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PREFACE

In this edition, Volume 28 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:

“Influence of Sperm Number and Antioxidant Melatonin in Extender on the Quality of Post-Thawing Sheep Spermatozoa”; “Nuclear Maturation Competence and Pronuclear Formation in Ovine Oocytes Supplemented with *L-ergothioneine* in Maturation Medium”; “Rumen Fermentation Profiles of Protein-Energy Synchronization Index-Based Ration: an *In Vitro* Study”; “Phylogeography of the Maleo Senkawor (*Macrocephalon maleo* Sal. Muller 1846) Based on Cytochrome B Gene in Sulawesi And Their Sex Determination using Molecular Sexing”; “Performance and Growth Curve Prediction of Crossbred Chickens from Crossing between Local Roosters and Layer Hens”; “Effect of Carbohydrate Type and Phenotype on the Quality of Post-Thawing Frozen Semen of KUB Chicken; “Effect of Dietary with Different Energy and Protein Levels on Laying Quails Performance”; “Quality of Physical and Sensory of Super Native Chicken Breast Marination with Herbs and Spices with Different Levels of Marination Concentration”; and Farmers' Perceptions and Behavior which Affect the Adoption Rate of KUB Chickens.

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 2023

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Influence of Sperm Number and Antioxidant Melatonin in Extender on the Quality of Post-Thawing Sheep Spermatozoa

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ABSTRAK

Putri F, Karja NWK, Setiadi MA, Kaiin EM. 2023. Pengaruh konsentrasi spermatozoa dan penambahan antioksidan melatonin pada pengencer terhadap kualitas spermatozoa domba post-thawing. JITV 28(1):1-10. DOI: <http://dx.doi.org/10.14334/jitv.v28.i1.3069>.

Penelitian ini bertujuan untuk melihat pengaruh konsentrasi spermatozoa dan efektifitas suplementasi melatonin pada pengencer terhadap kualitas semen post-thawing. Semen ejakulat dikoleksi dengan menggunakan metode vagina buatan (MVB). Penelitian dilakukan dalam dua tahap, pertama semen dibekukan dalam pengencer andromed dengan konsentrasi yang berbeda dalam satu straw (50, 100, 200 juta perstraw), yang kedua semen dibekukan dalam pengencer yang disuplementasi melatonin dengan dosis berbeda (0, 0,5, 1,0 dan 1,5 mM melatonin). Parameter yang diamati adalah pergerakan spermatozoa dengan menggunakan Computer Assisted Sperm Analysis, keutuhan membran dan keutuhan akrosom. Data dianalisis menggunakan Anova dan di uji lanjut menggunakan uji Duncan. Hasil penelitian menunjukkan bahwa kualitas dan pola pergerakan semen domba tidak terdapat perbedaan signifikan saat dibekukan dengan konsentrasi 50, 100, maupun 200 juta perstraw ($P>0,05$). Penambahan melatonin dengan konsentrasi 1,0 mM pada pengencer memberikan pengaruh terhadap motilitas total dan motilitas progresif spermatozoa ($P<0,05$) tetapi tidak memberikan pengaruh yang signifikan terhadap persentase karakteristik pola gerak spermatozoa. Dari hasil penelitian dapat disimpulkan bahwa konsentrasi spermatozoa perstraw tidak memberikan pengaruh terhadap kualitas semen domba dan suplementasi melatonin dalam pengencer dapat mengurangi efek negatif selama proses frozen-thawed terhadap motilitas, tudung akrosom dan integritas membran plasma pada semen domba. Suplementasi melatonin dengan konsentrasi 1 mM pada bahan pengencer merupakan konsentrasi dengan kualitas terbaik pada penelitian ini.

Kata Kunci: Kriopreservasi, Konsentrasi Semen, Melatonin, Kualitas Post-thawing

ABSTRACT

Putri F, Karja NWK, Setiadi MA, Kaiin EM. 2023. Influence of sperm number and antioxidant melatonin addition in extender on the quality of post-thawing sheep spermatozoa. JITV 28(1): 1-10. DOI: <http://dx.doi.org/10.14334/jitv.v28.i1.3069>.

This study aimed to examine the effect of spermatozoa concentration and the effectiveness of melatonin supplementation in diluent on the quality of post-thawing semen. Ejaculated semen was collected using the artificial vaginal method (MVB). The study was carried out in two stages, firstly semen was frozen in andromed diluent with different concentrations in one straw (50, 100, 200 million per straw), and the second was frozen semen in diluent supplemented with melatonin with different doses (0, 0.5, 1, 0 and 1.5 mM melatonin). Parameters observed were the movement of spermatozoa using Computer Assisted Sperm Analysis, membrane integrity, and acrosome integrity. Data were analyzed using ANOVA and further tested using Duncan's test. The results showed no significant difference in the quality and movement pattern of sheep semen when frozen at concentrations of 50, 100, or 200 million per straw ($P>0.05$). Furthermore, adding melatonin to the diluent in this study affected spermatozoa's total motility and progressive motility at a concentration of 1.0 mM ($P<0.05$) but did not significantly affect the percentage of spermatozoa motility pattern characteristics. From the results, it can be concluded that the concentration of per straw spermatozoa does not affect the quality of sheep semen, and melatonin supplementation in diluent can reduce the effects of the frozen-thawed process on motility, acrosome cap, and plasma membrane integrity in sheep semen. Melatonin supplementation with a concentration of 1 mM in the extender was the highest quality concentration in this study.

Key Words: Cryopreservation, Melatonin, Post-thawing Quality, Sperm Number

INTRODUCTION

Cryopreservation of semen is an efficient technique to save sperm cells, allowing the preservation of gene

pools and the expansion of desired reproductive merits (Salmani et al. 2013). However, the cooling, freezing, and thawing processes cause physical and chemical stress on the spermatozoa membranes, reducing their

viability and fertility (Chatterjee et al. 2005). One cause of this decline is due to the action of the reactive oxygen species (ROS) generated by the cellular components of semen, abnormal spermatozoa, and neutrophils, namely, a superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) (Perumal, 2014). Spermatozoa are susceptible to OS because their plasma membrane contains an unusually high percentage of polyunsaturated fatty acids (PUFAs) exposed to a damaging process called lipid peroxidation. Lipid peroxidation occurs as electrons from plasma membrane lipids are stripped away by ROS, which propagates a chain of redox reactions that eventually generates highly mutagenic and genotoxic electrophilic aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and acrolein (Bui et al. 2018). 4-HNE is derived from lipid hydroperoxides of ω -6 fatty acids such as linoleic acid, and arachidonic DNA damage observed in spermatozoa results from OS. It is an essential characteristic of semen quality and, therefore, a useful marker in diagnosing male infertility (Bui et al. 2018).

The semen sample must be appropriately diluted to allow enough per straw sperm for artificial fertilization (Kalita et al. 2019). The combination of different storage temperatures and reduced sperm concentration at refrigerated insemination doses has recently been studied in cattle (Murphy et al. 2013; Murphy et al. 2016; Murphy et al. 2018). In rabbits, after 48 hours of refrigerated storage, higher sperm concentrations (30 and 60×10^6 spermatozoa/mL) were associated with higher overall motility (Johinke et al. 2015). Another study stated, In bovine, no significant reduction in fertility of liquid semen when sperm concentration was reduced from 10 to 1.5×10^6 sperm per insemination dose (Shanon et al. 1984). The suggested dose of semen is 0.1 to 0.2 ml with about 50 to 100 million active spermatozoa. About 50 to 100 million spermatozoa are needed in one dose for vaginal insemination, but 25 to 50 million are suggested for cervical insemination, and 15 to 20 million are enough for laparoscopic AI (Yusuf et al. 2005; Gergátz 2007). However, several scientists have used much higher numbers: 100 (Fernandez-Abella et al. 2003), 50 to 300 (Ehling et al. 2003), and 400 (King et al. 2004) million spermatozoa in one dose of semen. Because there is no straightforward enterprise standard, it is necessary to research whether there is an effect of concentration differences on the quality of post-thawing sheep spermatozoa.

Several studies were carried out to improve semen quality by adding antioxidants such as vitamin C (Trilaksana et al. 2016), glutathione (Salmani et al. 2013), and melatonin (Succu et al. 2011). In recent years, melatonin has been widely used as an additional antioxidant in cryopreservation. Ashrafi et al. (2013) reported that supplementation of melatonin in the freezing medium could counteract the adverse effects of the freeze-thawing process on the motility, viability,

normal morphology, and plasma membrane integrity in bull spermatozoa with a concentration of 2 mM. At the same time, Succu (2011) reported that doses of one mM of melatonin led to higher viability rates and higher percentages of total motile and progressive motility. These results align with Pang et al. (2016), that reported melatonin can improve the quality of bovine frozen-thawed semen. The pineal gland releases melatonin (N-acetyl-5-methoxy tryptamine) in the brain; it is involved in many physiological functions, including regulation of seasonal reproduction, and influences the immune system and circadian rhythms (Awad et al. 2006; Jang et al. 2010). On the other hand, the antioxidant property of melatonin has been demonstrated in vivo. Melatonin can also maintain the semen characteristics of goats (Ramadan et al. 2009), rats (Sönmez et al. 2007), wild boars (Jang et al. 2010), and humans (Espino et al. 2010; Ortiz et al. 2011).

Differences in concentrations in extenders and melatonin supplementation as an antioxidant in extenders still need to be explored; because there is a lack of information regarding the effect of sperm concentration on freezing without the reduction in their post-thaw survival of spermatozoa and melatonin supplementation as an antioxidant in extenders on sheep. Hence the present study was taken up.

MATERIALS AND METHOD

Ethical approval

This research was approved by The Institution of Research and Community Services, Animal Care Use Committee Bogor Agricultural University, 231 – 2021 IPB.

Research animal

Two sheep from the Rehabilitation and Reproduction Unit of IPB were used in this study, aged 18-24 months and weighing 30-40 kg. Feed was given in the morning and evening as forage as much as 3 kg/head/day and concentrate with 11% protein as much as 0.5 kg/head/day. Drinking water is provided ad libitum.

Semen collection and cryopreservation

Spermatozoa collection from ejaculate was carried out once a week for two months with the artificial vaginal method (MVB) performed six times as the number of replications. The research was conducted in two steps. In the first step, the semen was diluted with an Andromed diluent at three different concentrations of 50, 100, and 200 million spermatozoa. Semen samples were aspirated into 0.25 ml straws. The second stage of semen was diluted with Andromed with the best concentration in the previous step and supplemented with antioxidant

melatonin at different doses (0, 0.5, 1.0, and 1.5 mM melatonin). Diluted semen samples were evaluated for quality; only semen with more than 70% motility was used in this study.

Furthermore, the packaged semen was equilibrated in a refrigerator at 5°C for 4 hours. After equilibration, each semen sample of each treatment was evaluated for quality. Freezing of semen was done by placing straws 10 cm above the surface of liquid nitrogen in tightly closed styrofoam for 20 minutes. Furthermore, the straws were put into liquid nitrogen (-196°C) and stored in a liquid nitrogen container for quality evaluation.

Semen evaluation

Spermatozoa Movement Assessment. The movement of spermatozoa was observed using Computer Assisted Sperm Analysis (CASA, Sperm Vision Minitube) at the Animal Reproductive Biotechnology Laboratory, Research Center for Biotechnology, BRIN. Observing the motility of spermatozoa refers to the modified Hariono (2021), in which the straws for each treatment were thawed by immersing them in water at 37°C in a water bath for 30 seconds. First, an evaluation was carried out by dripping 10 L of cement on an object glass and covered with a cover glass. Then, the sample was placed on a microscope (Zeiss Axio Scope A1). The following parameters were evaluated for post-thawed semen, namely: Motility: the percentage of motile sperm; Progressive motility: the percentage of progressively motile sperm; Curvilinear velocity (VCL: $\mu\text{m}/\text{second}$); Straight line velocity (VSL: $\mu\text{m}/\text{second}$); Average Path Velocity (VAP: $\mu\text{m}/\text{second}$), Amplitude of Lateral Head Displacement (ALH: μm) and Straightness (STR: %).

Membrane Integrity. Examine spermatozoa plasma membrane integrity (PMI) using the Hypoosmotic Swelling Test (HOS-Test). The composition of the HOS solution was 0.135 g fructose and 0.0737 g trisodium citrate $2\text{H}_2\text{O}$ in 10 mL milli-Q water. A sample of 20 L of cement was added to 80 L of HOS solution and left for 30 minutes in a water bath (37°C). After incubation, 10

μL of cement was placed on a glass object, covered with a cover glass, then observed under a light microscope (Nikon FDX-35) with a magnification of 400x. Straight tails characterize spermatozoa with plasma membrane damage. In contrast, circular or bulging tails characterize spermatozoa with plasma membrane integrity. The number of spermatozoa observed was at least 200 cells in 10 fields.

Acrosome Integrity. TBG staining begins with dripping cement and trypan blue solution, dissolved using 0.81% NaCl simultaneously and then slowly homogenized, made into smear preparations, and dried vertically. Next, the preparations were stained using a neutral red solution consisting of 86 ml 1 N HCL and 14 ml 37% formaldehyde solution, and 0.2 g Neutral Red (Sigma N-2880). The preparations were stained for 3 minutes, then dried, rinsed using running water, and dried again. In the next staining stage, the preparations were immersed in a jar containing 5% Giemsa solution, left at room temperature for three days, and then rinsed again by dipping into a container filled with water for 2 minutes. After drying, it was warmed using a heating table (40°C) (Nofa et al. 2018). An evaluation was conducted on 200 cells using a light microscope with an objective lens of 400x magnification. Spermatozoa with acrosome integrity (AIn) are purple on the head, while spermatozoa with incomplete acrosomes will have a pale lavender or faded color. Acrosome status was calculated by counting the number of spermatozoa with AIn divided by the total number of spermatozoa multiplied by 100%.

Statistical analysis

The data of spermatozoa assessment were shown as the mean $\pm$ SD. Each experiment was repeated five times. The data were analyzed using One-Way Analysis of Variance (ANOVA), and Duncan's Multiple Range Test (DMRT) was performed to determine if there was any significant difference among the treatments at $P < 0.05$. Statistical analysis was conducted using IBM SPSS® Statistics version 22.0 (IBM Corp., Armonk, NY, US).

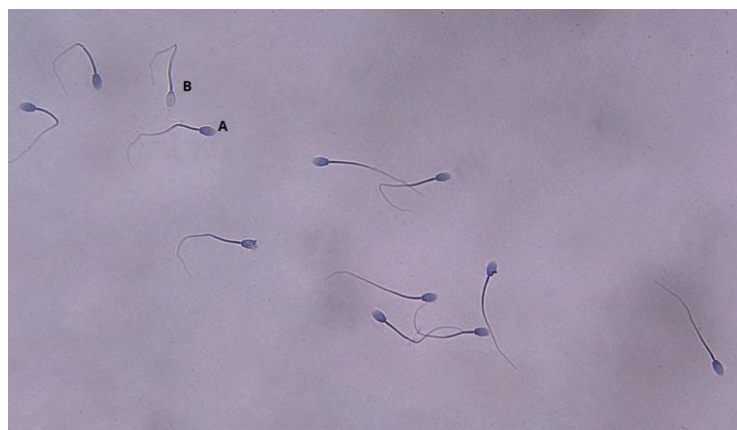


Figure 1. Acrosome staining using trypan blue-Giemsa examined at 400x: (A) acrosome integrity, (B) damaged acrosome

RESULTS AND DISCUSSION

Quality of frozen-thawed sheep spermatozoa with different concentrations per straw

Motility is an essential indicator of successful fertilization. Prior to cryopreservation, the quality of the fresh semen will determine whether the semen is suitable for processing. Toelihere (1985) stated that semen could be continued for processing: if it has a high mass movement value and motility, a concentration of 1500 million to 3000 million, and an abnormality value below 15 percent. Based on observation, high fresh semen motility (80.00 ± 0) indicates that spermatozoa will survive through cooling or cryopreservation. The sperm motility percentage of frozen semen after thawing was $>40\%$. In general, freezing and thawing procedures significantly reduced the quality of sheep semen. The percentages of total motility, progressive motility, plasma membrane integrity, and acrosome integrity of spermatozoa immediately after thawing are presented in Table 1.

The quality of frozen semen is influenced by the freezing process but is also closely related to the quality of fresh semen produced before the freezing process is carried out (Awad et al. 2006; Salmani et al. 2013; Nofa et al. 2018). The results showed that the quality after thawing of ejaculated spermatozoa met the requirements for artificial insemination based on the Indonesian National Standard number SNI 4869.3-2014 part 3: Goats and Sheep with the condition that the percentage of frozen semen motility was at least 40% or mass movement ++ (Badan Standardisasi Nasional 2014). The percentage of total motility was higher at a concentration of 100 million spermatozoa per straw, 45.74 ± 3.70 , and did not show significant differences between treatment groups. These findings align with Holt (2000) and Lemma (2011) that the freezing and thawing process causes damage to spermatozoa, resulting in a decrease in post-thawing quality. It is well known that sperm can be affected by several factors during the freeze-thaw process, leading to loss of plasma membrane integrity and damage to the acrosome, mitochondrial dysfunction leading to irreversible loss of motility and fertility (Karja et al. 2013). The success of cryopreservation was also assessed by recovery rate (RR), the percentage of spermatozoa that recovered from the freezing process, which was calculated by comparing motile spermatozoa in fresh semen and after thawing (Arifiantini et al. 2007).

Table 1 shows a higher percentage of total motility at a concentration of 100 million per straw spermatozoa and does not show a significant effect between treatments ($P > 0.05$). De Graft (2007) reported successful *in vivo* fertilization at 100 million spermatozoa per dose. In addition, this fertility rate is comparable to a dose of 50

million sperm; this supports the results of this study that lowering or increasing the sperm concentration per dose will not harm the fertility rate *in vivo*. Progressive motility percentage (Table 1) did not show a significant effect between the treatment groups. Progressive motility is one of the essential criteria for spermatozoa to fertilize oocytes (Kato et al. 2011). In *in vivo* fertilization, the spermatozoa ejaculated in the vagina and moved forward in the oviduct via the cervical canal and uterus (Hyakutake et al. 2018). Furthermore, spermatozoa change movement patterns to become non-progressive, and changes in movement patterns are required for spermatozoa to pass through the utero-tubal junction and enter the oviduct (Muiño et al. 2008). Progressive motility may decrease rapidly due to the depletion of glycolytic substrates and decreased energy (Ahmad et al. 2015).

Based on table 1, the value of acrosome integrity of sheep spermatozoa did not show any significant difference. The results showed that the acrosome integrity had different concentrations per straw spermatozoa, 45.00 ± 5.00 ; 46.36 ± 3.34 ; 46.17 ± 5.39 , respectively. The low percentage of acrosome integrity is associated with a low percentage of intact plasma membrane and motility (Sitepu et al. 2018). Based on table 1, sheep spermatozoa's plasma membrane integrity values showed no significant difference. The results showed that the acrosome integrity had different concentrations per straw spermatozoa, 46.50 ± 3.50 ; 48.33 ± 4.80 ; 48.00 ± 4.27 , respectively. Changes in the plasma membrane play an essential role in the capacitation process (Dapino et al. 2009) and cause a decrease in spermatozoa fertility (Thomas et al. 2006). Damaged spermatozoa and physiological changes in the membrane can be described in the motility of the spermatozoa (Gervasi & Visconti, 2017). In addition, an intact plasma membrane can reduce the lipid phase transition, protect the membrane's ion-exchange function, and decrease intracellular $[Ca^{2+}]$ (Muiño et al. 2008). Changes in membrane permeability during cooling can cause an increase in intracellular $[Ca^{2+}]$ (Fuller & Whittingham, 1996). Peroxidation of fatty acids of spermatozoa due to damage to the plasma membrane can cause loss of motility (Sundararaman et al. 2012). The integrity of the plasma membrane is related to the metabolic ability of spermatozoa in energy production and ATP synthesis, not generally if there is damage to the membrane, which results in decreased motility and survival of spermatozoa (Nofa et al. 2018).

The movement patterns of post-thawing sheep spermatozoa with different concentrations are shown in table 2. The movement patterns evaluated were VCL, LIN, and ALH. Based on these results, it was found that the VCL value did not differ between all treatment groups, the lower VCL value was found in the group with a concentration of 50 million, and the highest VCL value

Table 1. Characteristics of sheep spermatozoa after freezing with different concentrations

Parameter (%)	Concentration (10 ⁶ /straw)		
	50	100	200
Total Motility	44.10±1.68 ^a	46.36±3.34 ^a	45.74±3.70 ^a
Progressive Motility	29.36±6.94 ^a	33.43±9.26 ^a	32.08±4.23 ^a
Plasma Membrane Integrity	46.50±3.50 ^a	48.33±4.80 ^a	48.00±4.27 ^a
Acrosome integrity	45.00±5.00 ^a	46.36±3.34 ^a	46.17±5.39 ^a

The same superscript letters following numbers in the same column indicate a non-significant difference (P<0.05)

Table 2. The movement pattern of sheep spermatozoa after being frozen at different concentration

Parameter	Concentration (10 ⁶ /straw)		
	50	100	200
VCL (μL/s)	92.02±8.71 ^a	98.94±29.02 ^a	95.48±25.19 ^a
VSL (μL/s)	46.50 ±2.33 ^a	48.33±3.20 ^a	48.00±2.85 ^a
VAP (μL/s)	65.28±3.86 ^a	68.50±14.70 ^a	66.79±12.72 ^a
LIN (%)	0.49±0.30 ^a	0.49±0.04 ^a	0.51±0.05 ^a
STR (%)	0.69±0.01 ^a	0.69±0.02 ^a	0.73±0.02 ^a
ALH (μm/s)	5.94±0.74 ^a	5.81±0.35 ^a	6.03±0.56 ^a

VC= Curvilinear Velocity, VSL= Straight Line Velocity, VAP= Average Path Velocity, LIN= Linearity, STR= Straightness, ALH= Lateral Amplitude of Head Displacement. The same superscript letters following numbers in the same column indicate a non-significant difference (P<0.05)

was found in the group with a concentration of 100 million. At the same concentration, there was no difference in the percentage of LIN between the 50 million, 100 million, and 200 million concentration groups. Meanwhile, the percentage of LIN in the 50 million and 100 million groups was lower than in the 200 million groups. The percentage of spermatozoa LIN at different periods did not differ between the treatments. The results showed no effect of spermatozoa concentration on changes in the percentage of LIN. The values for the amplitude of lateral head displacement (ALH) are presented in Table 2. The ALH values of spermatozoa in the 200 million group were higher than the 50 million and 100 million groups (P<0.05). Hyperactivation is part of the capacitation process (Colás et al. 2009). Spermatozoa undergo motility hyperactivation due to molecular changes in the flagellum (Abou-hailed & Tulsiani, 2009). Spermatozoa of various species have altered patterns of flagellar motion during the final stages of capacitation. This hyperactivated motility is characterized by large-amplitude solid flagellar beats and erratic swimming trajectories; the most common expression of hyperactivation appears to involve a decrease in the progressive movement and an increased bending of the flagellar midpiece while maintaining vigorous motility

(Taitzoglou et al. 2004). Hyperactivation motility can be calculated using CASA by looking at the VCL, ALH, and LIN. (Hinrichs and Loux 2012). Based on Table 2, it can be seen that the spermatozoa did not show any hyperactivity, as Shojaei et al. (2012) explained that the determination of spermatozoa undergoes a change in movement to hyperactivation when the ALH value is >7 μm.

