damayanti R, Wiyono A, Dharmayanti N. 2021. Pathogenicity study of ducks infected with a local isolate of highly pathogenic avian influenza-H5N1-clade 2.3. . In: Inounu I, Priyanti A, Burrow H, Morris S, Min R, Suhubdy, Sutaryono Y, editors. Proc 4th Int Semin Livest Prod Vet Technol. Bogor (Indones): Indonesian Center for Animal Research and Development; p. 277–288.

Thesis:

Mwasame DB. 2020. Analysis of the socioeconomic contribution of donkey ownership and use to houshold livelihoods in Kiambu country, Kenya (Thesis). Nairobi (KE). University of Nairobi

Electronic magazines:

Maranga B, Kagali R, Omolo K, Sagwe P. 2022. Effect of growth substrates on water quality, catfish (*Clarias gariepinus*) culture, and spinach (*Spinacia oleracea*) propagation under the aquaponic system. Livest Res Rural Dev.:82. http://www.lrrd.org/ lrrd34/9/3482mara.html.

Institution:

a. [PSA] Philippine Statistics Authority. 2016. Dairy Industry Performance Report, January – December 2015. Quezon City (Philiphine): Philippine Statistics Authority. P. 1-11
b. [FAO] Food and Agriculture Organization. 2021. Gateway to dairy production and products. Food Agric Organ United Nations. [accessed August 10, 2021]. https://www.fao.org/dairy-production-products/production/feed-resources/en/.

Patent:

Raab RM, Lazar G, Shen B. 2022. AGRIVIDA Inc, assignee. Engineered phytases in animal feed. 2022 Feb 8.

10. **Citation in text:**

The citation consists of the author's last name and publication year.

Example:

a. One author: .... ranging from 84 to 135 per minute (Scott 2015). Scott 2015 stated..........
b. Two authors: .... in glucocorticoid production, primarily cortisol (Narayan & Parisella 2017). Narayan & Parisella (2017) stated that stress caused an increase in ……
c. Three authors or more: .... in milk production without affecting the ewe's weight (Barbato et al. 2021). Barbato et al (2012) reported .....
d. The same author cited from 2 different papers: (Purwadaria et al. 2022a, 2022b).
e. The author with the same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
f. Several different authors are written consecutively: (Damayanti et al. 2021; Leonard et al. 2022; Motlagh RK 2022).
g. Institution: FAO (2021)............

11. **Table:**

a. The standard word used is Times New Roman with 1 space distance and 11 of the font size.
b. The title is a simple, clear, and understandable sentence without reading the manuscript.
c. Each column from the table should have a heading. Its unit is separated from the title by a comma, in parentheses, or at its bottom.
d. The table description is written under the table with 1 space distance and 11 of the font size. The data source is written under the table or in the table in its header.
e. The dividing line is made in the form of horizontal.

12. **Figure and graphic:**

a. The title uses Times New Roman with 1 space distance and 11 of the font size. It is a simple and clear sentence that is laid under the figure or graphic.
b. Line in the graphic should show clearly the difference between one and others if there is more than one curve.
c. Clear contrast figure with proportionate size and high resolution to present the best performance.
d. Write a figure or graphic source under the title.

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2. The complete manuscript is sent in three copies to the Editorial Board of IJAVS and its electronic file, or by online:

http://medpub.litbang.pertanian.go.id/ index.php/jitv.

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