Quality of frozen-thawed sheep spermatozoa with different doses of melatonin

Cryopreservation is a method that can support the conservation of animal genetic materials by freezing semen. The use of the conserved semen and spermatozoa is not limited by geographical distance and time (Pamungkas et al. 2014). The use of cement cryopreservation technology is quite often used for fish cement (Martínez-Páramo et al. 2017; Magnotti et al. 2018), livestock (Lv et al. 2019; Salinas et al. 2021; Yáñez-Ortiz et al. 2021), and human (Tao et al. 2020). It is well known that the cryopreservation process of semen can lead to the formation of reactive oxygen species (ROS). ROS are formed due to an imbalance between the antioxidant content and free oxides, resulting in oxidative stress. Oxidative stress damages the plasma

membrane because phospholipids' membrane structure is sensitive to lipid peroxidation (Bucak et al. 2010). Total motility, progressive motility, plasma membrane integrity, and acrosome integrity of spermatozoa incubated with melatonin are presented in Table 3. Percentages of total motility and progressive motility in this study were assessed using CASA, and significant differences were found between treatments at different doses of melatonin. The percentages of total motility and progressive motility were higher ($P<0.05$) in the diluents supplemented with 1.0 and 1.5 mM melatonin (62.68 ± 2.72 and 38.91 ± 2.68 ; 58.11 ± 2.40 and 35.05 ± 1.68 , respectively). The results showed a decrease in the integrity of the plasma membrane after the cryopreservation process, but the diluent supplemented with melatonin showed a higher value than the control. Melatonin protects mouse spermatozoa from the side effects of the peroxidative agent homocysteine reported by Sönmez et al. (2007). Melatonin has also been shown to protect spermatozoa from mercury oxidative damage (Rao & Gangadharan, 2008) and increase the hyperactivation of hamster spermatozoa (Fujinoki, 2008). Furthermore, Ramadan et al. (2009) reported that melatonin maintains the sperm quality of Damascus goats.

The percentage of progressive motility showed no significant difference ($P>0.05$) between the control

group (45.74 ± 3.70) and the diluent supplemented with 0.5 mM melatonin (48.63 ± 3.01). Melatonin supplementation of 1 mM in the diluent increased the percentage of PMI and AIn ($P<0.05$) (62.22 ± 0.30 and 67.23 ± 2.6) compared to other groups. Several studies have confirmed that melatonin can reduce oxidative stress (Reiter, 1998; Jang et al. 2010). Pang et al. (2016) reported that melatonin could improve plasma membrane and acrosome integrity in frozen-thawed bovine semen. The beneficial effect of melatonin on plasma membranes is that melatonin stimulates the activity of enzymes involved in ROS metabolism and maintains cell membrane integrity (Izadpanah et al. 2015). In addition, melatonin can be an effective anti-apoptotic agent (Tsantariotou et al. 2008). Other studies have shown that melatonin can prevent apoptosis by controlling Bcl-2 expression and ameliorating oxidative stress in the testes (Othman et al. 2016). Supplementation with one mM of melatonin was reported to prevent capacitation and apoptosis of mouse spermatozoa (Casao et al. 2010). This anti-apoptotic ability may cause increased motility and plasma membrane integrity. In addition, our study also found that melatonin supplementation at a dose of 1.5 mM can reduce the motility determined; this decrease in quality is thought to be due to melatonin at high concentrations can have a negative effect. Erez et al. (1992) found that high doses of melatonin in

Table 3. Characteristics of sheep spermatozoa after freezing with different doses of melatonin

Parameter (%)	Doses (mM)			
	0	0.5	1.0	1.5
Total Motility	45.74 ± 3.70^a	48.63 ± 3.01^a	62.68 ± 2.72^b	58.11 ± 2.40^b
Progressive Motility	32.08 ± 4.23^{ab}	28.02 ± 1.13^a	38.91 ± 2.68^c	35.05 ± 1.68^{bc}
Plasma Membrane Integrity	48.00 ± 4.27^a	59.24 ± 2.67^b	62.22 ± 0.30^b	61.16 ± 1.22^b
Acrosome integrity	46.17 ± 5.39^a	59.88 ± 1.70^b	67.23 ± 2.61^c	66.61 ± 1.52^c

Different letters at different doses show significant differences ($P<0.05$)

Table 4. The movement pattern of sheep spermatozoa after being frozen with different doses of melatonin

Parameter	Doses (mM)			
	0	0.5	1.0	1.5
VCL ($\mu\text{L/s}$)	95.48 ± 25.19	78.14 ± 1.52	86.75 ± 3.51	90.19 ± 4.75
VSL ($\mu\text{L/s}$)	48.97 ± 8.29	41.33 ± 0.84	42.90 ± 1.14	43.33 ± 2.23
VAP ($\mu\text{L/s}$)	66.79 ± 12.72	60.90 ± 1.71	60.79 ± 2.85	61.45 ± 3.88
LIN (%)	0.51 ± 0.05	0.52 ± 0.02	0.49 ± 0.01	0.47 ± 0.03
STR (%)	0.73 ± 0.26^b	0.68 ± 0.20^a	0.70 ± 0.02^{ab}	0.69 ± 0.01^{ab}
ALH ($\mu\text{m/s}$)	5.96 ± 0.72	6.16 ± 0.50	6.24 ± 0.07	6.42 ± 0.26

VCL= Curvilinear Velocity. VSL= Straight Line Velocity. VAP= Average Path Velocity. LIN = linearity. STR= Straightness. ALH= Lateral Amplitude of Head Displacement. Different letters at different doses show significant differences ($P<0.05$)

reproductive fluids can decrease sperm motility. Excessive levels of melatonin in semen affect the physiology of the human spermatozoa (Yie et al. 1991). Incubation of bull semen with melatonin between 0.5 and 4 mM for 60 min significantly reduced spermatozoa motility (Tanyildizi et al. 2006). This may be due to different cement processing procedures, different proportions of components in the plasma membrane, and different species (Zhang et al. 2015). However, these results do not fully explain the negative effect of high concentrations of melatonin on semen, and therefore further research should be carried out. In some literature, different doses are optimal for semen cryoprotection. For example, one showed that 1 and 2 mM were optimal concentrations, while another study found that the best concentration was 0.25 mg/mL in various species of fields (Appiah et al. 2019).

Total motility, progressive motility, PMI, and AIn of spermatozoa incubated with melatonin are presented in Table 3. Percentages of total motility and progressive motility in this study were assessed using CASA, and significant differences were found between treatments at different doses of melatonin. The percentages of total motility and progressive motility were higher ($P < 0.05$) in the diluents supplemented with 1.0 and 1.5 mM melatonin (62.68 ± 2.72 and 38.91 ± 2.68 ; 58.11 ± 2.40 and 35.05 ± 1.68 , respectively). The percentage of progressive motility showed no significant difference ($p > 0.05$) between the control group (45.74 ± 3.70) and the diluent supplemented with 0.5 mM melatonin (48.63 ± 3.01). Melatonin supplementation of 1 mM in extender increased the percentage of PMI and AIn (62.22 ± 0.30 and 67.23 ± 2.6 , respectively) compared to other groups.

The description of the acrosome status of the spermatozoa in Figure 1 uses TBG staining: (A) spermatozoa with AIn absorb purple color (B) spermatozoa with damaged acrosomes will have a pale lavender/faded color. Thus, if part of the spermatozoa acrosome is damaged or incomplete, the cells cannot absorb color correctly, and the staining results will look faded. Irregularity in the head can also indicate that the plasma membrane has been damaged and has the potential for acrosomal damage. The results showed that the acrosome status of spermatozoa supplemented with melatonin after thawing showed a reasonably high percentage ranging from 59-67%. The acrosome integrity percentage indicated no damage to the spermatozoa that could reduce its fertilization ability. Spermatozoa cannot fertilize if acrosomal damage exceeds 50% (Hernández et al. 2012).

The movement pattern of post-thawed spermatozoa with melatonin supplementation is shown in Table 4. There was no significant difference between VCL, ALH, and LIN values. Velocity and linearity contribute to

essential characteristics of the spermatozoa function (Shibahara et al. 2003). By definition, linearity is linearly moving in a straight line, affecting progressive motility (Sarastina et al. 2007). Sarastina et al. (2007) state that if $LIN > 35\%$, the average spermatozoa move linearly straight ahead. The results of this study describe the LIN value ranging from 47 to 52%; this indicates that the spermatozoa move linearly straight ahead. The ALH value in this study showed no difference ($P > 0.05$) with a value of 5 to 6 μm . All frozen semen had ALH values $< 5 \mu\text{m}$, meaning the spermatozoa were not hyperactive (Sarastina et al. 2007; Kathiravan et al. 2011).

The relationship between the CASA variable and the fertilization ability indicates that metabolically active spermatozoa have high penetration potential. Good motility is needed for the ability of spermatozoa to penetrate the cumulus and reach the zona pellucida. Furthermore, the intact membrane will bind to the zona pellucida (Ducha et al. 2012; Okabe 2013). Conversely, Spermatozoa with damaged plasma membranes cannot bind to ZP (binding zone) because the membrane that binds the zona pellucida has lost its receptors (Ducha et al. 2012).

CONCLUSION

In conclusion, differences in spermatozoa concentration did not significantly affect the quality of sheep semen. They demonstrated that adding one mM of melatonin increased the effect of the frozen-thawed process on motility, acrosome integrity, and plasma membrane integrity in sheep semen.

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Nuclear Maturation Competence and Pronuclear Formation in Ovine Oocytes Supplemented with *L-ergothioneine* in Maturation Medium

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ABSTRAK

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Tujuan dari penelitian ini yaitu untuk mengetahui efikasi penambahan *L-ergothioneine* (LE) pada medium maturasi terhadap kompetensi pematangan inti dan pembentukan pronukleus pada oosit domba *in vitro*. Penelitian tahap 1 dilakukan untuk mengevaluasi tingkat maturasi inti oosit. Oosit dibagi menjadi empat kelompok dan dimatangkan selama 24 jam dalam medium maturasi yang disuplementasi 0 mM (kontrol), 10 mM, 15 mM, dan 20 mM LE. Penelitian tahap 2 dilakukan untuk mengevaluasi tingkat fertilisasi oosit. Oosit dibagi menjadi dua kelompok dan dimatangkan selama 24 jam dalam medium maturasi yang disuplementasi 0 mM LE (kontrol) dan 10 mM LE (dosis optimal dari penelitian tahap 1) kemudian diko-inkubasi dengan sperma dalam medium fertilisasi selama 12 jam. Hasil penelitian tahap 1 menunjukkan bahwa tingkat maturasi inti oosit dengan suplementasi LE 10 mM ($88,74 \pm 1,79\%$) dan 15 mM ($87,52 \pm 2,30\%$) pada medium maturasi berbeda nyata ($P < 0,05$) dibandingkan dengan kelompok kontrol ($78,55 \pm 1,78\%$) dan LE 20 mM ($77,37 \pm 1,21\%$). Hasil penelitian tahap 2 menunjukkan bahwa tingkat fertilisasi oosit dengan suplementasi LE 10 mM pada medium maturasi menunjukkan persentase pembentukan dua pronukleus secara signifikan lebih tinggi ($75,55 \pm 3,47\%$) daripada kelompok kontrol ($63,78 \pm 3,49\%$). Kesimpulannya, suplementasi LE 10 mM pada medium maturasi mampu meningkatkan kompetensi pematangan inti oosit dan pembentukan pronukleus.

Kata Kunci: Domba, Fertilisasi *In vitro*, Maturasi *In vitro*, *L-ergothioneine*, Oosit

ABSTRACT

Agustin RP, Setiadi MA, Supriatna I. 2023. Nuclear maturation competence and pronuclear formation in ovine oocytes supplemented with *L-ergothioneine* in maturation medium. JITV 28(1):11-21. DOI: <http://dx.doi.org/10.14334/jitv.v28.i1.3120>.

This study aimed to determine the efficacy of supplementing maturation medium with *L-ergothioneine* on nuclear maturation competence and pronuclei formation of ovine oocytes *in vitro*. In the first study, oocytes were divided into four groups and matured for 24 hours in a maturation medium supplemented with 0 mM (control), 10 mM, 15 mM, and 20 mM LE. In the second study, oocytes were divided into two groups and matured for 24 hours in a maturation medium supplemented with 0 mM LE (control) and 10 mM LE (optimal dose from the first study), then co-incubated with sperm in a fertilization medium for 12 hours. The results of the first study showed that the maturation rate of oocytes enriched with LE 10 mM ($88.74 \pm 1.79\%$) and 15 mM ($87.52 \pm 2.30\%$) in maturation medium was significantly different ($P < 0.05$) compared to the control ($78.55 \pm 1.78\%$) and LE 20 mM ($77.37 \pm 1.21\%$). Furthermore, the results of the second study revealed that the fertilization rate of oocytes with 10 mM LE ($75.55 \pm 3.47\%$) supplementation in maturation medium had a significantly higher percentage of two pronuclei formation than control ($63.78 \pm 3.49\%$). In conclusion, supplementing 10 mM LE in a maturation medium improved oocyte nuclear maturation competence and fertilization rates.

Key Words: *In vitro* Maturation, *In vitro* Fertilization, *L-ergothioneine*, Oocyte, Ovine

INTRODUCTION

Some research has been conducted to develop genetic improvements and accelerate population growth in small ruminants such as sheep through reproductive technology. Sheep are domesticated, and ruminant mammals commonly kept as livestock and have tremendous potential for genetic improvement in meat

and milk production. The genetic material of these animals needs to be improved to increase fertility and productivity in small animal farms. An assisted reproductive technology known as *in vitro* embryo production (IVEP) consists of direct follicle retrieval of immature oocytes for *in vitro* maturation (IVM), *in vitro* fertilization (IVF), i.e., co-incubation of *in vitro*-matured oocytes with capacitated sperm, and *in vitro* culture

(IVC) of zygotes to the blastocyst stage (Mondal et al. 2019; Soto-Heras & Paramio 2020). It is a valuable technique for understanding early embryonic development, with applications ranging from treating reproductive disorders to preserving gametes from animals with high genetic value (Rizos et al. 2008; Zhu et al. 2018). IVEP enables the birth of offspring from infertile, prepubertal, pregnant, lactating, and even dead or slaughtered animals. Moreover, this technology can be used as an action plan to save some endangered species (Paramio & Izquierdo 2016).

Usually, the oocytes for IVEP are obtained from the ovarian waste of slaughterhouses. Mammalian ovaries contain many immature oocytes that can be used for reproductive technology, such as IVF (El-raey & Nagai 2014). Nevertheless, *in vitro*-matured oocytes usually have less developmental capacity than *in vivo*-matured oocytes due to abnormalities that occur during cytoplasmic maturation (Quan et al. 2017). Meanwhile, oocyte quality after *in vitro* maturation is essential in supporting fertilization and embryo development (Gulo et al. 2020). It is generally known that *in vitro* oocyte maturation is the crucial process in the whole procedure of IVEP since immature oocytes have to undergo synchronized nuclear and cytoplasmic maturation to acquire fertilization and developmental competence (Paramio & Izquierdo 2014; Soto-Heras & Paramio 2020).

Furthermore, compared to the developability between *in vivo* and *in vitro* generated oocytes, improper *in vitro* maturation is the leading cause of polyspermy after IVF (Zhu et al. 2018). During IVM, significant changes occur in the cytoplasm and nucleus of the oocytes. Subsequently, oocytes expel the first polar body and enter metaphase II (Zhu et al. 2018). These changes are crucial for promoting successful fertilization and embryo development (Quan et al. 2017). Therefore, IVM is essential for producing high-quality oocytes for IVF and determines the potential developmental ability of the oocyte (Zhu et al. 2018).

Shortly after the animal is slaughtered, most organs undergo ischemia, which reduces oxygen and energy supply, forcing a switch to anaerobic metabolism to maintain ATP and increase ROS production (Soto-Heras & Paramio 2020). Oxygen tension in the oviduct is 3–4 times lower than in typical IVM culture (3–9% vs. 20% respectively), and such high oxygen tension is thought to cause intolerable creation of ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals, and peroxy radicals (Rocha-Frigoni et al. 2016). When ROS production exceeds a cell's antioxidant defense mechanisms, it leads to oxidative stress and disrupts the redox system's processes (reduction/oxidation). Furthermore, these radicals can interact with some molecules to accept electrons and stabilize due to their high reactivity and instability. These interactions, in turn, can trigger a series of interrelated events that can ultimately lead to

cellular damage, such as the oxidation of amino acids and nucleic acids and lipid peroxidation (especially of membrane phospholipids). In addition, excessive amounts of ROS cause DNA damage by rupturing mitochondrial membranes, releasing cytochrome C and activating caspase cascades. These processes lead to apoptosis and unsuccessful IVEP (Rocha-Frigoni et al. 2016). Several studies have been performed to control ROS levels during *in vitro* systems. One of them is to change the composition of the maturation medium by adding an antioxidant to increase intracellular antioxidant capacity and neutralize free radicals (Gulo et al. 2020).

The natural amino acid L-ergothioneine (LE) is a potent OH^- scavenger and an inhibitor of iron- or copper ion-dependent OH^- production from H_2O_2 (Zullo et al. 2016). LE is a histidine-derived amino acid, having a sulfur atom in the second position of the imidazole ring. Mammals cannot produce ergothioneine; only several organisms synthesize this compound, notably filamentous fungi and *actinobacteria* (Bazela et al. 2014; Mishra et al. 2018). For this reason, mammals obtain ergothioneine exclusively from their feed, which includes oats, meat, corn, and mushrooms. Ergothioneine has been shown to neutralize singlet oxygen, hydroxyl radicals, hypochlorous acid, and peroxy radicals and prevent the nitration of proteins and DNA caused by peroxynitrite (Zullo et al. 2016; Mishra et al. 2018). Compared to other traditionally known antioxidants such as glutathione (GSH), uric acid, and Trolox, LE has recently been shown to have the highest antioxidant activity and to remove the most active free radicals (Zullo et al. 2016). Notably, the two functional groups in LE significantly impacting *in vitro* oocyte maturation are the quaternary ammonium group $N-(CH_3)_3$ and L-thiol (SH) group. The quaternary ammonium group increases the ATP level in cells by transporting fatty acids into the mitochondria to support the oocyte's nuclear maturation (Bazela et al. 2014).

Meanwhile, the L-thiol group increases glutathione level to prevent oxidative stress and support pronuclear formation after *in vitro* fertilization (Bazela et al. 2014; Mishra et al. 2018). In addition, recent research suggests that altering the maturation medium can significantly affect the developmental capability of oocytes *in vitro* (Quan et al. 2017). Therefore, to determine the effects of L-ergothioneine supplementation in the maturation medium, this study examined the enhancement of nuclear maturation competence and fertilization ability characterized by forming two pronuclei.

MATERIALS AND METHODS

This study was conducted at the *In vitro* Fertilization (IVF) Laboratory, Division of Reproduction and Obstetrics, Department of Veterinary Clinic,

Reproduction and Pathology, School of Veterinary Medicine and Biomedical Science, IPB University, Bogor. The bioethical committee of IPB University approved all experimental procedures in this study (No. 218 – 2021 IPB).

Research design

This research was divided into two experiments. In the first experiment, we evaluated the efficacy of L-ergothioneine (LE) (Sigma-Aldrich, E7521) supplementation in a maturation medium on the nuclear maturation competence of ovine oocytes. The selected oocytes were collected, divided into four groups, and then matured in a maturation medium supplemented with LE 0 mM (control), 10 mM, 15 mM, and 20 mM for 24 hours in a CO₂ 5% incubator at 38.5°C. Afterward, the oocyte's nuclear status was observed under a phase contrast microscope (Olympus IX 70, Japan). The doses used in this research referred to the previous study by Öztürkler et al. (2010) and Mishra et al. (2018). The optimum dose obtained from experiment 1 was then used in experiment 2. Ten replications were carried out in the first experiment, and the experimental design used in experiment 1 was a completely randomized design (CRD).

In the second experiment, we assessed the potency of LE supplementation in a maturation medium on the pronucleus formation of ovine oocytes after *in vitro* fertilization (IVF). Oocytes were collected, selected, and then *in vitro*-matured in a maturation medium supplemented with LE 0 mM (control) and 10 mM (the best dose obtained from the first experiment). Oocytes were then fertilized *in vitro* for 12-13 hours in a CO₂ 5% incubator at 38.5°C. Pronucleus formation was observed under a phase contrast microscope (Olympus IX 70, Japan). Eleven replications were carried out in the second experiment, and the experimental design used in experiment 2 was a completely randomized design (CRD).

Oocytes collection

In less than five hours, ovaries from sheep were collected from a slaughterhouse and transferred to the laboratory using a transport medium at 35-37 °C. Streptomycin sulfate (MEIJI, Indonesia) 0.1 mg/mL and penicillin-G (MEIJI, Indonesia) 100 IU/mL are added to 0.9% sodium chloride (WIDATRA, Indonesia), which serves as the transport medium. The method of oocytes collection (cumulus-oocyte complexes or COCs) refers to Bhakty et al. (2021), which is performed by slicing and flushing on a collection medium containing Phosphate-Buffered Saline (PBS) enriched with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, F7524), 100 IU/mL penicillin-G (Sigma-Aldrich, P4687) and 0.1 mg/mL

streptomycin sulfate (Sigma-Aldrich, S9137). Oocytes were collected in a Petri dish containing 2 mL of collection medium and selected under a stereo-microscope according to the evaluation criteria. Oocytes with at least three layers of compact cumulus cells and homogeneous cytoplasm should be used for maturation.

Oocyte maturation *in vitro* (IVM)

The maturation medium was equilibrated for two hours in a CO₂ incubator. Then, the selected COCs were washed three times with a maturation medium before the maturation process. The maturation medium contained Tissue Culture Medium 199 (TCM-199) (Sigma-Aldrich, M4530) supplemented with 10 % FBS, 0.1 IU/mL Follicle Stimulating Hormone (FSH) (Vetoquinol N-A inc, Canada), 10 IU/mL Human Chorionic Gonadotrophin (hCG) (ChorulonTM, MSD Animal Health), and 50 g/mL gentamycin (Sigma-Aldrich, G1264). COCs were then placed in 100 µL droplets (10-15 oocytes) of maturation medium in Petri dishes covered with mineral oil (Sigma-Aldrich, M5310) and incubated in a 5% CO₂ incubator at 38.5°C for 24 hours.

Evaluation of oocyte maturation

After 24 hours of incubation, COCs were repeatedly pipetted into a PBS medium containing 0.25% hyaluronidase (Sigma-Aldrich, H3506) to remove cumulus cells and obtain denuded oocytes. The denuded oocytes were washed three times in collection medium, then placed in a drop of KCl on a glass slide padded with paraffin and vaseline (1:9) on the edges and covered with a coverslip. A fixative solution with a 3:1 ratio of absolute ethanol and acetic acid was used to fix the slide for 48-72 hours. The slide was then stained with 2% aceto-orcein (Sigma-Aldrich, St. Louis, O7380, USA) for 2-3 minutes, rinsed with acetoglycerol decolorizer (consisting of acetic acid, glycerol, and distilled water), and examined with a phase-contrast microscope (Olympus IX 70, Japan). Depending on the stages of nuclear maturation, oocytes were classified as a germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase and telophase I (A/T), and metaphase II (MII). Oocytes that reached the M II stage were indicative of mature oocytes.

In vitro fertilization (IVF) of oocytes

Matured oocytes were fertilized using frozen semen from one ram obtained from an artificial insemination center in Lembang, Bandung, West Java, Indonesia. The frozen semen was thawed in water at 37°C bath for 30 seconds and then centrifuged at 500 x g at 28°C for five minutes in PBS media enriched with 0.2% Bovine Serum

Albumin (BSA) (Sigma-Aldrich, A7030). The supernatant was discarded; approximately 200 μ L remained homogenized with the pellet. Sperm concentration was calculated to determine the volume of IVF medium and sperm to obtain a final sperm concentration of 5×10^6 sperm/mL (Bhakty et al. 2021). In addition, 0.1 IU/mL heparin (Sigma-Aldrich, H5515) and 10 μ M hypotaurine (Sigma-Aldrich, H1384) was added to the sperm suspension and then kept in a CO₂ incubator until the matured oocytes were washed three times in IVF medium. Finally, droplets containing 100 μ L of processed sperm were co-incubated with matured oocytes covered with mineral oil in a 5% CO₂ incubator at 38.5°C for 12-13 hours.

Evaluation of oocyte fertilization

The formation of the pronucleus was used to evaluate the fertilization rate. Normal fertilization was distinguished by forming two pronuclei (2PN) after staining with 2% aceto-orcein, while polyspermy refers to oocytes containing more than two pronuclei (Catalá et al. 2012).

Statistical Analysis

Nuclear maturation rate data were expressed as percentages and statistically analyzed using Analysis of Variance (ANOVA) with a significance level of 95%. Duncan's Multiple Range Test (DMRT) is used when there is a difference between the treatments. In addition, data on fertilization rates were also presented as percentages and statistically analyzed using an independent t-test. Data were processed using SPSS version 27 software.

RESULTS AND DISCUSSION

Nuclear maturation competence of ovine oocytes supplemented with LE in the maturation medium

In this experiment, comparisons were made between oocytes matured in four doses of LE. Regarding the assessment of nuclear maturation (germinal vesicle, germinal vesicle breakdown, metaphase I, anaphase/telophase I, and metaphase II), after 24 hours of incubation, the percentage of oocyte maturation reaching metaphase II (MII) between experimental groups are shown in Table 1. In addition, the microscopic representation of the stages of nuclear maturation of the ovine oocyte in this study is shown in Figure 1.

The data in Table 1 show that the percentage of oocytes that could reach the MII stage ranged from 77.37 to 88.74%. The highest percentage of oocytes that

reached the MII stage was shown in group LE 10 mM group (88.74 ± 1.79), while the lowest percentage was shown in LE 20 mM group (77.37 ± 1.21). In this study, the addition of LE 10 mM in IVM medium was significantly different ($P < 0.05$) from control and LE 20 mM but showed no significant difference ($P > 0.05$) if compared with LE 15 mM (87.52 ± 2.30). At the same time, the percentage of oocytes reaching MII in the LE 20 mM group showed a downward trend ($77.37 \pm 1.21\%$) compared to the other groups. Therefore, supplementation of 10 mM to 15 mM LE in the maturation medium most likely promoted the nuclear maturation of sheep oocytes *in vitro*. In comparison, supplementation of 20 mM LE demonstrated a decrease. Therefore, instead of 15 mM LE, supplementation with 10 mM LE in IVM medium was chosen for further study (*in vitro* fertilization) since the smallest dose already gave the best result.

The chromatin configuration allows for evaluating and classifying the oocyte nuclear maturation status into various stages. A large nucleus with an intact envelope and filamentous chromatin characterizes oocytes at the germinal vesicle (GV) stage. In contrast, oocytes with slightly condensed or clumped chromatin without a visible nuclear membrane are classified as germinal vesicle breakdown (GVBD). After GVBD, oocytes enter the metaphase I (MI) stage, characterized when chromosomes become strongly condensed and positioned in the ooplasm on the peripheral of the metaphase plate with no polar body (PB). Furthermore, the complete sets of chromosomes are pulled to each opposite pole or toward the centrioles. A new nuclear membrane is formed around the clumps of chromosomes (anaphase-telophase I). This phase occurs briefly, and it is rarely found in this study. Later, the existence of a metaphase plate with two chromatin masses positioned in the ooplasm's periphery followed by the ejection of the first polar body (represented by a dense chromosome set), categorized as a matured metaphase II (MII) oocyte. Those with altered or absent nuclear structures were considered degenerated (Shirazi et al. 2010; Landim-Alvarenga and Maziero 2014). Immature oocytes (GV-A/TI) are incapable of fertilization and embryo development. Hence immature oocytes must undergo proper oocyte nuclear (MII) and cytoplasmic maturation to obtain their developability (Zhu et al. 2018).

The present study aimed to attenuate OS during *in vitro* maturation in an ovine model by LE supplementation (10 mM, 15 mM, and 20 mM) to improve oocyte quality and successful *in vitro* fertilization. In this study, different concentrations of LE during IVM showed that supplementation of LE at doses of 10 and 15 mM significantly ($P < 0.05$) improved the nuclear maturation of sheep oocytes compared with other concentrations (0 mM and 20 mM). This finding agrees with Öztürkler et al. (2010), who found that the percentage of oocyte maturation was significantly higher

Table 1. Nuclear maturation rate of ovine oocytes supplemented with LE in maturation medium*

Treatment	Number of oocytes	Status of nuclear maturation (% mean±SEM)					D
		GV	GVBD	MI	A/T	MII	
LE 0 mM	175	0.00±0.00 ^a	4.65±1.15 ^{ab}	15.60±2.03 ^b	1.20±0.84 ^a	78.55±1.78 ^a	0.00±0.00 ^a
LE 10 mM	177	0.00±0.00 ^a	0.50±0.50 ^a	10.76±1.74 ^{ab}	0.00±0.00 ^a	88.74±1.79 ^b	0.00±0.00 ^a
LE 15 mM	172	0.67±0.67 ^a	2.10±1.15 ^a	8.55±1.68 ^a	1.17±0.79 ^a	87.52±2.30 ^b	0.00±0.00 ^a
LE 20 mM	179	0.71±0.71 ^a	8.14±2.32 ^b	12.96±2.31 ^{ab}	0.82±0.54 ^a	77.37±1.21 ^a	0.90±0.60 ^a

LE= L-ergothioneine; GV= germinal vesicle; GVBD= germinal vesicle breakdown; MI= metaphase I; A/T= anaphase-telophase I; MII= metaphase II; D= degeneration. ^{a, b} different superscripts in the same column showed significant differences (P<0.05). *This study was carried out with ten replications

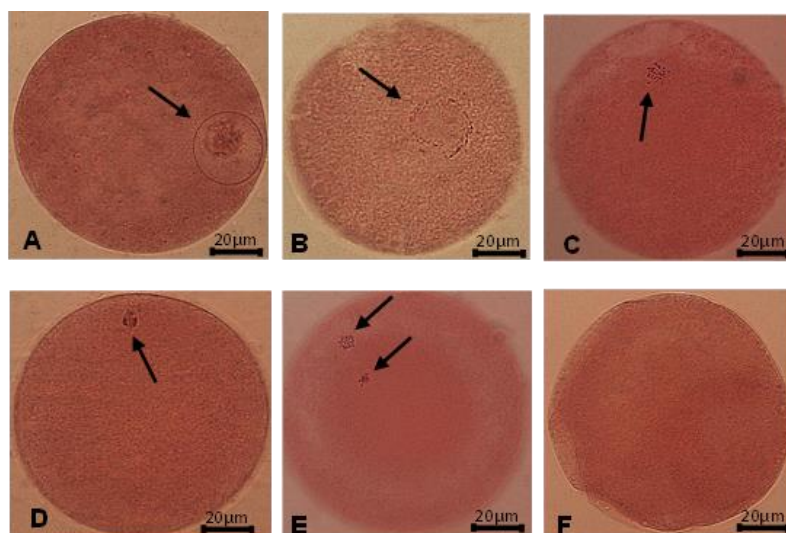


Figure 1. Nuclear maturation status of oocyte using 2% aceto-orcein staining. A= germinal vesicle; B= germinal vesicle breakdown; C= metaphase I; D= anaphase-telophase I; E, metaphase II; F= degeneration. Images were taken using a phase-contrast microscope. Arrows indicate the oocyte's nuclear maturation status.

in the LE 10 mM group compared with other groups (control and L-ascorbic acid 10 mM). The possible explanation is the role of LE as a direct ROS scavenger (Franzoni et al. 2006) and its ability to prevent DNA damage and apoptosis (Paul & Snyder 2010). Secondly, proteins involved in antioxidant cellular pathways, such as superoxide dismutase (SOD), GSH reductase, and catalase, are upregulated by LE (Li et al. 2014; Kerley et al. 2018). In contrast, Mishra et al. (2018) showed that the maturation rate was not affected by LE supplementation (5 mM and 10 mM) in the maturation medium (82.96-84.11%). However, supplementation of LE (10 mM) during the maturation period showed a significantly higher blastocyst rate than the other groups with lower concentrations (0 mM and 5 mM).

As we know, LE is a naturally occurring, water-soluble amino acid found mainly in fungi and derived from histidine, which mammals ingest exclusively through their diet (Bazela et al. 2014; Mishra et al. 2018). LE has two functional groups: the quaternary ammonium group $N-(CH_3)_3$ and the L-thiol (SH) group. The quaternary group of LE transports fatty acids to mitochondria and increases cellular ATP levels. The

thiol group suggests an antioxidant function (Bazela et al. 2014). Amino acids play an essential role in cellular processes serving as the basis for producing proteins, energy, organic osmolytes, and intracellular buffer (Sutton et al. 2003). Amino acids added to the maturation medium serve as a vital energy source for both cumulus cells and oocytes to promote nuclear maturation (Songsasen 2012; Bahrami & Cottee 2022). In addition, amino acids act as glutathione (GSH) precursors and increase GSH levels to prevent oxidative stress in oocytes (Bahrami & Cottee 2022), and apparently, ATP is necessary for GSH production (Xie et al. 2016).

Oocyte maturation is highly dependent on energy metabolism because all the dynamic processes that occur during the development of the nucleus and cytoplasm require a lot of energy from various substrates such as carbohydrates, amino acids, and lipids (Songsasen 2012; Xie et al. 2016). It is well known that numerous organelles, such as the mitochondria, endoplasmic reticulum, and Golgi complexes, increase in number during oocyte growth, demonstrating the importance of protein synthesis and energy production in this process

(Songsasen 2012; Warzych & Lipinska 2020). Most of the cellular energy (about 90%) is generated by mitochondria through oxidative phosphorylation, which can cause a significant amount of ROS (Bazela et al. 2014). Meanwhile, increased oocyte ROS levels can disrupt the maturation-promoting factor and trigger programmed cell death (Rakha et al. 2022). The main cause of triggering apoptosis is often excessive ROS production and mitochondrial damage. ROS can cause the oxidation of mitochondrial pores due to the disruption of mitochondrial membrane potential, triggering the intrinsic apoptotic process (Simon 2000; García-Martínez et al. 2020). In addition, mitochondria possess DNA (mtDNA) that is distinct from nuclear DNA, and mtDNA is more prone to breakage because it lacks histones for its protection. In addition, mitochondria lack the DNA repair mechanisms of the nucleus (Paul & Snyder 2010). Although mitochondria have antioxidant defense mechanisms that can detoxify the amount of ROS produced, some ROS manage to evade these processes and damage proteins, lipids, and mtDNA (Bazela et al. 2014). The primary ergothioneine transporter is OCTN1 (organic cation transporter novel-type 1), encoded by the gene *slc22a4*, which has a beneficial role in LE activity (Cheah & Halliwell 2021). OCTN1 is abundantly expressed in mitochondria (Lamhonwah & Tein 2006; Paul & Snyder 2010). Therefore, supplementing the maturation medium with LE could protect mtDNA from OS and improve mitochondrial quality, which could support cytoplasmic maturation of the oocyte, as mitochondrial distribution is a dynamic process and one of the essential factors in oocyte quality (García-Martínez et al. 2020). Moreover, increasing the number and quality of mitochondria positively affects both the maturation rate and the formation of two pronuclei (Opiela & Kitska-Ksiizkiewicz 2013).

The L-thiol (SH) group of LE implies an antioxidant role and cytoprotective potentials against various cell-stressing factors (Bazela et al. 2014). Thus, LE acts as an antioxidant and effective free radical scavenger. Mishra et al. (2018) reported that LE supplementation during IVM can increase the activity of the enzyme GSH peroxidase, leading to a significant upsurge of GSH in oocytes and, consequently decrease in intercellular ROS, which prevents apoptosis in cells. Furthermore, the free radical scavenging properties of LE neutralize the production of ROS by oocytes throughout the maturation period and protect cell organelles such as mitochondria, which are the primary source of ROS, enabling oocytes to reach the next developmental stage (Bazela et al. 2014). Accordingly, LE acts as an antioxidant and an anti-apoptotic substance. From this study, adding 10-15 mM LE during IVM can promote the successful nuclear maturation of the ovine oocyte, making the oocyte suitable for fertilization by sperm and allowing the formation of two pronuclei achievable. This study

suggested that under *in vitro* conditions, the components of the maturation medium are essential in supporting oocyte maturation to the M-II stage, with antioxidants playing an important role because oocytes are highly susceptible to oxidative stress (Rakha et al., 2022). Therefore, enrichment of the maturation medium with antioxidants, co-enzymes, amino acids, or vitamins can enhance the success of oocyte maturation (Rao et al. 2002; ÖZtürkler et al. 2010). Moreover, the selection of antioxidants and their concentrations are essential. In addition, better information about antioxidants and their mechanisms is needed to support the production of high-quality oocytes and successful maturation and fertilization *in vitro*.

In this study, supplementation of 20 mM LE in the maturation medium shows a decrease in the percentage of oocyte maturation compared to lower doses (LE 10 and 15 mM). Most likely, it is because LE is primarily stored in the body with minimal metabolism (Gründemann 2012). Consequently, a change in the balance between antioxidants and prooxidants may lead to toxicity reported at greater doses. In addition, it is well known that too many antioxidants in the media can cause adverse effects by altering the redox state and weakening the disulfide bonds in proteins, leading to denaturation or inactivation (Guérin et al. 2001; Zullo et al. 2016).

Fertilization capability of ovine oocytes supplemented with LE in the maturation medium

The evaluation of pronucleus formation after fertilization of *in vitro*-matured oocytes by frozen sperm with a concentration of 5×10^6 sperm/mL and incubated for 12-13 h showed in Table 2. Fertilized oocytes are characterized by the formation of pronucleus (PN). Regular fertilization is characterized by two pronuclei (2PN) consisting of one female and one male pronucleus. Oocytes with more than two pronuclei (>2PN) were categorized as polyspermy (Catalá et al. 2012). A microscopic image of the formation of the ovine oocyte in this study is shown in Figure 2.

Table 2 shows that there was a significant difference ($P < 0.05$) in total fertilization, formation of two pronuclei (2PN), and one pronucleus (1PN) between the control group and LE 10 mM (optimum dose obtained from experiment 1). In contrast, there was no significant difference ($P > 0.05$) in the formation of more than two pronuclei (polyspermy) between the two groups. The supplementation of LE 10 mM during maturation resulted in a significantly ($P < 0.05$) higher percentage of total fertilization ($83.91 \pm 2.04\%$) and the formation of 2PN ($75.55 \pm 3.47\%$) compared with the control group ($74.07 \pm 1.06\%$ and $63.78 \pm 3.49\%$, respectively). At the same time, the percentage of 1PN was higher in the control group ($21.62 \pm 2.04\%$) than in the LE 10 mM group ($11.35 \pm 1.54\%$).

Table 2. Fertilization rate of ovine oocytes supplemented with LE in maturation medium*

Treatments	Number of oocytes	Total Fertilization (%mean±SEM)	Pronucleus formation (%mean±SEM)			D
			2PN (Normal)	>2PN (Polyspermy)	1PN	
LE 0 mM	152	74.07±1.06 ^a	63.78±3.49 ^a	10.29±3.91 ^a	21.62±2.04 ^a	4.32±1.56 ^a
LE 10 mM	150	83.91±2.04 ^b	75.55±3.47 ^b	8.36±2.58 ^a	11.35±1.54 ^b	4.73±1.46 ^a

LE= L-ergothioneine; 1PN= one pronucleus; 2PN=two pronuclei; >2PN= more than two pronuclei; D= degeneration. ^{a,b} different superscripts in the same column showed significant differences (P<0.05). *This study was carried out with eleven replications

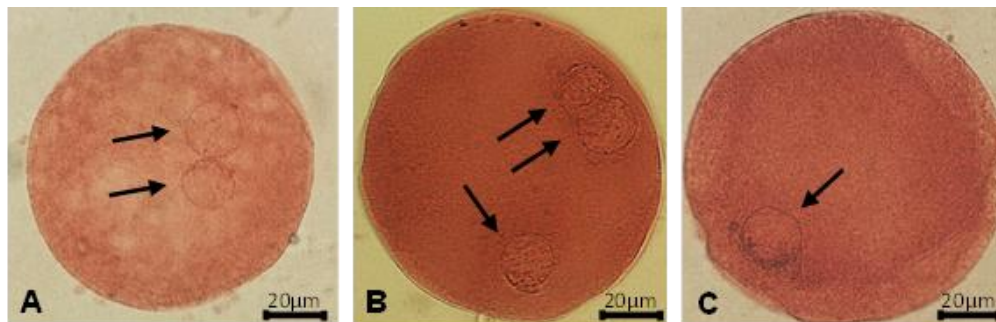


Figure 2. The pronucleus formation of ovine oocytes after *in vitro* fertilization. A. two pronuclei; B. more than two pronuclei (polyspermy); C. one pronucleus. Arrows indicate pronucleus.

In this study, the supplementation of 10 mM LE to the maturation medium significantly increased the formation of two pronuclei after IVF compared to the control group (0 mM LE). The plausible explanation is that LE increases the GSH content in oocytes during IVM, improving oocyte quality and supporting their subsequent development (Hidaka et al. 2018; Anchordoquy et al. 2019). An essential component of the successful formation of two pronuclei is proper cytoplasmic maturation supported by the synthesis of intracellular GSH during IVM (García-Martínez et al. 2020) as incomplete or abnormal oocyte cytoplasmic competence causes inhibition of sperm head decondensation (Catalá et al. 2012). Moreover, the formation of the male pronucleus after fertilization depends on the adequate amount of GSH reservoirs acquired during the maturation period since GSH plays an essential role in the development, upkeep, and defense of the meiotic spindle towards oxidative stress (García-Martínez et al. 2020). In addition, a study by Mishra et al. (2018) stated that LE supplementation in a maturation medium could support cytoplasmic oocyte maturation by increasing intracellular GSH. Increased intracellular GSH in matured oocytes can significantly increase the percentage of male pronucleus (MPN) formation after IVF (Urdaneta et al. 2004; Anchordoquy et al. 2019). In addition to oocyte activation, GSH is involved in sperm decondensation and conversion of the sperm head, which fertilizes the oocyte into the male pronucleus (De Matos et al. 2002; Rodríguez-González et al. 2003). GSH content has been shown to increase *in vivo* as the oocyte

approaches the time of ovulation, protecting it at later stages of fertilization (Anchordoquy et al. 2019). In addition, GSH in the intracytoplasmic protects cells from oxidative damage, and high intracytoplasmic GSH in oocytes after IVM remains constant after fertilization and disappears at the 6 to 8-cell embryonic stage (Urdaneta et al. 2004).

The incidence of polyspermy in our study ranged between 8.36–10.29%. In comparison, the polyspermy rate in the survey by Gulo et al. (2020) showed a range between 8.5–14.3%, while the range in the study by Anzalone et al. (2021) was 6.5–17.8%. This finding indicated that the polyspermy occurrence in this study is still in the normal range. The prevalence of polyspermic penetration in ovine oocytes has been known as a significant problem in IVF that also has been observed in certain other species. It results in early embryonic lethality or embryo developmental arrest, which reduces the fertilization rate of oocytes (Bragança et al. 2021).

Furthermore, Mondéjar et al. (2013) stated that the leading cause of polyspermy is mainly due to deficient or belated zona pellucida hardening, and it appears to be connected to deficiencies in oocyte cytoplasmic maturation, which leads to difficulties in cortical granules (CGs) exocytosis (Batista et al. 2016; Evans 2020; Rojas et al. 2021). Conversely, Wang et al. (2003) mentioned that immature oocytes displayed higher percentages of polyspermic penetration after IVF in most mammals than mature oocytes. Immature oocytes cannot prevent polyspermy due to the CGs' failure to release after sperm entry, resulting from incomplete

oocyte activation (Wang et al. 2003). Oocyte activation is a coordinated and orchestrated process with predetermined outcomes consisting of meiosis II (MII) resumption, the extrusion of second polar body (2ndPB), cortical granule exocytosis which alters zona pellucida to prevent polyspermy and cytoskeletal reorganizations (Swain and Pool 2008; Kashir et al. 2022). Additionally, Wang et al. (2003) explained that immature oocytes have less calcium oscillation in response to sperm penetration, calcium injection, and injection of inositol 1,4,5-trisphosphate (IP₃) since immature oocytes contain fewer IP₃ receptors in their cortex, which reduces their capacity to respond to activators. Furthermore, the amount of cortical endoplasmic reticulum responsible for calcium reserves is less in immature oocytes (Wang et al. 2003).

Cytoplasmic maturation is the fundamental process that has an impact on oocyte quality, involving the development of organelles, mainly cortical granules, Golgi complexes, endoplasmic reticulum (ER), and mitochondria, as well as the accumulation of mRNA, proteins, substrates, and nutrients (Sun et al. 2022). This circumstance demonstrated that reorganizing cytoplasmic organelles such as CGs is essential to produce more competent oocytes, which are required to induce cortical and zona reactions (Slavík et al. 2005). A proper CGs migration to the ooplasm is compulsory for upcoming exocytosis after sperm penetration, and this migration occurs during the final oocyte maturation (Wang et al. 2003). Contrarily, insufficient cytoplasmic maturation leads to improper fertilization due to abnormal signaling pathways and insufficient protein synthesis, which results in cortical response failure and polyspermic penetration (Swain & Pool 2008). In addition, Xia (2013) stated that there are three possible degrees of oocyte cytoplasmic maturation: immature, mature, and overmature. Oocytes with immature or over-mature cytoplasm (aging) tend to have polyspermy after IVF. Aging oocytes reveal biological abnormalities, including impairment in cortical granules reaction and zona pellucida hardening, mitochondrial malfunction, meiotic aberrations, and depleted ATP production and maturation-promoting factor (MPF) expression (Wang et al. 2017; Luo et al. 2020).

According to Bahrami et al. (2019) that the slicing technique to collect oocytes from the ovarium could be one of the reasons for polyspermy because this method might cause the release of oocytes from primordial follicles and only depend on the presence of thick layers of cumulus cells and homogeneous cytoplasm, allowing these oocytes to be classified as grade A. Consequently, 24 hours of IVM is likely insufficient to fully and concurrently mature the nuclear and cytoplasmic of these primary oocytes. Furthermore, oocytes that have reached nuclear maturity may not have reached cytoplasmic maturity (Lee et al. 2003; Abbara et al. 2018) since some aspects of cytoplasmic maturation are

independent of the nuclear component even though cytoplasmic components can be influenced by nuclear maturation (Swain & Pool 2008; Xia 2013). Consequently, depending on the oocyte's cytoplasmic maturation level, meiotically matured oocytes with first polar body appearance may not have all the essential maternal constituents needed for subsequent fertilization, PN formation, and embryo development (Swain & Pool 2008; Sun et al. 2022).

This study found one pronucleus (1PN) formation in control and LE 10 mM groups. The main reasons for unsuccessful IVF include failure in sperm chromatin decondensation, a lack of male pronuclear (MPN) formation, and ineffective oocyte activation (Shirazi et al. 2018). In theory, the failure of oocyte activation resulted from Phospholipase C zeta (PLC ζ) as a Sperm-borne Oocyte-activating Factor (SOAF) unable to permeate the sperm plasma membrane (Ramadan et al. 2012). Furthermore, the oocyte contents, particularly glutathione (GSH), appear to be necessary for weakening the disulfide bond in the sperm nucleus and facilitating the formation of male pronuclear throughout fertilization (Shirazi et al. 2018). Some possibilities have been reported that one pronucleus (1PN) zygotes might develop from parthenogenetic activation, aberrant formation of nuclear envelopes such as asynchrony of the pronuclear emersion, belated pronuclear formation, and fusion between male and female pronuclei (Van Der Heijden et al. 2009; Hirata et al. 2020). Parthenogenetically activated oocytes would result in a haploid embryo, and only maternal chromatin is decondensed due to the failure of sperm nucleus decondensation (Hirata et al. 2020). Contrarily, Van Der Heijden et al. (2009) revealed that several 1PN zygotes are of paternal origin and typically formed when parental chromatin fuses following sperm penetration. Furthermore, asynchronous pronuclear development and male and female pronuclear fusion indicate that fertilization has succeeded, and the following embryos could be diploid (Hirata et al. 2020). A report by Itoi et al. (2015) proposed that IVF-derived 1PN embryos are presumably generated as the male and female chromosomes are surrounded by the same nuclear membrane due to sperm entering the oolemma from various locations, including sites near the spindle apparatus in the ooplasm.

CONCLUSION

In conclusion, the supplementation of 10 to 15 mM LE in the maturation medium increases oocyte nuclear maturation of oocytes. In addition, supplementing 10 mM LE in the maturation medium improves the oocyte fertilization rate.

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Rumen Fermentation Profiles of Protein-Energy Synchronization Index-Based Ration: an *In Vitro* Study

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ABSTRAK

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Penelitian bertujuan untuk mengkaji pengaruh ransum berbasis indeks sinkronisasi protein-energi (SPE) terhadap profil fermentasi rumen. Materi yang digunakan yaitu cairan rumen Kambing Jawa Randu yang diambil sesaat setelah kambing dipotong. Komposisi ransum perlakuan terdiri dari rumput gajah, lamtoro, bungkil kelapa, ampas tahu, dedak, onggok, dan mineral mix yang disusun berdasarkan indeks SPE masing-masing bahan pakan. Pengujian dilakukan secara *in vitro* dan menggunakan rancangan acak lengkap (RAL). Perlakuan terdiri dari 4 indeks SPE yaitu 0,55 (R1); 0,6 (R2); 0,65 (R3); dan 0,7 (R4) dan masing-masing terdapat 5 ulangan. Data dianalisis dengan analisis variansi dan diuji menggunakan orthogonal polynomial (OP). Hasil analisis variansi menunjukkan bahwa indeks SPE tidak berpengaruh terhadap produksi asetat (C2) dan butir (C4), tetapi berpengaruh sangat nyata terhadap pencernaan bahan kering (KBK), pencernaan bahan organik (KBO), pH, volatile fatty acids (VFA) total, propionate (C3), C2:C3 rasio, metan (CH₄), amonia (NH₃), dan sintesis protein mikroba (SPM). Hasil uji OP menunjukkan pengaruh kuadrat pada semua variabel dengan persamaan yaitu $Y = -81,601X^2 + 375,04X - 310,78$ (KBK), $Y = -433,69X^2 + 522,69X - 128,75$ (KBO), $Y = -44X^2 + 54,04X - 9,9$ (pH), $Y = -2160X^2 + 2576,8X - 604,2$ (VFA Total), $Y = -481,8X^2 + 585,01X - 143,45$ (C3), $Y = 50,93X^2 - 60,177X + 21,067$ (C2:C3), $Y = 202,45X^2 - 223,18X + 103,41$ (CH₄), $Y = 436X^2 - 552,28X + 181,08$ (NH₃), dan $Y = -1012X^2 + 1260X - 311,64$ (SPM). Ransum dengan indeks SPE 0,6 memiliki profil fermentasi rumen terbaik. Formulasi ransum dengan indeks 0,6 lebih efektif dibandingkan lainnya berdasarkan pada hasil propionate yang tinggi, metan yang rendah, NH₃ yang rendah, dan SPM yang tinggi. Ransum dengan indeks 0,6 tersusun atas 30% rumput gajah, 30% lamtoro, 10% bungkil kelapa, 10% ampas tahu, 10% dedak, 9% onggok, dan 1% mineral mix.

Kata Kunci: Ransum, Indeks Sinkronisasi Protein-Energi, Fermentasi Rumen, *In Vitro*

ABSTRACT

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The study examined the effect of protein-energy synchronization (PES) index-based rations on the rumen fermentation profile. The material used was the Jawa Randu goat's rumen fluid, collected soon after the goat was slaughtered. The treatment ration consisted of elephant grass, lamtoro (*Leucaena leucocephala*), coconut meal, tofu waste, bran, cassava waste, and mineral mix arranged based on the PES index of each feed ingredient. The research was conducted *in vitro* using a completely randomized design (CRD). The treatment consisted of 4 PES indexes, namely 0.55 (R1); 0.6 (R2); 0.65 (R3); 0.7 (R4), and each had 5 replications. Data were analyzed by ANOVA and orthogonal polynomials (OP). The results of the ANOVA showed that the PES index did not affect the production of acetate (C2) and butyrate (C4) but had a highly significant effect on dry matter digestibility (DMD), organic matter digestibility (OMD), pH, a total of volatile fatty acids (VFA), propionate (C3), C2:C3 ratio, methane (CH₄), ammonia (NH₃), and microbial protein synthesis (MPS). The analysis of OP showed a quadratic effect on all variables with the equation $Y = -81,601X^2 + 375,04X - 310,78$ (DMD), $Y = -433,69X^2 + 522,69X - 128,75$ (OMD), $Y = -44X^2 + 54,04X - 9,9$ (pH), $Y = -2160X^2 + 2576,8X - 604,2$ (VFA Total), $Y = -481,8X^2 + 585,01X - 143,45$ (C3), $Y = 50,93X^2 - 60,177X + 21,067$ (C2:C3), $Y = 202,45X^2 - 223,18X + 103,41$ (CH₄), $Y = 436X^2 - 552,28X + 181,08$ (NH₃), dan $Y = -1012X^2 + 1260X - 311,64$ (MPS). Protein-energy synchronization (PES) index of 0.6 achieved the best rumen fermentability. The formulation of the PES index-based ration of 0.6 had the most effective compared to other indexes based on the high of propionate, the lowest methane, the lowest NH₃, and the best microbial protein synthesis. The ration with an index of 0.6 is composed of 30% elephant grass, 30% *Leucaena leucocephala*, 10% coconut meal, 10% tofu waste, 10% rice brand, 9% cassava waste, and 1% mineral mix.

Key Words: Ration, Protein-Energy Synchronization Index, Rumen Fermentation, *In Vitro*

INTRODUCTION

The principle of preparing rations for ruminants is to stimulate the optimal development of rumen microorganisms. The role of rumen microorganisms is crucial for the feed fermentation process and fulfills more than 60% of the protein requirement for the animal (Liu et al. 2021). The main growth factor for all types of rumen microbes is the availability of ammonia and energy, while the need for cofactors differs for each species. The two compounds have a positive associative relationship, so the availability of one compound affects the use of other compounds in microbial protein synthesis (MPS). Therefore, synchronization of the availability of the two compounds is essential to achieve optimizing MPS and can be done through the preparation of rations based on the protein-energy synchronization index (Zhang et al. 2020).

Preparing rations based on the protein-energy synchronization (PES) index differs from the Cornell net carbohydrate and protein system (CNCSP) and practical synchronization methods. This ration is compiled with a synchronization value measured quantitatively through indexation with a scale of 0-1. The higher the PES index of the ration, the more harmonious the supply of ammonia and energy in the rumen (Sinclair et al. 1993; Walidi et al. 2017). Although MPS is the main target, the final result of preparing this ration is feed use efficiency through increased rumen fermentation activity. Suhada et al. (2016) proved that the higher PES index of the ration resulted in higher efficiency of microbial protein synthesis and higher total volatile fatty acids (VFA) production. The best energy efficiency is also obtained from rations with the highest index based on the ratio of acetate: propionate and methane (CH₄) production, which decreases with increasing PES index.

Preliminary research has been carried out to inventory the protein-energy synchronization index of various types of local feed ingredients from grass, legumes, energy source concentrates, and protein source concentrates. The PES index of each feed ingredient is used as the basis for compiling the ration by setting the scale planned by the researcher. However, this method has not been widely used, especially on local feed ingredients with different characteristics than sub-tropical regions. Therefore, this study aimed to examine the effect of preparing rations based on the protein-energy synchronization index on the rumen fermentation profile.

MATERIALS AND METHODS

Materials

The material used in this study was the rumen fluid of the Jawa Randu Goat, which was fed with 14% protein

content and 65% TDN. The composition of the treatment ration consisted of elephant grass, lamtoro (*Leucaena leucocephala*), coconut meal, tofu waste, rice bran, cassava waste, and mineral mix.

Research variables

The variables measured in this study were dry matter and organic matter digestibility, pH, microbial protein synthesis (MPS), ammonia (NH₃), total volatile fatty acids (VFA), acetate (C2), propionate (C3), butyrate (C4), C2:C3 ratio, and methane (CH₄).

Methods

The research was conducted experimentally at the Laboratory of Nutrition and Animal Feed Science, Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, Indonesia, from April to September 2021.

The in vitro method of Tilley & Terry (1963) was used in this research.

The ration is formulated based on the PES index of each feedstuff used. First, it is obtained by measuring the hourly degradation of organic matter (OM) and protein. The times used to measure the degradation were 2, 4, 6, 8, 12, 24, 48, and 72 hours for forages and 2, 4, 6, 8, 12, and 48 hours for concentrate. Degradability at all time observations is then entered into a linear equation to obtain degradation of g protein or Kg OM hourly. Then the degradation of g protein and Kg OM hourly is used to calculate the PES index of each feedstuff with the following equation.

$$PES\ index = 20 - \frac{\sum_{1-24}^n \sqrt{(20 - \frac{N}{OM\ hourly})^2}}{24}$$

where n is observation time; N/OM hourly is the degradation rate of protein, compared to the organic matter hourly; 20 is 20 g N-protein/kg OM degradability on the rumen; 24 is 24 hours (Hermon et al. 2008).

The PES index of feedstuff used results from preliminary research. After the PES index of each feed ingredient is obtained, the ration formulation is compiled based on the PES index. First, it is determined by multiplying the grams used for each feed ingredient in the ration by each PES index. Then multiplication is performed for each synchronization. index is added up and divided by the total gram of ration. The PES index used in the study were 0.55, 0.6, 0.65, and 0.7.

Experiment design and data analysis

The completely randomized design (CRD) was used with four types of rations with different protein-energy

Table 1. Feed composition and nutrient content used as treatments

Feedstuff	R1	R2	R3	R4
<i>PES indexes</i>	<i>0.55</i>	<i>0.6</i>	<i>0.65</i>	<i>0.7</i>
Elephant grass (%)	20	30	40	50
<i>Leucaena leucocephala</i> (%)	40	30	20	8
Coconut meal (%)	5	10	11	11
Tofu waste (%)	15	10	7	8
Rice bran (%)	15	10	7	8
Cassava waste (%)	4	9	14	14
Mineral mix (%)	1	1	1	1
Total (%)	100	100	100	100
Nutritional content *				
Dry matter (%)	82.65	83.34	84.03	82.91
Moisture (%)	17.35	16.66	15.97	17.09
Organic matter (%/DM)	87.27	84.62	83.86	83.47
Ash (%/DM)	12.73	15.38	16.14	16.53
Crude protein(%/DM)	15.55	14.80	13.91	12.40
Crude fiber (%/DM)	33.39	33.65	29.44	29.30
Extract ether (%/DM)	6.32	4.30	3.60	3.24
NFE (%/DM)	32.01	31.87	36.90	38.53
TDN (%/DM)	52.46	50.05	51.81	50.99

R1= ration with PES index 0.55; R2= ration with PES index of 0.6; R3= ration with PES index of 0.65; R4= ration with PES index 0.7; NFE= nitrogen free extract; TDN= total digestible nutrients; TDN= (70.60 + 0.259 CP + 1.01 EE) – (0.76 CF + 0.0991 NFE)

synchronization indexes, each treatment consisting of 5 replications; thus, there were 20 experimental units. The data obtained were tabulated and then analyzed using ANOVA. The treatments that significantly affect the variable is tested using orthogonal polynomials. The treatments are described in Table 1.

Measuring nutrition ration treatment

The nutrition of the treatment ration was measured by proximate analysis (AOAC 2005) to determine the nutrient content of each ration. The calculated nutrient content was dry matter (DM), moisture, organic matter (OM), ash, fat (extract ether), crude fiber, crude protein, nitrogen-free extract (NFE), and total digestible nutrient (TDN).

Measuring digestibility

The in vitro method used to measure supernatant and treatment residues is the in vitro method from Tilley & Terry (1963). The first stage is taking rumen fluid using a thermos filled with hot water at 39°C. The rumen fluid

was taken immediately after the goats were slaughtered at the abattoir. The rumen fluid is squeezed and filtered using gauze and put in a warm thermos drained of water. Next, in vitro digestion was carried out using a 250 ml Erlenmeyer which had been filled with 2 g of sample, added 16 ml of rumen fluid and 24 ml of McDougall's solution, then put in a shaker bath with a temperature of 39°C, the Erlenmeyer was shaken with CO₂ flowing for 30 seconds, with a pH of 6.5 -6.9 and then covered with ventilated rubber, and fermented for 2 x 24 hours. After 24 hours, the fermenter tube was centrifuged at 4,000 rpm for 15 minutes. The substrate will separate into a precipitate at the bottom and a supernatant (liquid) at the top. The supernatant was taken for subsequent analyses (pH, NH₃, partial VFA, and CH₄). At the same time, the residue or precipitate was then incubated for 24 hours for hydrolytic incubation to measure the dry matter digestibility (DMD) and organic matter digestibility (OMD). Both were calculated based on the equation made by Tilley & Terry (1963) followed.

$$\text{Digestibility} = \frac{DM/OM \text{ sample} - (DM/OM \text{ residue} - DM/OM \text{ blank})}{DM/OM \text{ sample}}$$

Measuring pH

The rumen fluid's pH was measured using a pH meter. First, the pH meter is calibrated sequentially with an alkaline solution (buffer) and an acidic solution. Second, the tip of the pH meter flowed with distilled water to keep the pH neutral. Then the pH meter is inserted into the supernatant. The pH meter display will show the read pH number. The pH measurement activities were carried out individually for each sample, and at each sample change, the pH meter was rinsed with distilled water.

Measuring volatile fatty acids

The first step is boiling the water in a boiling flask and washing the sample holder with distilled water. Next, put 5 ml of rumen fluid into the sample holder, and then add 1 ml of 15% H₂SO₄. Next, the distillate was accommodated in a 250 ml Erlenmeyer flask filled with 0.5 N NaOH until the distillate volume reached 100 ml. Next, the distillate was added with two drops of phenolphthalein and titrated with 0.5 N HCl until a color change occurred. Finally, as a control, a blank was made with 5 ml of 0.5 N NaOH with 0.5 N HCl. After the sample data and the blank are obtained, enter them into the equation below.

$$VFA\ level = \left((Y - Z) \times NHCL \times \left(\frac{1000}{5} \right) \right) nM$$

where Y is ml of HCl required for blank titration, and Z is ml of HCl required for distillate titration.

The rumen fluid's partial VFA levels (acetate, propionate, butyrate) were measured using the gas chromatography technique described by Matsui et al. (1992). The standard solutions contained the following VFA: acetate 52.54% molar, propionate 13.42% molar, isobutyrate 5.40% molar, n-butyrate 10.89% molar, isovalerate 4.23% molar, n-valerate 4.61% molar. Calculations other than the previous explanation can also be carried out using this technique: VFA (% molar) = VFA area of the sample x standard VFA content divided by standard VFA area. Partial VFA was measured by using the gas chromatography technique by taking 4 ml of rumen fluid using a pipette and then centrifuging it at 10,000 rpm for 15 minutes. Next, the supernatant was added to as much as 2 ml into a 5 ml plastic tube. Next, add 30 mg of 5-sulphosalicylic acid. Then the solution is shaken, centrifuged at 3,000 rpm for 10 minutes at 4°C, then filtered with a millipore until a clear liquid is obtained. A total of 1 l of clear liquid was injected into the gas chromatography, which was previously injected with a standard solution of VFA. The concentration in the sample is calculated using the VFA formula = sample height divided by the standard height times the standard concentration, according to the individual solution to be measured.

Estimation of methane level

Methane gas (CH₄) levels were not measured directly through laboratory tests but were calculated based on the proportion of partial VFA obtained. The gas content of CH₄ is computed using the mM formula developed by Orskov & Ryle (1996), namely CH₄ = 0.5 a – 0.25 p + 0.5 b (a = acetate, p = propionate, and b = butyrate).

Measuring ammonia

NH₃ levels were measured by using the Conway micro diffusion technique. The first step is to prepare the Conway cup by smearing vaseline on the cup and lid. Next, 1 ml of boric acid was taken and dropped in the middle of the cup. Then on the skating to the right of the cup, 1 ml of rumen fluid was added. Next, the cup is tilted, then 1 ml of saturated Na₂CO₃ is added to the slide to the left of the cup. Next, the cup is closed and moved slowly so that all the liquid is mixed and left for 24 hours at room temperature. After 24 hours, the solution was titrated with 0.01 N H₂SO₄ until the color changed to pink. After that, it is calculated by the following equation, NH₃ = (ml titrant x N H₂SO₄ x (1000/1)) mM (National Bureau of Standards 1967).

Measuring MPS

According to Zinn & Owens (1986), measuring MPS has several stages. In the first stage, 0.5 g of dry residue sample (residue in the oven at 60°C for two days) was pulverized with mortar, then put into a closed 25 ml culture tube. The second stage involved adding 2.5 ml of HClO₄ (70%) into the culture tube. After that, the culture tubes were tightly closed and incubated in a water bath at 90-95°C for one hour. After completion of incubation (marked by a change in the color of the solution to black-like charcoal), the sample was crushed into a small powder with a glass rod so that the sample was easily extracted. Then, 17.5 ml of 0.0285M NH₄H₂PO₄ pH 2 buffer solution was added to the tube and stirred until homogeneous with a vortex stirrer. The solution was heated again in a water bath at a temperature of 90-95°C for 15 minutes. The heating results were filtered with Whatman paper no 4. The resulting filtrate was taken at 0.5 ml and put into a centrifuge tube. The 0.5 ml of 0.4 M AgNO₃ and 9 ml of 0.2 M NH₄H₂PO₄ buffer solution were added and allowed to stand for 30 minutes in the darkroom (Better precision is shown by incubating overnight in a refrigerator at 5°C. After that, the solution was centrifuged for at least 15 minutes at 5,000 rpm and decanted the supernatant. The precipitate formed was rinsed with distilled water pH 2 and centrifuged again. The results of the second centrifuge were added 10 ml of

0.5 N HCl, stirred evenly using a vortex, tightly closed the culture tube, then heated again in a water bath with a temperature of 90-95°C for 30 minutes. Light absorption (absorbance) was read at a wavelength of 260 on an ultraviolet spectrophotometer (Walker & Nader 1986). The standard is 100, 200, 300, and 400 mg yeast RNA. The procedure was the same as for the sample and diluted at 1:20 after seven steps.

$$Y = a + bx$$

where Y is Absorption spectrophotometer ultraviolet, a is Intercept, b is regression coefficient, and x is MPS.

RESULTS AND DISCUSSION

Digestibility value

The protein-energy synchronization (PES) index-based ration preparation aims to increase the quantity of rumen microbial synthesis and its ability to carry out optimal ration fermentation. Dietary nutrient digestibility is an essential indicator of rumen fermentability. High digestibility values indicate optimal microbial activity in utilizing and converting nutrients into fermented products needed by the animal. The results showed that dry matter digestibility (DMD) and organic matter digestibility (OMD) is relatively smaller than the standard digestibility, which can reach 50-80%, even more (Yanuartono et al. 2019). Mahyuddin (2008) explained that *in vitro* digestibility measurements resulted in lower digestibility compared to the actual conditions in the rumen. The results of *in vitro* digestibility are also very diverse, depending on the level of control of the factors that affect *in vitro* digestibility. Factors influencing *in vitro* digestibility are temperature stability, rumen fluid source, rumen fluid handling, artificial saliva, CO₂ supply, valve ventilation control, and others. However, the digestibility rhythm produced is a vital overview that can be used as a reference in determining the digestibility of the ration in the rumen.

The analysis of variance showed that the PES index-based ration had a highly significant effect ($P < 0.01$) on DMD and OMD. The Tukey analysis showed that the DMD in R1, R2, and R3 is not different and is higher than in R4. Meanwhile, the OMD on R2 and R3 were not different and higher than R1, then R1 was higher than R4. The orthogonal polynomial (OP) analysis showed a quadratic response to the DMD ($Y = -81.601X^2 + 375.04X - 310.78$; ($R^2 = 0.71$)) and OMD ($Y = -433.69X^2 + 522.69X - 128.75$; ($R^2 = 0.92$)). Figure 1a showed that DMD initially increased in rations from a PES index of 0.55 (R1) to a PES index of 0.6 (R2) and then continued to decrease in rations with a PES index of 0.65 (R3) and 0.7 (R4). The peak was in the ration with a PES index of 0.6 and a DMD of 51.55%. Similar graphic dynamics are shown in the OMD results for all treatments. The

quadratic graph (Figure 1b) is generated with the peak point at the PES index of 0.6 with an OMD of 48.74%.

Based on Tukey and OP analysis, it is known that R4 has the lowest DMD and OMD, which may be due to the ratio between protein and NFE rations being quite large; even the PES concept is also closely related to the balance in the availability of protein and NFE rations. R4 has the highest NFE but the lowest protein, with a ratio of 3.11. Walidi et al. (2017) and Indah et al. (2020) explained that a high proportion of NFE and protein could affect the availability of ammonia and energy, which needs to be balanced. This high proportion of NFE and protein will correlate with the slowdown in microbial protein synthesis (MPS) and ultimately affect the slowness of the DMD and OMD processes. NFE and protein ratios in R1, R2, and R3, respectively, were 2.06, 2.15, and 2.65, which makes it possible that there is no difference in the Tukey analysis. Although the Tukey analysis showed no difference, the OP analysis showed that the highest DMD was obtained in R2. Besides that, Tukey analysis showed that the OMD at R1 is lower than R2 and R3. Franco et al. (2016) stated that factors affecting the digestibility value are the amount and content of nutrients consumed. The high value of DMD on R2 is due to the relatively evenly distributed feedstuff compared to others in fiber, protein, and energy sources. Protein and carbohydrates (NFE) are nutrients that majorly affect digestibility. The composition of this ration resulted in crude protein content and NFE, respectively, 14.80% and 31.87%. Although R1 had a lower protein and NFE ratio than R2, the use of *Leucaena* in this treatment was the highest. Montoya-Flores et al. (2020) stated that adding *leucaena* to the treatment will affect digestibility. *Leucaena* is a legume that contains high protein but contains anti-nutrients, namely tannin, and mimosine, which can interfere with the performance of rumen microorganisms. According to Ariani et al. (2015), *leucaena* leaves should be no more than 40 percent to reduce the risk of poor performance of rumen microorganisms. The decrease in DMD in the R3 and R4 treatments on OP analysis was due to the higher ratio of protein and NFE. The increase in this ratio is in line with the lower crude protein content and higher NFE levels, so the synchronization of protein and energy becomes increasingly unbalanced, which is in line with the opinion of Syamsi et al. (2018) that the synchronization index is primarily determined by comparing protein levels and the NFE of the ration. Protein is the primary source of ammonia supply, and NFE is a fermentable carbohydrate that provides available energy in the rumen to increase the use of ammonia in MPS.

The digestibility of organic matter is closely related to the degradation of dry matter because most of the dry matter is a component of organic matter. Hartono et al. (2016) stated that DMD and OMD have a positive correlation, so OMD will follow the dynamics of decreasing or increasing DMD. The DMD and OMD

Table 2. Dietary digestibility profile based on protein-energy synchronization index *in vitro*

Digestibility	Treatment				Sig.
	R1	R2	R3	R4	
Dry matter (%)	50.76±0.52 ^a	51.24±0.82 ^a	51.18±1.00 ^a	48.55±0.42 ^b	**
Organic matter (%)	47.68±0.33 ^b	48.32±0.25 ^a	48.18±0.51 ^a	44.49±0.32 ^c	**

R1= index 0.55, R2= index 0.6, R3= index 0.65, R4= index 0.7. **highly significant effect

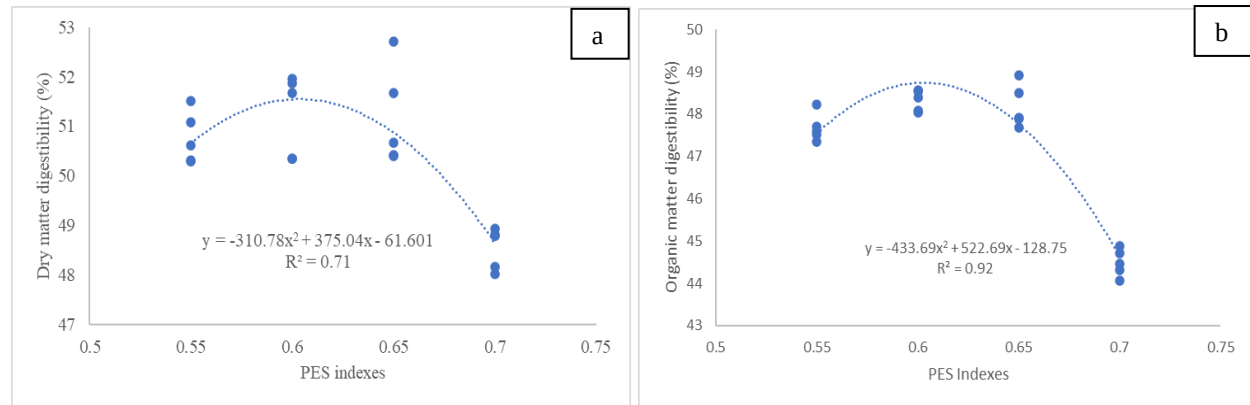


Figure 1. Digestibility graph of dry matter (a) and organic matter (b)

Table 3. Profile of rumen fermentation products in rations based on *in vitro* protein-energy synchronization index

Fermentation products	Treatment				Sig.
	R1	R2	R3	R4	
pH	6.5±0.07 ^b	6.72±0.11 ^a	6.6±0.10 ^{ab}	6.38±0.08 ^c	**
VFA Total (mM)	158.80±2.05 ^b	166.80±1.30 ^a	155.60±1.14 ^c	142.00±1.87 ^d	**
C2 (mM)	110.68±8.0	109.34±3.88	117.74±5.26	116.19±2.02	ns
C3 (mM)	32.33±2.46 ^b	34.78±0.99 ^a	32.56±1.30 ^b	30.19±0.60 ^c	**
C4 (mM)	9.71±0.45	10.15±0.75	10.72±1.16	9.30±1.05	ns
C2:C3 Ratio	3.43±0.90 ^c	3.15±0.18 ^d	3.62±0.16 ^b	3.85±0.11 ^a	**
CH ₄ (mM)	42.40±3.27 ^{bc}	40.90±1.82 ^c	45.37±2.05 ^{ab}	45.89±1.23 ^a	**
NH ₃ (mM)	9.32±2.89 ^a	6.16±2.03 ^c	6.72±0.43 ^{bc}	7.92±0.35 ^{ab}	**
MPS (g/ml)	74.49±0.48 ^c	82.08±4.27 ^a	77.65±3.87 ^b	75.12±1.53 ^c	**

R1= index 0.55, R2= index 0.6, R3= index 0.65, R4= index 0.7, ns= no significant effect, VFA= volatile fatty acids, C2= acetate, C3= propionate, C4= butyrate, CH₄= methane, NH₃= ammonia; MPS= microbial protein synthesis. **highly significant effect

values are also influenced by rumen environmental conditions, affecting rumen microbial activity. One of the main ones is the degree of acidity or rumen pH. Kitkas et al. (2022) stated that the rumen's pH standard was 5.5-6.8; the closer to 6.8, the better. Table 3 shows that the pH in R1, R2, and R3 is above 6.5, and R4 is below. It also makes it possible that there is no DMD difference in the Tukey analysis. Besides that, the pH of R2 was the highest, so R2 was the highest DMD and OMD on OP analysis, in line with the opinion of Li et al. (2021) that the lower the pH, the lower the digestibility

because microorganisms generally have optimum activity at a pH close to neutral.

A PES index-based-ration is based on the potential of a simultaneous ammonia and energy supply through an indexed overview. The PES ration index is set between 0.0 to 1.0, where the index closer to 1.0 indicates the most simultaneous potential in providing the two nutrients. This result is different from the research by Chumpawadee et al. (2005), which shows that the synchronization index has a linear effect on DMD and OMD, where the DMD and OMD will increase with

increasing PES index. Based on Table 1, the type of feed ingredient also needs to be considered. The preparation of the rations in Table 1 only focuses on achieving the ration index, which impacts nutrient levels that are different from what is expected. For example, the ratio between NFE proteins does not decrease with increasing index numbers. In addition, the use of feed ingredients such as legumes has an impact on the digestibility value, which is due to the anti-nutrients they carry. Then it gives non-linear results increasing in DMD or OMD.

Rumen fermentation products

The feed eaten by ruminants will undergo a fermentation process in the rumen. Most sources of protein and carbohydrates will degrade their fermentation into various fermentation products. The type of feed, temperature, and degree of acidity (pH) strongly influence rumen fermentation products. The optimal pH for rumen metabolism is in the range of 6.2-7. Therefore, a decrease in pH below 6.2 will cause a slowdown in the performance of fiber-digesting bacteria.

In contrast, a reduction in pH below 5.6 will cause the death of fiber-digesting bacteria, an increase in lactate-producing bacteria, and the appearance of early symptoms of acidosis (Kim et al. 2018; Li et al. 2021). The results showed that the average rumen pH of all

treatments ranged from 6.38-6.72 (Table 3). This condition indicates the rumen fluid is at an ideal pH for rumen microbe activity, suggesting that the PES index-based-ration can optimize the rumen pH conditions to be within the normal range.

The analysis of variance showed that the diet based on the PES index had a highly significant effect ($P < 0.01$) on rumen pH. Tukey's analysis showed that R2 and R3 had the highest pH, but R3 was not different from R1, and R4 was the lowest. The OP analysis showed a quadratic response of the PES index to pH (Figure 2a) with the equation $Y = -44X^2 + 54.04X - 9.9$ and a coefficient of determination (R^2) = 0.67. Figure 2a shows that the pH increased from R1 to R2, then decreased in R3 and R4. The peak reached P (0.61:6.69), meaning it obtained the highest pH at the index of 0.61 with a pH of 6.69. The degree of pH of the rumen fluid is a balance between the buffer capacity and the alkaline or acidic properties of the fermentation product. Cahyaningtyas et al. (2019) explained that carbohydrates are one of many factors influencing rumen pH. High fiber content can help improve the pH balance of the rumen. The R2 treatment in Table 1 has the highest fiber content (33.65%) but the lowest NFE content (31.87%). Jasin and Sugiyono (2014) stated that the high content of NFE in the ration causes the pH to be quiet because it is a fermentable carbohydrate quickly used by lactate-producing microorganisms. Tables 1 and 3 prove that

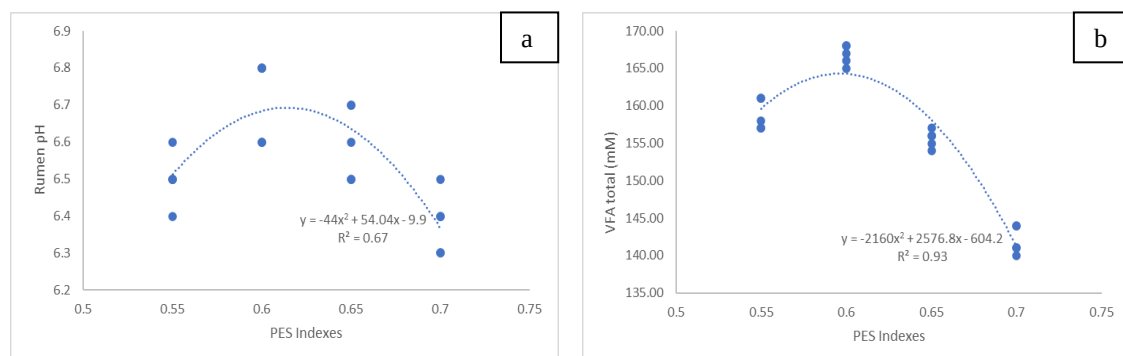


Figure 2. Graph of the pH (a) and a total of volatile fatty acids (b) on rumen fluid

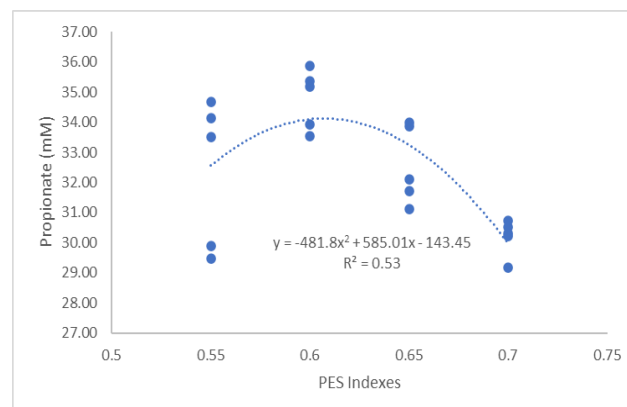


Figure 3. Graph of propionate production on rumen fluid

rations with decreasing fiber content and increasing NFE levels resulted in a lower pH. The crude fiber content of R1, R3, and R4 were 33.39%, 29.44%, and 29.30%; the NFE levels were 32.01%, 36.90%, and 38.53%, respectively.

VFA production describes the conversion rate of carbohydrates or organic matter into energy sources for ruminants and indicates the quality of feed fermentability in the rumen (Muchlas et al. 2014). The average concentration of the total VFA product in the study ranged from 142.00-166.80 mM (Table 3). These results indicate that the PES index-based ration can produce a reasonably high VFA because the standard VFA in rumen fluid ranges from 80-180 mM (Rahayu et al. 2018). The analysis of variance showed that the ration based on the PES index had a highly significant effect ($P < 0.01$) on the total VFA production. The Tukey's analysis showed that the highest VFA was R2, then followed by R1, R3, and R4. The orthogonal polynomial analysis indicates that the PES index has a quadratic impact with the equation $Y = -2160 X^2 + 2576.8 X - 604.2$ (Figure 2b) and a coefficient of determination (R^2) = 0.93. The peak reached the PES index of 0.6 with a VFA of 164.31 mM. Tukey's and OP's analysis showed that the R2 was the highest on VFA production. The R2 ration with an index of 0.6 (Table 1) was prepared with a balanced proportion of feedstuff and causes the nutritional content produced is also quite balanced, especially in NFE and protein. The balance of these two nutrients in R2 (with a low ratio) supports faster microbial development than other treatments. In addition, the R2 content of crude fiber is quite high, which has an impact on controlling rumen pH. It supports high DMD and OMD at an index of 0.6. Conversely, Li et al. (2021) explained that carbohydrates are the primary substrate in rumen fermentation. Therefore, VFA production is strongly influenced by the fermentability of this substrate. The optimum pH level allows amylolytic microorganisms and cellulose and hemicellulose digesters to work optimally. Therefore, the production of VFA in this study strongly correlates with both digestibility and the degree of rumen pH. VFA composition is acetate (C2), propionate (C3), and butyrate (C4), while the other small portion is formic acid, isocaproic acid, isobutyrate, isovalerate, and valerate. Concentrations of C2, C3, and C4 from the fermentation of feed ingredients describe the solubility of carbohydrates and proteins during the fermentation process (Wahyuni et al. 2014; Syamsi & Walidi, 2021). C2 levels were between 109.34-117.74 mM, C3 levels were 30.19-34.78 mM, and C4 levels were 9.30-10.72 mM. When calculated based on the average value of all treatments, the proportion of C2 is 72.76%, C3 is 20.86%, and C4 is 6.38%. Syamsi et al. (2018) stated that the expected proportion of partial VFA in the rumen consists of 65% C2, 20% C3, and 12% C4.

The analysis variance of the PES index on partial VFA showed no significant effect ($P > 0.05$) on the levels of C2 and C4 but a highly significant effect on the levels of C3 ($P < 0.05$). Syamsi et al. (2018) also prove that the PES index did not affect C2 and C4. These two compounds have different formation pathways with propionate. The production of C2 and C4 is closely related to fiber degradability. Table 1 shows that the crude fiber content of the treatment tends to decrease with increasing PES index. Nevertheless, it does not affect C2 and C3 production. Aling et al. (2020) stated that cellulolytic microorganisms digest fiber. These microorganisms require quite diverse metabolite compounds (cofactors). The availability of these cofactors dramatically affects its performance in producing C2 and C3. The composition of the rations in Table 1 only focuses on achieving the PES index, but there is no addition of specific metabolites to improve cellulolytic microbial performance.

In contrast to acetic and butyric acids, the PES index-based ration significantly affected propionate. Tukey's analysis showed that R2 was the highest C3 production, followed by R1 and R3 (not different), and the lowest was R4. The orthogonal polynomial analysis on propionate shows a quadratic effect with the equation $Y = -481.8 X^2 + 585.01 X - 143.45$ and the coefficient of determination (R^2) = 0.53. Propionate is the primary glucogenic fatty acid produced in the digestion of carbohydrates by ruminants. Propionate production has the same dynamics as total VFA production, namely an increase in R2 and a decrease in R3 and R4 (Figure 3), with the peak at R2 (0.61; 34.13%). The different formation pathways compared to C2 and C4 lead to significant C3 production. The NFE in R4 was the highest, followed by R3, R1, and R2. Although the NFE level of R2 is the lowest, DMD and OMD are the highest and achieve the same index of 0.61. In addition, propionate formation is closely related to the degradation of carbohydrates from the starch or NFE that occurs in 2 pathways, succinate, and acrylate (Syamsi et al. 2018; Isipato et al. 2020). The formation of C3 through the acrylic pathway utilizes lactic acid in the rumen fluid. This theory is very much in line with the measurement of pH degrees (Figure 2a) which shows a similar graphic rhythm. The higher the pH conditions obtained, the higher C3, namely the ration with an index of 0.61. The high pH indicates that the utilization of lactate in the production of C3 through the acrylate pathway also increases.

The study shows that the ratio of C2:C3 is between 3.15-3.85. it was high compared to the research result by Syamsi & Walidi (2021), which was between 1.77-1.94. The average C2 production is relatively high, with a calculation reaching 72.76%, and an increase does not follow it in the average propionate production. Therefore there is a high gap between C2 and C3. Ifani et al. (2021)

stated that in the stoichiometry of carbohydrate conversion to VFA, acetate production had a higher tendency, among others. This condition is referred to as acetogenic, where the activity of cellulolytic bacteria is more dominant than amylolytic. The analysis of variance on the PES index-based ration showed a highly significant effect ($P < 0.01$) on the C2:C3 ratio and methane production. Tukey's analysis showed the order of C2:C3 from low to high, i.e., R2, R1, R3, and R4. The orthogonal polynomial analysis on the C2:C3 ratio indicates a quadratic effect with the equation $Y = 50.93X^2 - 60.177X + 21.067$ and the coefficient of determination (R^2) = 0.66 (Figure 4a). The lowest point of the graph is obtained at the ratio of 3.29 and the index of 0.59. Although the PES index had no significant effect on C2, Table 1 shows that the production of C2 in R2 had a reasonably low production compared to other treatments. In addition, the production of C3 in R2 was the highest. Therefore, the lowest C2:C3 ratio is achieved in this index range.

The research got methane production ranging from 40.90-45.89 mM. Tukey's analysis showed that R3 and R4 had the highest CH_4 production, but R3 was not different from R1, then R1 and R2 were the lowest. The quadratic effect influenced methane with the equation $Y = 202.45X^2 - 223.18X + 103.41$ and a coefficient of determination $R^2 = 0.38$. The lowest point was at an index of 0.55, with a methane production of 41.90 mM (Figure 4b). Mitsumori and Sun (2008) stated that methane production highly correlates with the C2:C3 ratio. It is evidenced in Figures 4a and 4b, which show the same graphic trend. The higher C2:C3 gap ratio indicates the low use of hydrogen (H_2) in the propionate formation pathway, so it is more widely used in methanogenesis. For every 1 mole of methane, it takes 1 mole of CO_2 and 4 moles of H_2 . These compounds are commonly produced in the C2 and C3 formation pathways from Ac-CoA. Each production of 1 mole of C2 will make 2 moles of CO_2 and 4 moles of H_2 , while each output of 1 mole of butyrate will have 2 moles of CO_2 and 4 moles of H_2 . On the other hand, the production of C3 makes more use of these two compounds, where 1 mole of C3 requires 3 moles of CO_2 and 5 moles of H_2 . Therefore, increased C3 output will limit the availability of CO_2 and H_2 , reducing the methanogenesis process (Syamsi et al. 2018; Yanuartono et al. 2019). Therefore, the lowest methane production was in the R2 ration index range because it had the highest C3 production with a low C2 average output compared to the others. Ammonia (NH_3) concentration is one of the indicators to determine feed protein degradability, microbial activity, and rumen microbial population (Susilo et al. 2019). The analysis of variance showed that the PES index-based ration had a highly significant effect on the concentration of NH_3 . Tukey's analysis showed that R1 was the highest NH_3 production, but R1 was not different from R4, R4 was

not different from R3, then R3 and R2 were the lowest. Analysis of the orthogonal polynomial shows a quadratic effect with the equation $Y = 436 X^2 - 552.28 X + 181.08$ with a coefficient of determination (R^2) = 0.52 (Figure 5a). The NH_3 graph decreases in the diet with a PES index of 0.6 (R2) at 6.16 mM to 0.55 (R1) at 9.32 ± 2.89 mM. Then the concentration of NH_3 increased again to 0.65 (R3) and 0.7 (R4). According to Arias et al. (2020), the concentration of NH_3 in rumen fluid is influenced by protein degradation, rumen villi absorption, and microbial protein synthesis (MPS). This research was carried out using the in vitro method so that the observed concentration of NH_3 could only be associated with protein degradability and MPS. Figures 1a and 1b show that DMD and OMD produce closed quadratic graphs with the highest digestibility at an index of 0.61.

In contrast, the concentration of NH_3 produces an open quadratic graph with the lowest attendance at the index of 0.6. These results are contradictory, but high feed degradability does not determine the concentration of NH_3 because most of these compounds are also used in MPS. If MPS activity is high, then the use of NH_3 will also be increased so that its concentration is observed to be low in the rumen. The analysis of variance showed that the ratio based on the PES index had a highly significant effect on the MPS. Tukey's analysis showed that the highest MPS was R2, followed by R3, and the lowest was R1 and R4. MPS had a quadratic effect with the equation $Y = -1012 X^2 + 1260X - 311.64$ with a coefficient of determination (R^2) = 0.59 (Figure 5b). The MPS increased from the index of 0.5 (R1) to 0.6 (R2). They then decreased with the lowest production in the index of 0.7 (R4). These results indicate an association with low NH_3 production in the same index range. It means that the activity of MPS is high, so many utilize NH_3 as a source of the N chain. High MPS in the rumen can describe the high colony and rumen microbial activity. Ifani et al. (2021) stated that factors affecting microbial protein synthesis were dry matter consumption, nitrogen compounds, fermented energy supply, the ratio of forage to concentrate in the ration, synchronization of nitrogen and energy, rumen environment, rate of food, vitamins, and minerals.

The complete results of the research show that there is an interrelated relationship between each variable. However, the results of this study are conceptually different from the basic concept developed by the inventor of this synchronization method, namely Sinclair et al. (1993). The basic idea of the synchronization index states that the digestibility and fermentability of the rumen will increase along with the increase in the PES index of the ration. Syamsi and Walidi (2021) said that studies on preparing the PES index-based ration showed varied results. Some studies show a positive effect with a linear effect, some have a quadratic effect, and some even have no significance. This research results from a

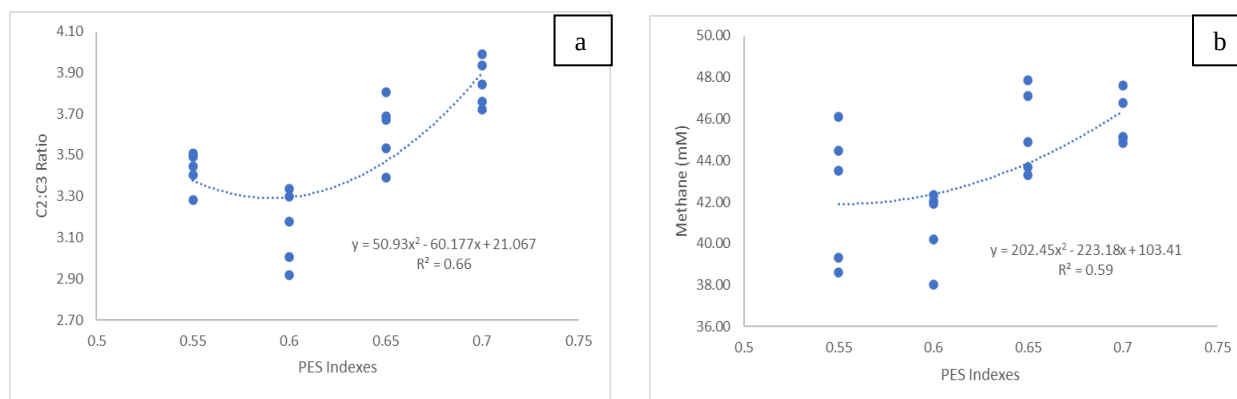


Figure 4. Graph of C2:C3 ratio (a) and methane production (b) of rumen fluid

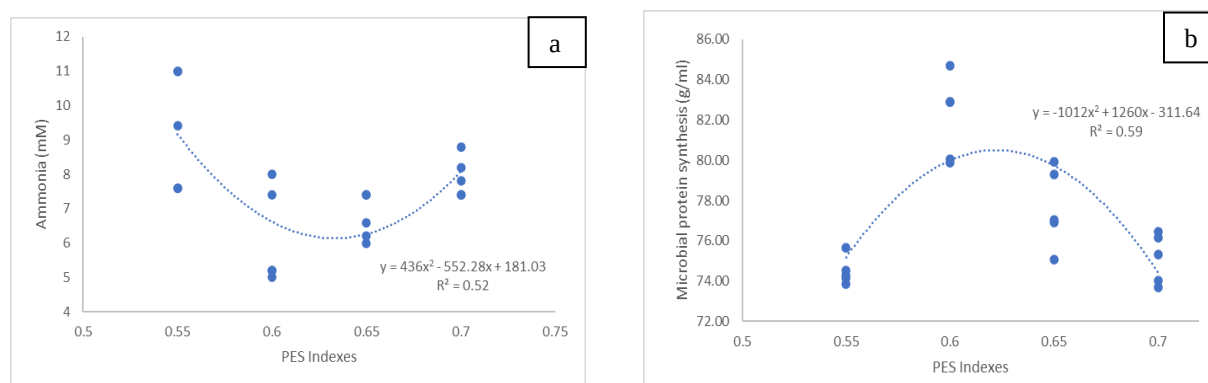


Figure 5. Graph of NH₃ (a) and microbial protein synthesis (b) of rumen fluid

follow-up study from a series of previous studies, namely the inventory of the PES index on various ruminant feedstuff carried out *in vitro*. Therefore, developing a list of feed ingredients PES index research needs to be done in Sacco. Then, the results can be tested again to be compared with the results of this study. Some critical notes observed from previous studies and other research results are that the preparation of rations based on the PES index must still pay attention to the nutrient composition of the resulting rations. The balance between protein, fiber, and NFE levels must still be considered to achieve a harmonious supply of ammonia and energy in the rumen fluid. On the other side, the rations in this study were prepared by calculating the individual index of each feedstuff. Therefore, designing rations must focus on achieving a predetermined index, which is also an important note; different formulating mechanisms must be compared. It means that the ration is prepared with a specific ratio of protein and NFE, then the PES index of the ration is determined through the in-sacco process. The development of this research is still very possible and produces more valid data.

CONCLUSION

Protein-energy synchronization (PES) index of 0.6 achieved the best rumen fermentability. Furthermore, the

provision of the PES index-based ration of 0.6 was the most effective compared to other indexes based on the high propionate, the lowest methane, the lowest NH₃, and the best microbial protein synthesis. The ration with an index of 0.6 is composed of 30% elephant grass, 30% *Leucaena leucocephala*, 10% coconut meal, 10% tofu waste, 10% rice brand, 9% cassava waste, and 1% mineral mix.

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Phylogeography of the Maleo Senkawor (*Macrocephalon maleo* Sal. Muller 1846) Based on Cytochrome B Gene in Sulawesi And Their Sex Determination using Molecular Sexing

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ABSTRAK

Samad A, Solihin DD, Sumantri C, Purwantara B. 2023. Filogeografi maleo senkawor (*Macrocephalon Maleo* Sal. Muller 1846) berdasarkan gen Cytochrome B di Sulawesi dan identifikasi jenis kelaminnya menggunakan teknik sexing molekuler. JITV 28(1):34-44 DOI: <http://dx.doi.org/10.14334/jitv/v28i1>. 3107.

Burung Maleo Senkawor (*Macrocephalon maleo*) merupakan hewan endemik dan terdistribusi luas di Sulawesi. Burung Maleo Senkawor adalah monomorfik sehingga sulit dibedakan antara individu jantan dan betina. Tujuan penelitian ini adalah mengungkap hubungan kekerabatan *M. maleo* di berbagai wilayah geografis di Sulawesi melalui penggunaan penanda gen *Cytochrome-b* (Cyt-b), mengetahui rasio jenis kelamin, dan menguji keandalan primer 2550F/2718R. 15 sampel (bulu dan cangkang telur) berhasil dianalisis dari koleksi di Sulawesi Utara, Sulawesi Tenggara, dan Sulawesi Tengah. Isolasi DNA total dilakukan dengan menggunakan Dneasy® Blood and Tissue kit (Cat. No. 69504) mengikuti protokol Qiagen yang telah dimodifikasi. Amplifikasi PCR (35 siklus) menggunakan primer *forward* MMCytb_F (5'- GAAAATCCCACCCCTACTA-3') dan primer *reverse* MMCytb_R (5'- GTTGGCTACGAGGAGTCAGA-3') serta primer untuk sexing 2550F/2718R. Analisis dilakukan pada sekuens parsial gen Cyt-b mtDNA *M. maleo* sepanjang 903 bp beserta sekuens asam aminonya (301 AA) dan sekuens gen *Chromo Helicase DNA-Binding* (CHD). Jarak genetik model Kimura -2 Parameter dan *p-distance*, pada interpopulasi *M. maleo* di Sulawesi (populasi Sulawesi Tenggara dipisahkan dari populasi Sulawesi Tengah dan Sulawesi Utara) dengan nilai 0,002 (0,2%) – 0,003 (0,3%). Tiap-tiap populasi telah terbentuk haplotipe yang berbeda. Primer 2550F/2718R mampu mengamplifikasi gen CHD dan berhasil mengidentifikasi jenis kelaminnya. Individu jantan lebih dominan dibandingkan individu betina dengan rasio jenis kelamin 6,5:1

Kata Kunci: *Macrocephalon maleo*, Phylogeography, Gen Cyt-b, Sex identifikasi, Sulawesi

ABSTRACT

Samad A, Solihin DD, Sumantri C, Purwantara B. 2023. Phylogeography of the Maleo Senkawor (*Macrocephalon maleo* Sal. Muller 1846) based on Cytochrome B Gene in Sulawesi and their sex determination using molecular sexing. JITV 28(1):34-44. DOI: <http://dx.doi.org/10.14334/jitv/v28i1>. 3107.

The Maleo Senkawor bird (*Macrocephalon maleo*) is endemic and widely distributed in Sulawesi. Maleo Senkawor bird is monomorphic so it is difficult to distinguish between male and female individuals. This study aimed to determine the kinship relationship among *M. maleo* in various geographic areas in Sulawesi through the use of the gene marker *Cytochrome-b* (Cyt-b), determine the sex ratio, and examine the reliability of the 2550F/2718R primer. Fifteen (feather and eggshell) samples from North Sulawesi, Southeast Sulawesi, and Central Sulawesi were collected. Total DNA isolation was performed using the Dneasy® Blood and Tissue kit (Cat. No. 69504) following a Qiagen protocol with modification. PCR amplification (35 cycles) used a forward primer MMCytb_F (5'- GAAAATCCCACCCCTACTA-3'), a reverse primer MMCytb_R (5'- GTTGGCTACGAGGAGTCAGA-3') and a primer for sexing used 2550F/2718R. Analysis was performed on the length of 903 bp Cyt-b mtDNA gene sequences of *M. maleo* along with their amino acid sequences (301 AA) and *Chromo Helicase DNA-Binding* (CHD) gene sequences. Based on the genetic distance of the Kimura 2-Parameter model and *p-distance*, the interpopulation of *M. maleo* in Sulawesi (the population of Southeast Sulawesi was separated from the population of Central Sulawesi and North Sulawesi) was 0.002 (0.2%) – 0.003 (0.3%). Each population has formed a different haplotype. Primer 2550F/2718R was able to amplify the CHD gene and could distinguish the sex identification. Male individuals are more dominant than female individuals with a sex ratio of 6.5:1.

Key Words: *Macrocephalon maleo*, Phylogeography, Cyt-b gene, Sex determination, Sulawesi

INTRODUCTION

The Maleo Senkawor bird (*Macrocephalon maleo*) is a species of the Megapodiidae family endemic in Sulawesi island of Indonesia and threatened species by extinction. Many populations were lost due to over-exploitation of their eggs and also lost connectivity between the forest and nesting sites (Arista et al. 2015; Froese & Mustari 2019).

The dispersal of *M. maleo* in Sulawesi formed population groups in nature. Thus, it is suspected that there has been local adaptation and contributed to the emergence of population diversity in each geographical distribution area. There is a need application of genetic marker candidate that able to reveal variations in the interpopulation and conserve requirements for nucleotides characterizing the species. The Cytochrome-b (Cyt-b) gene is one of the genetic markers that have potential to be applied in this study. The *Cytochrome-b* gene is widely used as a marker of differences in geographic distribution and population origin of a species (Çoraman et al. 2013; Ibis et al. 2014; Petrova et al. 2014; Yong et al. 2015; Lapinski et al. 2016; Kato et al. 2016; Anucherngchai et al. 2019; Amiri et al. 2021).

Sources of DNA in birds that are commonly used for genetic analysis can be obtained through non-invasive such as feathers, eggshells, and feces (Begović et al. 2017; Kaunisto et al. 2017; Maia et al. 2017; Drahulian et al. 2018; Rytönen et al. 2019; Peters et al. 2020; Demarchi et al. 2022). In the present study, DNA was obtained from the feather and eggshell material of the *M. maleo* bird. The use of non-invasive samples was more safe for *M. maleo* compared using a blood sample.

Differentiation and identification of species as well as sex ratios are important steps in conservative interventions (Awad et al. 2015). Therefore, research involving molecular in sex determination of *M. maleo* is important to determine the range of sex ratios in both natural and captive populations. Accurate sex determination is crucial for population development so that it can support the success of conservation programs for endangered species (Purwaningrum et al. 2019). Besides, the activity of egg taking and forest encroachment carried out by humans had a significant effect (87.94%) on the decline in the population of this

species (Arista et al. 2015) and also large egg size (length 10.18 cm; width 6.12 cm; weight 211.70 grams) (Widnyanya 2017) attractive for the community to be used as a food.

The Maleo Senkawor is one of the species that have similar phenotypic characteristics between sex. Some basic methods that can be used for sex identification include differences in morphometric characteristics, laparoscopy for sex determination, sex chromosome examination, behavioral observations, and the presence of brooding patches. However, these methods have disadvantages. Sex identification through molecular techniques is more accurate than the other methods. The use of Polymerase Chain Reaction (PCR) method by utilizing specific primers for sex determination has been commonly used in various bird species (Khaerunnisa et al. 2013; Çakmak et al. 2017; Purwaningrum et al. 2019; Osman et al. 2020), but not much has been done on *M. maleo*, especially using materials from feathers and egg shells. One of the genes used in sex determination is Chromo Helicase DNA-Binding (CHD). This CHD gene was amplified by primer 2550F-2781R (Fridolfsson and Ellegren 1999).

The aimed of this study was therefore to reveal the *M. maleo* kinship relationships in different geographic areas in Sulawesi through the use of the gene marker Cytochrome-b (Cyt-b) from Mitochondria DNA with fragment size (903 bp). This Study was also aimed to determine the sex ratio and to examine the reliability of primers in amplification CHD gene with DNA material from feather and eggshell samples of *M. maleo*. The results are expected to support the conservation and development of the *M. maleo* population in Sulawesi island of Indonesia.

MATERIALS AND METHODS

Study area

This research was carried out from July 2019 to January 2021. A total of 15 samples were collected, consisting of 14 feather samples and 1 eggshell sample (hatched). The research location covered 3 regions of North Sulawesi (7 samples), Southeast Sulawesi (5 samples), and Central Sulawesi (3 samples).

Table 1. Primers used in the study

Primers	Sequences (5'– 3')	Genes	Fragment length (bp)
MMCytb_F	GAAATCCCCACCCCCTACTA	Cyt-b	903
MMCytb_R	GTTGGCTACGAGGAGTCAGA		
2550F	GTTACTGATTCTGCTACGAGA	CHD-Z	586
2718R	ATTGAAATGATCCATGCTTG	CHD-W	436

Procedures

Total DNA isolation

Total DNA isolation was carried out from feathers and eggshells using the *Dneasy® Blood and Tissue Kit* (Cat. No. 69504). The procedure used followed on the Spin-Column Protocol procedure from Qiagen (2003) which has been modified by addition of buffer A and buffer B. The sample was then incubated at a temperature of 56 °C (overnight).

Amplification and aequencing of PCR products

The DNA replication process of the Cyt-b gene and CHD gene was carried out through the *Polymerase Chain Reaction* (PCR) technique. A pair of Cyt-b gene primers were designed based on the *M. maleo* genome data in *GenBank* (access code: MW574376.1). The primers were designed through the primer3 program (<http://bio-info.ut.ee/primer3-0.4.0/primer3>). Meanwhile, the primers used to amplify the CHD gene are universal primers, namely 2550F/2718R designed by (Fridolfsson and Ellegren 1999). The primers can be seen in the following Table 1.

PCR conditions during the Cyt-b gene amplification process included Pre-denaturation at 94°C for 3 minutes, Denaturation at 94°C for 45 seconds, annealing at 54,45°C for 45 seconds, extension at 72°C for 1 minute with a total of 35 cycles, and post extension 72°C for 6 minutes. Meanwhile, the PCR conditions during the CHD gene amplification process included pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 51,5°C for 45 seconds, an extension at 72°C for 1.5 minutes with a total of 35 cycles, and post extension at 72°C for 10 minutes. Detection of PCR products between the Cyt-b gene and the CHD gene was carried out through electrophoresis on 1.2% agarose gel using TBE-1x buffer. Good DNA bands on target were sequenced through commercial services (*First Base Malaysia*). Identification of the sex of each individual was based on the number of emerged DNA bands. One (1) visible band was identified as a male individual and two (2) bands were identified as female individuals (Vucicevic et al. 2013; Çakmak et al. 2017).

Data analysis

Sequence data of the Cyt-b gene and the CHD gene were aligned using MEGA (*Molecular Evolutionary Genetics Analysis*) software version 7.0 (Kumar et al. 2016). Furthermore, the primer was used to correct the nucleotide sequence to obtain a good sequence. Subsequent sequences were BLAST-n at the National Center for Biotechnology Information (NCBI) site. Reconstruction of the phylogenetic tree was carried out

using the *Neighbor-Joining* (NJ) and UPGMA Unweighted Pair Group Method with Arithmetic Average (UPGMA) Kimura 2 – parameter model and p-distance at 1000 bootstraps repetitions. Observation of haplotype diversity used the DNAsp program version 6 (Rozas et al. 2017) and construction of phylogenetic networks was carried out using the Median-Joining method (Bandelt et al. 1999) using the Network program version 10.2 (Fluxus Technology Ltd c2021). Furthermore, 2 sequences from GenBank as an ingroup, namely *M. maleo* (accession number: AM236881.1 and MW574376.1), and 1 sequence from GenBank as an outgroup, namely *Alectura lathami* (accession number: AY346091.1).

RESULTS AND DISCUSSION

Result

DNA purity

Quantification of the extracted genomic DNA using the *Dneasy® Blood and Tissue Kit* (Cat. No. 69504) was carried out to obtain the concentration and purity values of DNA before PCR was performed. The average value of the concentration and purity of DNA from samples of feathers and eggshells of *M. maleo* can be seen in Table 2.

The quantification results in Table 2 showed that the average level of DNA purity is good (1.8-1.9). DNA can be said to be good if the value of the A260/A280 ratio is between 1.8-2.0 (Işçi et al. 2014; Yalçınkaya et al. 2017).

BLASTn result analysis

Sequence data were obtained after being BLASTn on the NCBI site to determine the similarity value to the The search results can be seen in (Tables 3 and Table 4). Table 3 showed that the samples analyzed are Maleo Senkawor birds (*M. maleo*) with a closeness of more than 99%.

Data in Table 4 and Figure 1 showed that seven (7) individuals in the population from North Sulawesi were identified as male. The population from Southeast Sulawesi identified 1 female and four (4) males. Meanwhile, in the population of Central Sulawesi, one (1) female and two (2) male individuals were identified. These results indicated that the ratio of male and female individuals in the analyzed sample is 6,5:1.

Sex determination of *M. maleo* showed that the sequence sizes of the CHDZ and CHDW genes were 586 bp and 436 bp, respectively (Figure 1). In Figure 1, it can be seen that the female individual with 2 bands (CHDZ/CHDW) in the samples from Southeast Sulawesi (MKT1) and Central Sulawesi (MS3) had 586 bp (CHDZ) and 436 bp (CHDW), while the male individual (CHDZ/CHDW) has only a single band (586 bp).

Table 2. Average concentration and purity of DNA

No	Source of DNA	N	concentration (µg/mL)	Purity (A ₂₆₀ /A ₂₈₀)
1	Feather	14	71.27	1.956
2	Eggshell	1	21.07	1,840

Table 3. Species identification based on BLASTn results in NCBI

Locations	Query cover (%)	E-value	Similarity %	Species	Accession number
North Sulawesi	100	0.0	99.67	<i>M. maleo</i>	MW574376.1
	100	0.0	99.56		AM236881.1
Southeast Sulawesi	100	0.0	100	<i>M. maleo</i>	MW574376.1
	100	0.0	99.89		AM236881.1
Central Sulawesi	100	0.0	99.78	<i>M. maleo</i>	MW574376.1
	100	0.0	99.67		AM236881.1

Table 4. Sex determination based on BLASTn results in NCBI

Species	Sample code	Gene	Fragment length (bp)	Accession number GenBank	Similarity (%)
<i>M. maleo</i> (North Sulawesi-1)	MT1	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (North Sulawesi-2)	MT2	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (North Sulawesi-3)	MT3	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (North Sulawesi-4)	MT4	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (North Sulawesi-5)	MT5	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (North Sulawesi-6)	MT6	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (North Sulawesi-7)	MT7	CHD1-Z	586	MT074328.1	98.35
<i>M. maleo</i> (Southeast Sulawesi-1)	MKT1	CHD1-W	436	MT074330.1	94.10
<i>M. maleo</i> (Southeast Sulawesi-2)	MBHM1	CHD1-Z	586	MT074328.1	95.08
<i>M. maleo</i> (Southeast Sulawesi-3)	MTB1	CHD1-Z	586	MT074328.1	95.23
<i>M. maleo</i> (Southeast Sulawesi-4)	MTB2	CHD1-Z	586	MT074328.1	95.23
<i>M. maleo</i> (Southeast Sulawesi-5)	MTB3	CHD1-Z	586	MT074328.1	95.23
<i>M. maleo</i> (Central Sulawesi-1)	MS1	CHD1-Z	586	MT074328.1	97.80
<i>M. maleo</i> (Central Sulawesi-2)	MS2	CHD1-Z	586	MT074328.1	97.80
<i>M. maleo</i> (Central Sulawesi-2)	MS3	CHD1-W	436	MT074330.1	94.64

Nucleotide sequence polymorphism

The results of multiple alignments of the partial Cyt-b gene (903 bp) Maleo Senkawor (*M. maleo*) obtained 898 bp (99.4%) conservative sites, 5 bp (0.6%), parsimony

sites 4 bp (0.5%) and 1 bp singleton (0.1%). In this study, 5 polymorphic nucleotide sites were found in the population from Sulawesi and 3 of them indicated specific locations at sites of 678 (Central Sulawesi), 890, and 891 (North Sulawesi) (Table 5).

Haplotype

Based on the data in Tables 5 and Table 4 haplotypes consisted of 3 interpopulation haplotypes and 1 haplotype based on sequences from *GenBank*.

The results of the analysis of Cyt-b gene sequences from 15 samples and 2 comparison sequences from *Genbank* (AM236881.1 and MW574376.1) found 4 haplotypes consisting of 3 interpopulation haplotypes and 1 haplotype in the *GenBank* standard sequence (Figure 2). Haplotype-1 is the sequence from *GenBank* (AM236881.1). Haplotype-2 is the population of Southeast Sulawesi and sequences from *GenBank* (MW574376.1), Haplotype-3 in the population of North Sulawesi, and haplotype-4 from the population of Central Sulawesi. The value of nucleotide diversity (π) in the intrapopulation of *M. maleo* was 0.00000 (0%) and the haplotype diversity (H_d) was 0.00000 (0%). Meanwhile, the interpopulation (3 geographic locations) nucleotide diversity (π) was 0.00218 (0.218%) and haplotype diversity (H_d) was 0.71324 (71.324%).

Amino acid sequence polymorphism

The translation results of 903 bp of nucleotide sequences of the partial Cyt-b gene of *M. maleo* obtained 301 amino acid (AA) sites. The results of this analysis provided information that there are 298 conserved AA sites and 2 varied AA encoding sites. The various AA sites are at sites 297 and 300 which are non-synonymous AA (coding nucleotides changed, amino acids changed). Changes occurred at 2 sites (297 and 300) are transitional substitutions that occurred in pyrimidine bases (Thymine to Cytosine or vice versa) and also in purine bases (Guanine to Adenine or vice versa). Substitution transition occurs at the triplet of codon 2 (890th site of T>C) and the triplet of codon 1 (898th site of G>A). AA substitution occurs at site 297 (from L>P or Leucine to Proline) AA substitution at this site occurs only in populations from North Sulawesi. Meanwhile, the 300th site (A>T or Alanine to Threonine) occurred in populations from North Sulawesi and Central Sulawesi (Table 6).

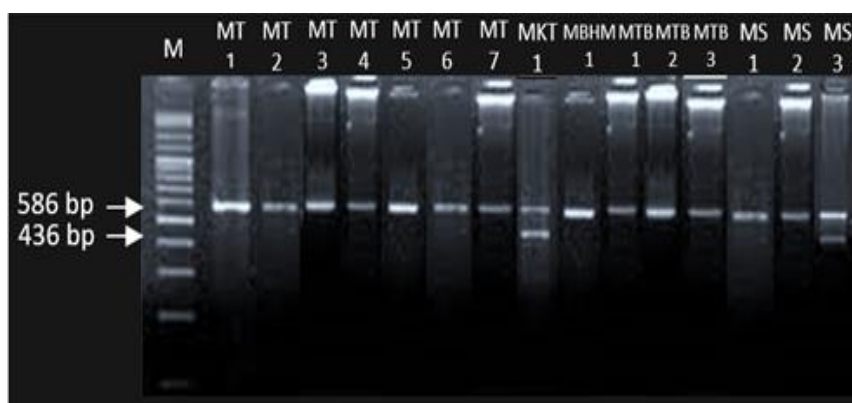


Figure 1. Differences in the size of the *Chromo Helicase DNA-binding* (CHD) gene in *M. maleo*. (M: DNA Ladder 100 bp); MT= Maleo Tambun (North Sulawesi); MKT= Maleo Kolaka Timur (Southeast Sulawesi); MBHM= Maleo Blok Hutan Mempah (Southeast Sulawesi); MTB= Maleo Tanjung Batikolo (Southeast Sulawesi); MS= Maleo Saluki (Center Sulawesi)

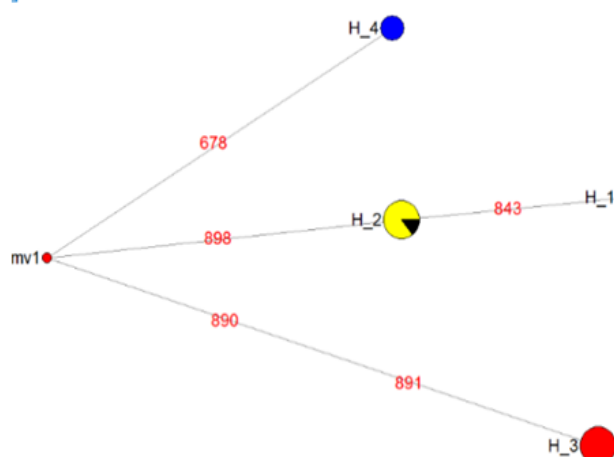


Figure 2. Median-joining network of *M. maleo* interpopulation haplotypes based on Cyt-b gene. where ● is H-1 (haplotype-1) *M. maleo* from *GenBank* (AM236881.1); ● is H-2 (haplotype-2) *M. maleo* from Southeast Sulawesi and sequences from *GenBank* (MW574376.1); ● is H-3 (haplotype-3) *M. maleo* from North Sulawesi; ● is H-4 (haplotype-4) *M. maleo* from Central Sulawesi

Table 5. Nucleotide sequence polymorphisms (SNPs) of the *M. maleo* interpopulation based on the Cyt-b gene

Species	Accession number and sample number	Nucleotide site				
		678 [771]	843 [936]	890 [983]	891 [984]	898 [991]
<i>M. maleo</i>	AM236881.1	T	A	T	C	G
<i>M. maleo</i>	MW574376.1	T	G	T	C	G
<i>M. maleo</i> (North Sulawesi-1)	MT-1	.	.	C	T	A
<i>M. maleo</i> (North Sulawesi-2)	MT-2	.	.	C	T	A
<i>M. maleo</i> (North Sulawesi-3)	MT-3	.	.	C	T	A
<i>M. maleo</i> (North Sulawesi-4)	MT-4	.	.	C	T	A
<i>M. maleo</i> (North Sulawesi-5)	MT-5	.	.	C	T	A
<i>M. maleo</i> (North Sulawesi-6)	MT-6	.	.	C	T	A
<i>M. maleo</i> (North Sulawesi-7)	MT-7	.	.	C	T	A
<i>M. maleo</i> (Southeast Sulawesi-1)	MKT-1	.	.	.	.	.
<i>M. maleo</i> (Southeast Sulawesi-2)	MBHM-1	.	.	.	.	.
<i>M. maleo</i> (Southeast Sulawesi-3)	MTB-1	.	.	.	.	.
<i>M. maleo</i> (Southeast Sulawesi-4)	MTB-2	.	.	.	.	.
<i>M. maleo</i> (Southeast Sulawesi-5)	MTB-3	.	.	.	.	.
<i>M. maleo</i> (Central Sulawesi-1)	MS-1	C	.	.	.	A
<i>M. maleo</i> (Central Sulawesi-2)	MS-2	C	.	.	.	A
<i>M. maleo</i> (Central Sulawesi-3)	MS-3	C	.	.	.	A

Site-specific nucleotides are marked in black boxes; bold numbers represent nucleotide sequences based on the partial sequence of the Cyt-b gene; the number in brackets is the nucleotide sequence based on the complete genome sequence of the Cyt-b gene.

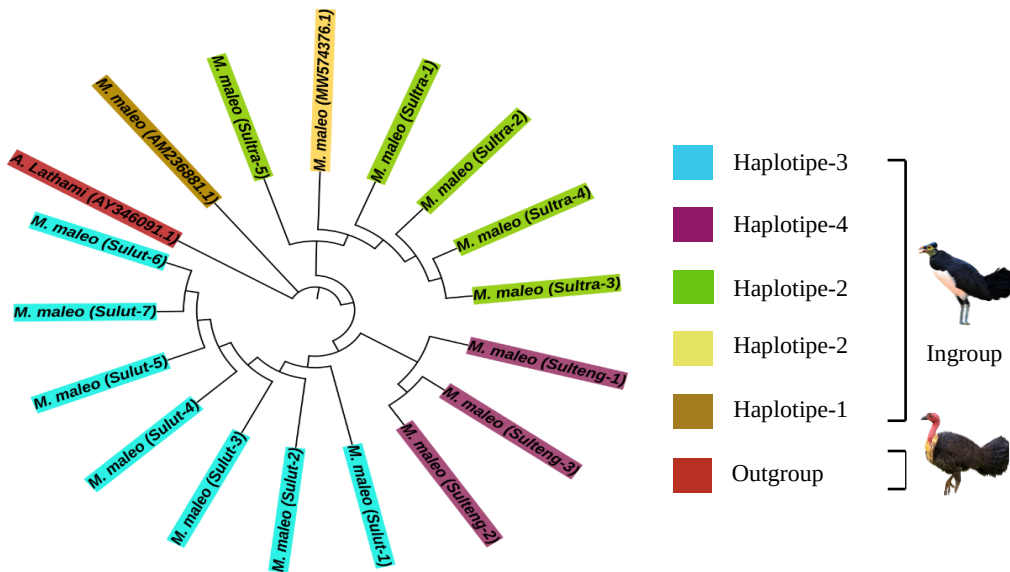


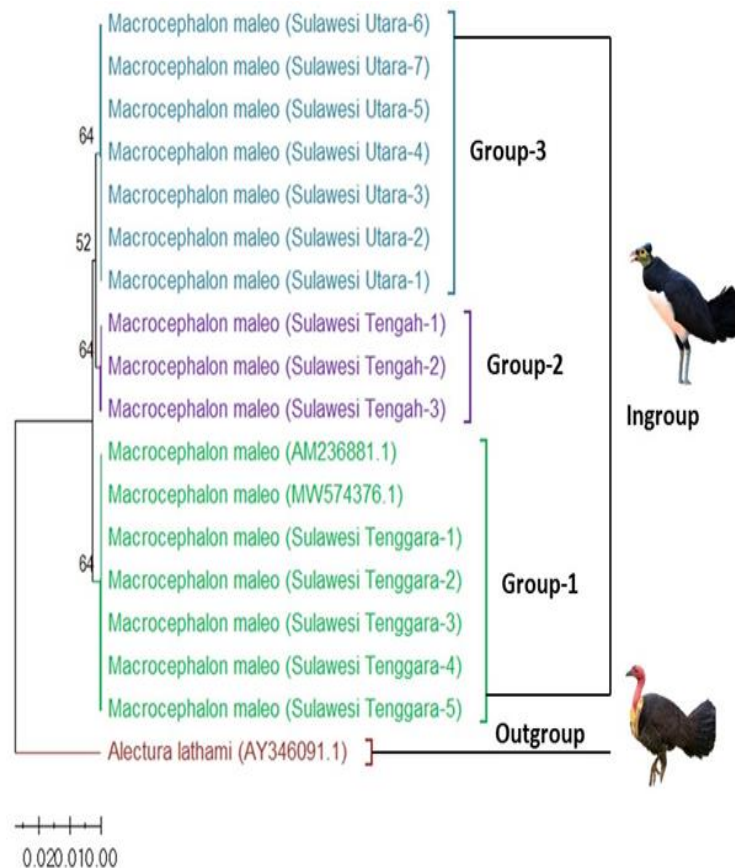
Figure 3. The relationship between populations of *Macrocephalon maleo* based on the Neighbor-Joining (NJ) method, bootstrap 1000 repetitions with the Kimura 2-parameter model on the nucleotide sequence of the Cyt-b gene

Table 6. Codon coding sites and amino acids that changed in the Cyt-b gene of *M. maleo*

Population and accession number	Codon site	
	297 (890,891)	300 (898)
AM236881.1	CTC	GCC
MW574376.1	...	...
North Sulawesi Utara	.CT	A..
Southeast Sulawesi	...	...
Central Sulawesi	...	A..

Population and accession number	amino acid site	
	297	300
AM236881.1	L	A
MW574376.1	.	.
North Sulawesi	<u>P</u>	<u>T</u>
Southeast Sulawesi	.	.
Central Sulawesi	.	<u>T</u>

The dot symbol (.) is an identical sequence to *M. maleo* from Genbank (AM236881.1 and MW574376.1); the bold and underlined numbers = non-synonymous change; the number in parentheses () = sequence of nucleotide sites of the Cyt-b *M. maleo* gene

**Figure 4.** The population between relations of *Macrocephalon maleo* based on the UPGMA (Unwight Pair Group Method with Arithmetic Average) method, bootstrap 1000 repetition with the *p*-distance model in the amino acid (AA) sequence

Genetic distance

The genetic distance between species used the Cyt-b *M. maleo* gene in 3 study populations of North Sulawesi, Southeast Sulawesi, and Central Sulawesi with values between 0.002 (0.2%) and 0.003 (0.3%). The genetic distance of the 3 populations when compared with the distance from the *M. maleo* sequence in *GenBank* (AM236881 and MW574376.1) is 0.000 (0.0%) – 0.004 (0.4%). Meanwhile, the genetic distance of *M. maleo* compared to *A. lathami* species (outgroup) was 0.144 (14.4%) – 0.146 (14.6%). The genetic distance based on AA in the 3 study population areas has a value of 0.003 (0.3%)-0.007 (0.7%). The genetic distance in the 3 study populations with comparison sequences from *GenBank* (AM236881 and MW574376.1) was 0.000 (0.0%) – 0.007 (0.3%). Meanwhile, the genetic distance between *M. maleo* and *A. lathami* species (outgroup) was 0.051 (5.1%) - 0.058 (5.8%).

Phylogenetic tree construction

The phylogenetic tree topology of *M. maleo* interpopulation in Sulawesi and 2 reference sequences from *GenBank* (AM236881.1 and MW574376.1) based on nucleotide sequences of the Cyt-b gene are still in the same cluster (1 cluster). Even though they are in the same cluster, it appears that the populations of *M. maleo* from North Sulawesi and Central Sulawesi tend to have a closer kinship relationship (Figure 3). The phylogenetic tree topology based on AA also shows the same pattern as the nucleotide sequences of the Cyt-b gene and it is known that populations from North Sulawesi and Central Sulawesi tend to have a closer kinship than populations from Southeast Sulawesi (Figure 4)

Discussion

Variations in nucleotide bases in the intrapopulation of *M. maleo* indicated that there is no substitution. Meanwhile, the interpopulation occurs based on a transitional substitution. The results of the analysis showed that transition substitution occurred 4 times and no transversion substitution occurs. In *Luscinia calliope*, the dominant transition substitution is based on the Cyt-b gene (Spiridonova et al. 2013). The occurrence of nucleotide base substitution in the interpopulation resulted in the percentage of *M. maleo* genetic distance between North, Southeast, and Central Sulawesi (0.2% - 0.3%). The low value of the genetic distance of *M. maleo* in the interpopulation indicates that there are no significant genetic differences between the populations of North, Southeast, and Central Sulawesi. This result is presumably because genetic mixing between sub-populations (gene flow) continues to occur so that the

differences between different geographic distributions are not significant (the geographic distribution barrier is relatively small). However, geographical variations have occurred between the origin of the geographic distribution of North Sulawesi, Central Sulawesi, and Southeast Sulawesi.

The value of nucleotide diversity and haplotype diversity based on intrapopulation obtained the same value, namely 0.00000 (0%), the low value is suspected whether the population size in each habitat is low or indeed the genetic diversity is low in nature. However, these results provided information that there is a negative impact that could threaten the survival of the *M. maleo* population due to the low intrapopulation nucleotide and haplotype diversity. Low genetic diversity may appear in small populations through inbreeding, thereby triggering negative traits, including decreased fitness, adaptation to environmental changes, reproductive problems, disease susceptibility, and other similar conditions that can reduce population persistence (Pekkala et al. 2014; Fünfstück and Vigilant 2015; Hedrick and Garcia-Dorado 2016; Harrison et al. 2019).

Based on the pattern formed in the haplotype network, the Southeast Sulawesi sub-population and the sequence from *GenBank* (MW574376.1) are combined into the same haplotype, namely Haplotype-2. This condition is because this sub-population is still composed of common nucleotides found in all populations. Meanwhile, the sub-populations of Central Sulawesi, North Sulawesi, and sequences from *Genbank* (AM236881.1) appeared to form separate sub-populations.

The existence of *M. maleo* in Sulawesi as a species in the Family Megapodiidae that has evolved, it might be thought to have originated from an ancestor in Australia. Megapodiidae existed in Australia in the late Oligocene and there is general agreement that Megapodiidae were isolated in Australia-Papua for a long time (Harris et al. 2014). In addition, *M. maleo* reaching the island of Sulawesi is inseparable from the process of forming this island. It is suspected that Sulawesi originated from Mainland Asia, Mainland Australia, and lands arising from the ocean floor during the Miocene to Pleistocene epochs (Hall 2001; Shekelle and Leksono 2019). *M. maleo* colonization on Sulawesi Island is directly related to the geological formation and source of its initial population and evolution of development.

Based on the results of sex identification, it is known that from the 15 samples analyzed, 13 individuals were male and 2 individuals were female. The dominance of male individuals over female individuals occurred in 3 study population areas. The sex ratio plays an important role and influences sex roles and the reproductive system (Szekely et al. 2014). An unbalanced sex ratio in a small population can result in a decline of population that leads to the extinction of the species (Kus et al. 2017) and this

certainly harms *M. maleo* as an endangered species. Sex determination using primers 2550F and 2718R (Fridolfsson and Ellegren 1999) was able to distinguish the sex of the *M. maleo* bird species with DNA sources from feathers and eggshells. The size of the CHDW and CHDZ fragments through the primary determination of 2550F/2718R varied, namely 440 bp -705 bp (Çakmak et al. 2017), 600 bp-100 bp (Ravindran et al. 2019). The results of this study were not significantly different from those that had been done previously on CHD-Z *M. maleo* (Yuda and Saputra 2021).

Habitat damage, especially in coastal areas in Sulawesi, which is one of the spawning habitats of *M. maleo* is very massive because it is used as an area for agriculture, aquaculture, and settlements. The decreasing number of nests of *M. maleo* is thought to be strongly influenced by the deterioration of conditions and the loss of the ideal nesting habitat for regeneration. Thus, the population declined and the limited geographical distribution of this animal are becoming increasingly limited and population development is increasingly worrying. Many areas where previously this animal species could be found, are now empty. In addition to habitat destruction, egg hunting is also a serious threat to the survival of this species, because the egg size is large so it is interesting for human consumption.

CONCLUSION

Based on genetic markers, the Cyt-b gene is large (79% of total length) indicating that populations from North Sulawesi and Central Sulawesi have a closer kinship with a genetic distance marker of 0.2%. Meanwhile, the population from Southeast Sulawesi is seen to be separated from other populations with a genetic distance of 0.3%. Although they are still in the same cluster (populations of North Sulawesi and Central Sulawesi), each population has its haplotype, namely haplotype-3 in populations from North Sulawesi and haplotype-4 in populations from Central Sulawesi. The Southeast Sulawesi population belongs to the sequence from GenBank (MW574376.1) has its haplotype, namely haplotype-2, and is in a separated cluster. Primers 2550F and 2718R were able to amplify the CHD gene in *M. Maleo* with fragment sizes for CHDZ and CHD W; were 546 bp and 436 bp, respectively. Meanwhile, sex identification showed that the male sex was dominant over the female sex with a ratio of 6,5:1

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Performance and Growth Curve Prediction of Crossbred Chickens from Crossing between Local Roosters and Layer Hens

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ABSTRAK

Dakhlan A, Sutrisna R, Santosa PE. 2023. Penampilan ayam persilangan antara ayam kampung dan ayam petelur dan prediksi kurva pertumbuhannya. JITV 28(1):46-56. DOI: <http://dx.doi.org/10/14334/jitv.v28i1.3122>.

Permintaan ayam silangan (ayam Kampung x ayam petelur) terus meningkat. Tujuan dari penelitian ini adalah untuk mendapatkan ayam silangan terbaik dari hasil persilangan antara ayam lokal/asli/kampung dan ayam petelur yang menghasilkan bobot badan tertinggi sampai umur delapan minggu dan prediksi pertumbuhannya menggunakan fungsi Gompertz. Pada penelitian ini digunakan ayam kampung jantan sebanyak 18 ekor (9 ayam Bangkok, 9 ayam Pelung) dan 36 ayam betina petelur galur Isa brown, Lohman brown, dan Hyline brown (masing-masing 12 ekor) untuk menghasilkan 360 ekor ayam silangan. Faktorial 2x3 (2 ayam Kampung jantan versus 3 galur ayam petelur) menggunakan rancangan acak lengkap dengan 3 ulangan diterapkan dalam penelitian ini. Hasil penelitian menunjukkan bahwa terdapat interaksi antara ayam Kampung dan ayam petelur terhadap bobot badan ayam silangan umur 8 minggu. Ayam silangan hasil persilangan antara ayam Pelung dan ayam petelur galur Isa Brown memiliki bobot badan terbaik pada umur 8 minggu. Ayam silangan ini akan mencapai bobot dewasa 3026,89 g dengan bobot pubertas 1113,53 g dan umur pubertas 7,40 minggu. Kesimpulan penelitian ini adalah bahwa ayam silangan hasil persilangan antara ayam Pelung jantan dan ayam betina petelur galur Isa brown merupakan ayam silangan terbaik dengan bobot badan tertinggi pada umur 8 minggu. Ayam silangan ini bisa menjadi salah satu alternatif untuk pemenuhan kebutuhan ayam lokal di Indonesia karena umur potong dapat dipersingkat dari 8 bulan menjadi 8 minggu.

Kata Kunci: Persilangan. Ayam Kampung Jantan. Ayam Petelur, Bobot Badan, Fungsi Gompertz

ABSTRACT

Dakhlan A, Sutrisna R, Santosa PE. 2023. Performance and growth curve prediction of crossbred chickens from crossing between local roosters and layer hens. JITV 28(1):46-56. DOI: <http://dx.doi.org/10/14334/jitv.v28i1.3122>.

Demand for crossbred chicken (Indonesian native chicken x laying hens) continues to increase. This study aimed to find the best-crossbred chicken resulting from crossing between local/native roosters and laying hens which produce the highest body weight up to eight weeks of age, and its growth prediction using the Gompertz function. Eighteen (18) native roosters (9 Bangkok roosters, 9 Pelung roosters) and 36 laying hens of strains Isa brown, Lohman brown, and Hyline brown (12 each) were used in this study to produce 360 crossbred chickens. Factorial 2x3 (two male local roosters versus three strains of laying hens) using a completely randomized design with 3 replications was applied in this study. The results showed an interaction between native roosters and layer hens on the body weight of crossbred chickens at eight weeks of age. Crossbred chicken from crossing between Pelung rooster and Isa brown layer hen was the best in body weight at eight weeks of age. This crossbred chicken would reach a mature weight of 3026.89 g with an inflection weight of 1113.53 g and an inflection age of 7.40 weeks. In conclusion, crossbred chicken from crossing between Pelung roosters and layer hens of Isa brown strain was the best crossbred with the highest body weight at eight weeks of age. This crossbred chicken could be an alternative for fulfilling the demand for local chicken in Indonesia because the slaughter age could be shortened from 8 months to 8 weeks.

Key Words: Crossbreeding, Local Rooster, Layer Hen, Body Weight, Gompertz Function

INTRODUCTION

According to a report from the Central Statistics Agency (BPS), in 2021, the average consumption of chicken meat in Indonesia reached 0.14 kg per capita per week or 7.28 kg per year. This figure increased by 7.69% compared to 2020. Furthermore, the consumption of

local/native chicken and broiler meat based on data from the Ministry of Agriculture of The Republic of Indonesia in 2018 was 782 grams and 5.68 kg per capita per year, respectively. Therefore, consumption of chicken meat increased by 24.9% (local chicken) and 11.2% (broiler) compared to the previous year. Meanwhile, the consumption of local/native chicken eggs and layer

chicken eggs was 4,067 and 106,418 eggs per capita, respectively. This egg consumption rate also increased by 14.71% (local chickens) and 6.64% (layer) compared to the previous year. Based on these data, it can be seen that the increase in consumption of local chicken meat and eggs was higher than that of broiler meat or layer eggs; this is due to the increasing awareness of the community to live a healthy lifestyle, considering that local chicken meat and eggs contain lower fat and cholesterol and the taste of local chicken meat and eggs are more delicious (savory) compared to broiler meat and layer chicken eggs (Siburian et al. 2021; Setyanovina et al. 2021; Wardiny et al. 2020). In addition, local chicken eggs contain higher levels of omega-3 fatty acids and vitamins E, A, and D than layer chicken eggs. However, the increased demand for local chicken meat and eggs cannot be fulfilled by the availability of meat or egg supply from broiler or layer.

The low contribution/availability of local chicken meat and eggs compared to broiler meat and layer chicken eggs might be because of the low population and production capacity of local chickens compared to broilers and layers chicken. Meat productivity indicated by the length of time to reach a certain body weight in local chickens is lower (360 grams/head / eight weeks) than in broilers that reach a body weight of 1.3 kg/head / four weeks. Meanwhile, the productivity of local chicken eggs is lower (30-80 eggs/head/year) compared to layers (351 eggs/head/year) (Rajab & Papilaya 2012; Lapihu et al. 2019). This condition is an opportunity to spur and develop market prospects and the local chicken farming industry. One of the solutions to improve the productivity of local chickens is by crossing local chickens with layers.

Increasing the population and productivity of local chicken meat and eggs through crossing with layers is an inexpensive and easy way to do it at the small and medium-scale farmer and livestock industry level. Moreover, this business is strongly supported by the high wealth and biodiversity of local chickens in Indonesia. Indonesia has 33 types of local chickens scattered in various regions, including Nunukan chicken, Kedu chicken, Merawang chicken, Pelung chicken, Bekisar chicken, Cemani chicken, and Bangkok chicken. On the other hand, research on crossbred performance and growth curves resulting from crosses between native and layer hens was limited.

Lapihu et al. (2019) reported that the body weight of chicken resulting from crossing between male Bangkok, Sabu, and Bare Neck (Legund) chickens with laying hens (strain Isa brown) were 1204.85 ± 137.33 g, 1154.00 ± 190.06 g, and 1085.89 ± 111.45 g, respectively. However, those authors included only one strain of layer hens, without other large local chicken (Pelung rooster) (Puspita et al. 2021) was not included in the experiment. Furthermore, many researchers reported that the

Gompertz function was the best growth predictor for the chicken growth curve (Akinsola et al. 2021; Hoang et al. 2021; Putra & Fajrina 2021; Sariyel et al. 2017). Therefore, the objective of the current study was to find the best crosses between local roosters and layers hens that produce the highest body weight and to predict its growth curve using the Gompertz function.

MATERIALS AND METHODS

This research was conducted on the Zumar breeder farm in Tulungagung Village, Gading Rejo District, Pringsewu Regency. The research material used in this study were 54 chickens consisting of Pelung roosters and Bangkok roosters (1-2 years old), nine each, and three strains of laying hens, 12 each. Factorial 2x3 with a completely randomized design and three replications was assigned to this experiment. The first factor was two roosters of local chickens (Pelung and Bangkok roosters) aged 1-1.5 years with an average body weight of 3.25 kg for the Bangkok rooster and 4.53 kg for the Pelung rooster, and the second factor was three strains of laying hens (Isa brown, Lohman brown, and Hyline brown) aged 29-30 weeks with an average body weight of 1.86 kg/head.

Chickens were placed in 18 cages measuring 1 m long x 1 m wide by 2 m high so that one rooster mates with two laying hens with three replications. Chickens are fed (L-83-1) to produce eggs by supplying drinking ad-libitum. The resulting eggs were hatched using a hatching machine with ten eggs for each parent so that the number of eggs hatched was 360 eggs. In this study, the hatchability of eggs reached 76%. After these eggs were hatched, DOC (day-old chick) was weighed to determine the hatching weight every week for eight weeks. The DOC was fed with BR-1 feed (starter feed) for 28 days, then continued with BR-2 feeding until eight weeks.

The variables observed were hatching Weight and weekly chicken weight for eight weeks. The research data in the form of body weights from 0-8 weeks were tabulated using the Excel program and then analyzed for variance using the R program to determine the influence of the main factors and the interaction between the two factors. The growth curve of the best crossbred was described and predicted using the Gompertz function ($y \sim a \cdot \exp(-b \cdot \exp(-c \cdot x))$) with y as body weight at age x , a as mature body weight, b as the first part of growth before inflection point and c as the second part in which growth rate decreases until the animal reaches the mature Weight (a), inflection weight was calculated as a/e and age of inflection was calculated as $(\ln(b))/c$ (Akinsola et al. 2021; Arando et al. 2021; Hrncar et al. 2021; Hoang et al. 2021; Putra & Fajrina 2021; Tjørve & Tjørve 2017; Nguyen et al. 2023; Afrouziyeh et al. 2021).

RESULTS AND DISCUSSION

Body weight of crossbred chickens

The growth of crossbred chickens due to crossing between male Bangkok chickens and three female laying hens is shown in Table 1 and Figure 1. The results of this study showed that the crosses between the Bangkok rooster and layer hens of the Isa brown strain produced male crossbred chickens with the highest body weight from 0-4 weeks of age and at 7-8 weeks of age, followed by crosses between male Bangkok chickens with Lohman brown and Hyline brown.

Based on Table 1 and Figure 1, it can be seen that, at first, growth was relatively slow, followed by a high growth rate at a later age; this can be seen at the age of 3-4 weeks, for example, the increase in body weight of male chickens from crossing Bangkok roosters and Lohman layer strain increases sharply (104.40 g, from 183.20 g - 287.60 g), while at week 1-3, the body weight

gain of chickens is 20.77, 59.63 and 65.33 g, respectively. Likewise, the growth of 4-8 weeks has increased sharply and can reach above 1000 g (1 kg). The results of this study are higher than those of Kholik et al. (2019), who reported that the increase in body weight at week 4 of Garut super chicken (crossbred from crossing Pelung, Kedu, and Sentul roosters and layer hens) fed with Pasak Bumi meal (*Euricoma longifolia* Jack) ranged from 74.30 g - 83.60 g.

Similar to the Bangkok rooster, the results of crosses between the Pelung rooster and three strains of layer hens showed that the offspring of crosses between the Pelung rooster and layer hens of the Isa brown strain produced the highest body weight from 0-4 weeks of age, followed by Lohman brown strain, and Hyline brown strain. The growth of crossbred resulting from crossing between the Pelung rooster and three strains of layer hens can be seen in Table 2 and Figure 2, which was similar to crossbred resulting from crossing between the Bangkok rooster and the three strains of layer hens.

Table 1. The growth of crossbred chickens as the result of crossing between Bangkok rooster and three strains of laying hens (g)

Age (week)	Male crossbred			Female crossbred		
	1	2	3	1	2	3
0	38.40	37.47	35.33	34.90	34.13	38.40
1	60.63	58.23	55.60	56.23	53.50	60.63
2	124.20	117.87	111.50	112.30	107.33	124.20
3	189.23	183.20	174.27	179.10	173.03	189.23
4	292.33	287.60	277.37	277.73	274.40	292.33
5	508.33	499.67	482.67	427.67	425.33	508.33
6	743.33	740.00	708.33	628.00	624.00	743.33
7	1018.00	980.33	929.00	827.33	823.00	1018.00
8	1205.33	1190.67	1021.33	977.33	968.00	1205.33

Crossbred 1= Bangkok x Isa brown, Crossbred 2= Bangkok x Lohman brown, Crossbred 3= Bangkok x Hyline brown

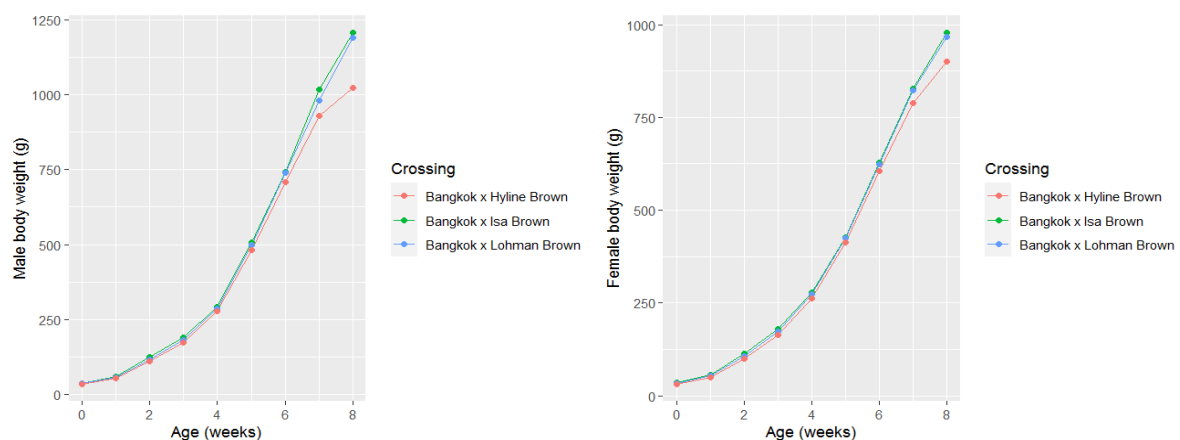


Figure 1. Graph of growth of male and female crossbred chickens (aged 0-8 weeks) resulted from crosses between Bangkok roosters and three strains of layer hens

Table 2. The growth of crossbred chickens as the result of crossing between Pelung rooster and three strains of laying hens (g)

Age (week)	Male crossbred			Female crossbred		
	1	2	3	1	2	3
0	39.73	38.47	35.73	36.10	34.77	39.73
1	62.20	61.00	55.77	56.93	55.70	62.20
2	129.20	124.67	109.53	115.07	114.50	129.20
3	191.93	186.47	180.93	176.73	177.47	191.93
4	296.33	291.17	277.60	282.17	274.17	296.33
5	519.00	513.33	495.33	434.00	424.67	519.00
6	755.33	751.33	709.33	632.67	626.33	755.33
7	1084.67	994.00	940.67	843.67	831.67	1084.67
8	1227.67	1212.67	1040.67	983.00	973.67	1227.67

Crossbred 1= Pelung x Isa brown, Crossbred 2= Pelung x Lohman brown, Crossbred 3= Pelung x Hyline brown

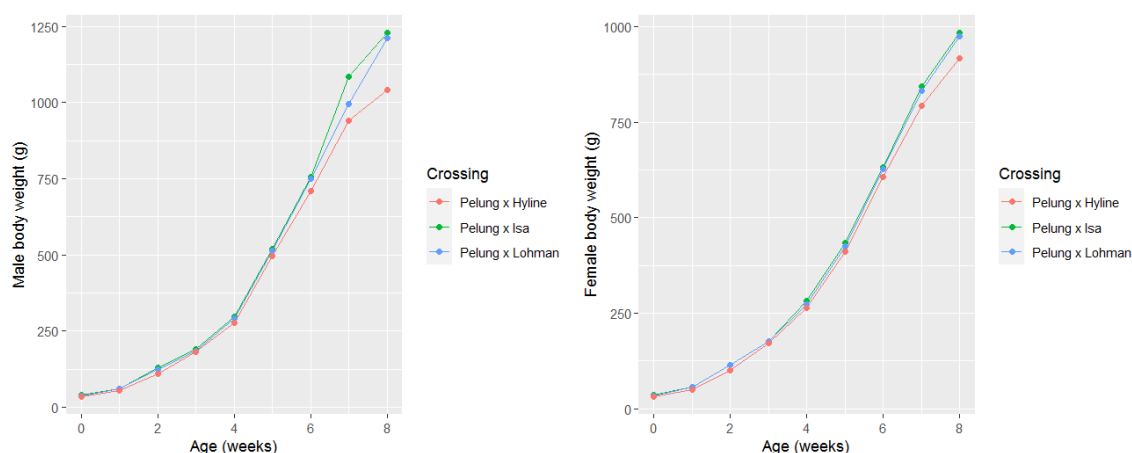


Figure 2. Graph of growth of male and female crossbred chickens (aged 0-8 weeks) resulted from crosses between Pelung roosters and three strains of layer hens

The result of this study was comparable with the result reported by Jacob et al. (2019) that the body weight of super native chicken at 12 weeks of age added with Gedi leaf juice (*Abelmoschus manihot L. medik*) in drinking water ranged from 1226.6 g to 1287.8 g. Munira et al. (2016) reported that Super native chicken fed ration substituted with different fermented rice bran resulted in body weight at ten weeks of age ranging from 837.50 g to 903.89 g. Anggraini et al. (2019) reported that Super native chicken with turmeric powder addition in diets resulted in body weight at eight weeks of age (56 days of age) ranging from 724.92 ± 39.00 g - 853.67 ± 62.72 g.

The effect of treatment on body weight of crossbred chickens

The results showed that there was an interaction between the treatment of local roosters (Bangkok and

Pelung roosters) and the treatment of layer hen strains (Isa brown, Lohman brown, and Hyline brown) on body weight of male and female crossbred chicken (0-8 weeks of age). Based on Table 3 and Table 4 (for example), crossbred chicken resulted from crossing between Pelung rooster and Isa brown strain of layer hen was the highest in body weight at 0 and 8 weeks of age both in male and female crossbred chickens.

Crosses between male Pelung chickens and Isa brown layer strain produced the highest body weight of crossbred chickens (39.73 g in males and 36.10 g in females at 0 weeks of age (DOC); 1227.67 g for males and 983.00 g for females at eight weeks of age). The results also showed that for both Bangkok and Pelung roosters, using the Isa brown strain of layer hen produced the highest crossbred chickens.

The research aiming to evaluate the production and reproduction performance of crossed BL (male Bangkok x female layer) and LB (male Layer x female Bangkok) indicated that the hatching Weight of the crossbred DOC

was 29.67 ± 0.63 g (LB) and 38.49 ± 0.43 g (BL) (Badaruddin et al. 2017). The results of this study were higher than those of Kholik et al. (2019) on Garut super chicken (crossbred from crossing Pelung, Kedu, and Sentul roosters and layer hens) fed with Pasak Bumi meal (*Euricoma longifolia* Jack) ranged from 510.20 g to 657.00 g at week 8. The results of this study were also higher than the body weight results of crossing Bangkok roosters and layer hens reported by Pagala et al. (2019), namely 265.38 g in male crossbred chickens and 256.19 g for female crossbred chickens at five weeks of age, reported by Herlina and Ibrahim (2019) that Super Kampung chicken (crossbreed between a local male rooster and laying hens) with the addition of the salam leaf powder in the ration ranged from 797g to 869.75 g at slaughter age (45 days of age). Sopian et al. (2014)

reported that crossing between male Pelung x female Sentul chicken (PS) and between male Sentul x Kampung chicken (SK) resulted in body weight of 1237 g and 1096 g in male and female offspring at 12 weeks of age, for PS cross and 1009 g and 823 g in male and female offspring at 12 weeks of age, respectively for SK cross. Trisiwi (2016) evaluated various dietary protein levels' effects on compensatory growth at 9-12 weeks of female crossbred between male native chicken and laying hens whose body weight at nine weeks ranged from 447.8 g to 860.8 g. These differences in the body weight of crossbred chickens may be due to differences in the parents' body weight, which are genetically inherited/passed on to the offspring.

The performance of male and female crossbred chickens due to crossing between Bangkok rooster and

Table 3. Body weight of crossbred chicken at 0 weeks of age (g)

Layer hen strain	Local rooster		Average
	Bangkok	Pelung	
Male			
Isa brown	38.40 ^{ab}	39.73 ^a	39.07
Lohman brown	37.47 ^{bc}	38.47 ^{ab}	37.97
Hyline brown	35.33 ^d	35.73 ^{cd}	35.53
Average	36.66	37.14	
Female			
Isa brown	34.90 ^{ab}	36.10 ^a	35.50
Lohman brown	34.13 ^b	34.77 ^b	34.45
Hyline brown	31.47 ^c	32.00 ^c	31.73
Average	33.13	33.58	

Different superscripts showed significant differences ($P < 0.05$)

Table 4. Body weight of crossbred chicken aged eight weeks (g)

Layer hen strain	Local rooster		Average
	Bangkok	Pelung	
Male			
Isa brown	1205.33 ^{bc}	1227.67 ^a	1216.50
Lohman brown	1190.67 ^{cd}	1212.67 ^{ab}	1201.67
Hyline brown	1021.33 ^f	1040.67 ^e	1031.00
Average	1150.33	1168.25	
Female			
Isa brown	977.33 ^b	983.00 ^a	980.17
Lohman brown	968.00 ^d	973.67 ^{bc}	970.83
Hyline brown	900.67 ^f	916.67 ^e	908.67
Average	953.00	960.83	

Different superscripts showed significant differences ($P < 0.05$)

Table 5. Performance of male and female crossbred resulted from Bangkok rooster (g)

Age (weeks)	Male crossbred	Female crossbred
0	37.07	33.50
1	58.16	53.31
2	117.86	106.76
3	182.23	172.14
4	285.77	271.68
5	496.89	421.78
6	730.56	619.44
7	975.78	813.22
8	1139.11	948.67

Table 6. Performance of male and female crossbred resulted from Pelung rooster (g)

Age (weeks)	Male crossbred	Female crossbred
0	37.07	34.29
1	59.66	54.00
2	121.13	110.19
3	186.44	174.98
4	288.37	273.51
5	509.22	423.44
6	738.67	621.78
7	1006.44	822.67
8	1160.33	957.78

Table 7. Performance crossbred resulted from Bangkok and Pelung rooster (g)

(weeks)	Crossbred from Bangkok rooster	Crossbred from Pelung rooster
0	35.28	35.68
1	55.73	56.83
2	112.31	115.66
3	177.19	180.71
4	278.72	280.94
5	459.33	466.33
6	675.00	680.22
7	894.50	914.56
8	1043.89	1059.06

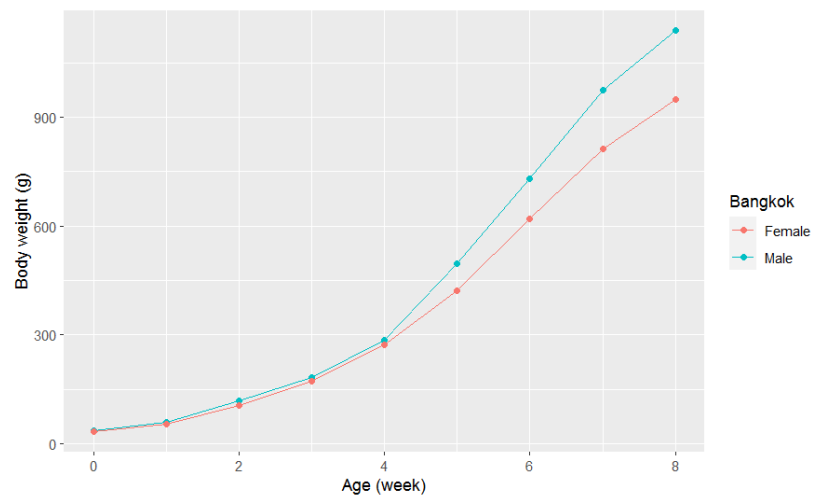


Figure 3. Graph of growth of male and female crossbred chickens (aged 0-8 weeks) resulted from crosses between Bangkok roosters and three strains of layer hens

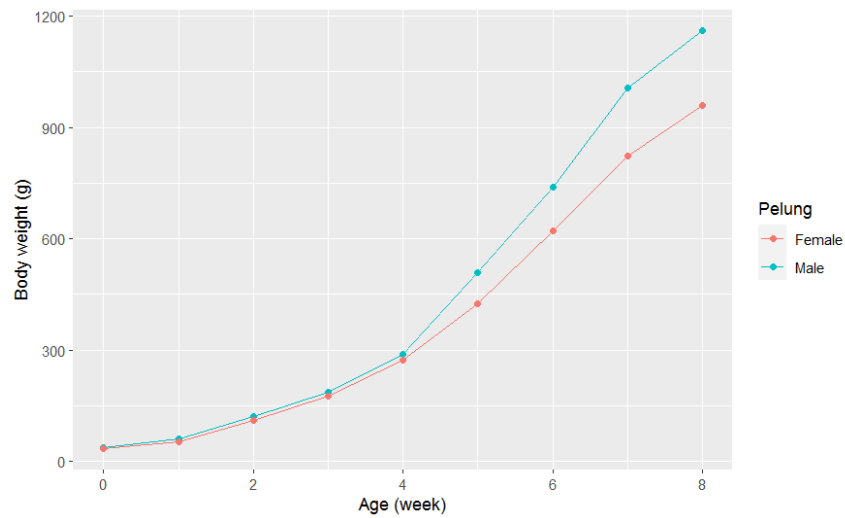


Figure 4. Graph of growth of male and female crossbreds resulted from crosses between Pelung rooster and three strains of layer hens aged 0-8 weeks

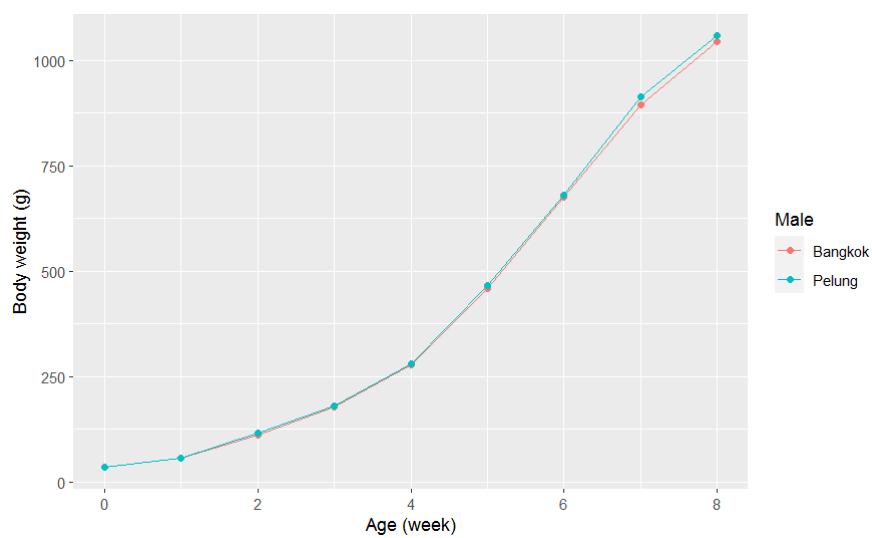


Figure 5. Graph of growth of crossbred chickens (aged 0-8 weeks) resulted from crosses of Pelung and Bangkok roosters with three strains of layer hens

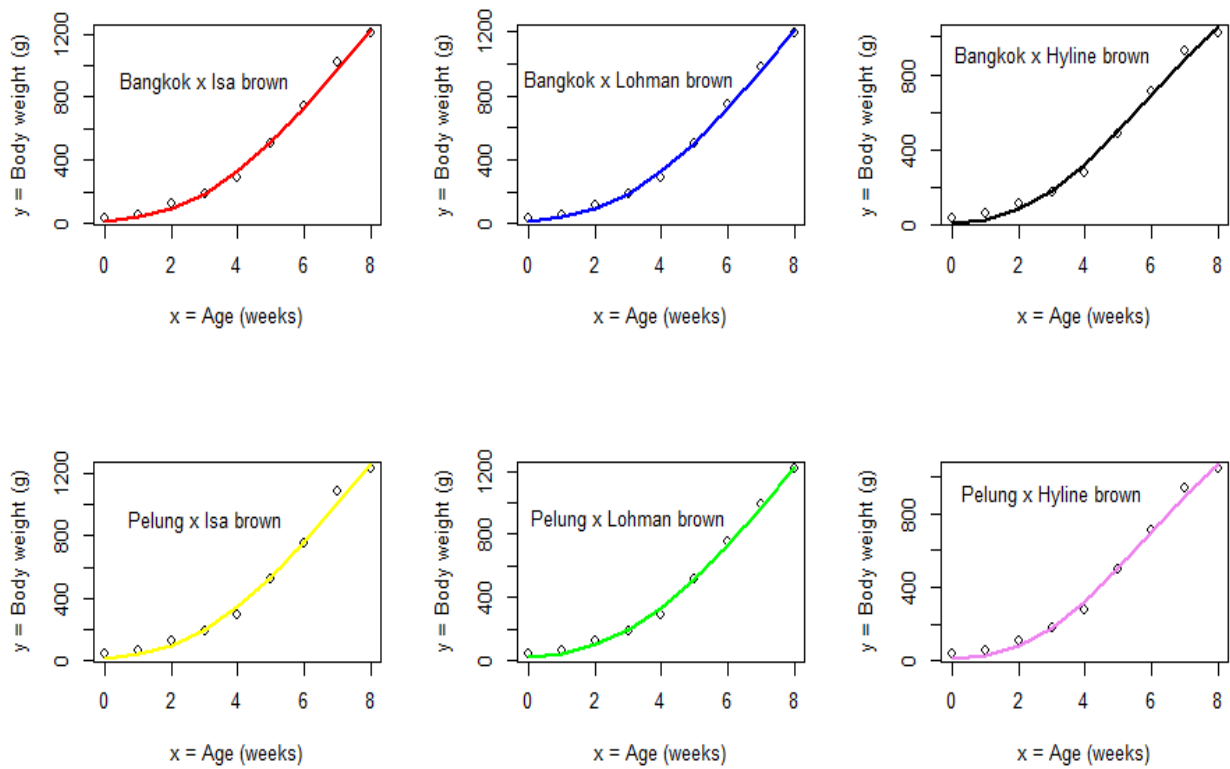


Figure 6. The growth curve of crossbred chicken (aged 0-8 weeks) resulted from crosses of Bangkok and Pelung roosters with three strains of layer hens, predicted using the Gompertz function

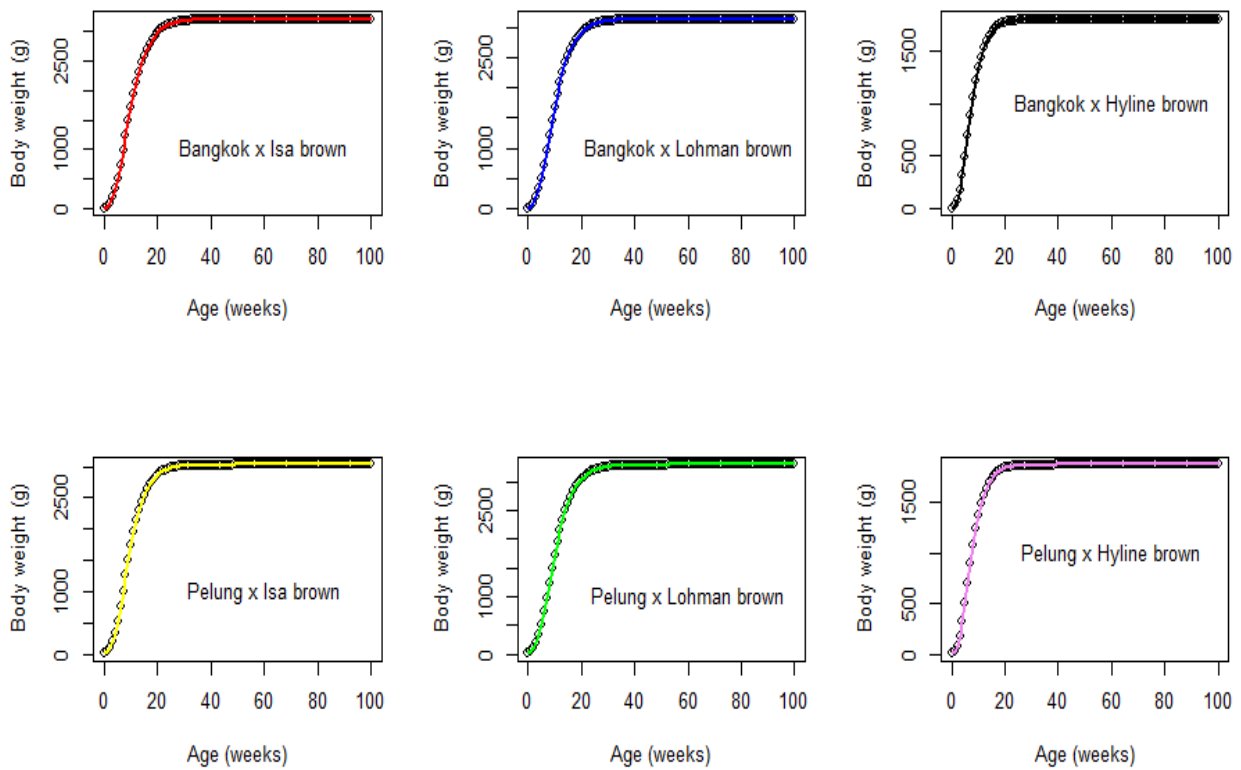


Figure 7. Growth curve prediction of crossbred chicken (0-100 weeks of age) resulted from crosses of Bangkok and Pelung roosters with three strains of layer hens, predicted using the Gompertz function

Table 8. Estimated growth curve parameters of crossbred chicken from 0 to 8 weeks

Crossbred	Parameters									
	a	b	c	WPOI	APOI	AIC	BIC	R ²	Adj.R ₂	RSE
Bangkok x Isa	3193.001	5.3585	0.2157	1174.64	7.78	91.4	92.19	0.9965	0.996	30.48
Bangkok x Lohman	3131.254	5.3751	0.2163	1151.92	7.78	88.21	88.99	0.9975	0.9972	25.53
Bangkok x Hyline	1800.041	5.5813	0.2936	662.20	5.86	95.22	96.01	0.9934	0.9924	37.7
Pelung x Isa	3026.898	5.4805	0.2299	1113.53	7.40	97.95	98.74	0.9932	0.9923	43.86
Pelung x Lohman	3296.985	5.3261	0.2107	1212.89	7.94	88.82	89.61	0.9974	0.997	26.41
Pelung x Hyline	1872.427	5.5275	0.2871	688.83	5.96	94.61	95.4	0.994	0.9931	36.43

a= mature body weight, b= the first part of growth before the inflection point, c= the second part in which the growth rate decreases until the animal reaches the mature Weight, WPOI= Weight at the point of inflection (in grams), APOI= age at the point of inflection (in weeks), AIC= Akaike Information Criterion, BIC= Bayesian Information Criterion RSE= Residual Standard Error, R²= coefficient of determination, Adj.R₂= adjusted coefficient of determination

Table 9. Predicted body weight (week 0-100) of crossbred chicken resulted from crosses between Pelung rooster and Isa brown strain of layer hen

Week	Predicted body weight (g)					
[0]	12.620	38.910	95.188	193.767	340.838	533.854
[6]	762.529	1012.226	1267.715	1515.943	1747.379	1956.201
[12]	2139.767	2297.819	2431.679	2543.573	2636.135	2712.074
[18]	2773.967	2824.148	2864.666	2897.272	2923.443	2944.405
[24]	2961.168	2974.554	2985.233	2993.745	3000.525	3005.924
[30]	3010.220	3013.637	3016.356	3018.517	3020.235	3021.602
[36]	3022.687	3023.550	3024.236	3024.781	3025.214	3025.559
[42]	3025.832	3026.049	3026.222	3026.359	3026.468	3026.555
[48]	3026.624	3026.679	3026.722	3026.757	3026.784	3026.806
[54]	3026.823	3026.837	3026.848	3026.856	3026.863	3026.869
[60]	3026.873	3026.877	3026.879	3026.882	3026.883	3026.885
[66]	3026.886	3026.887	3026.887	3026.888	3026.888	3026.889
[72]	3026.889	3026.889	3026.889	3026.889	3026.890	3026.890
[78]	3026.890	3026.890	3026.890	3026.890	3026.890	3026.890
[84]	3026.890	3026.890	3026.890	3026.890	3026.890	3026.890
[90]	3026.890	3026.890	3026.890	3026.890	3026.890	3026.890
[96]	3026.890	3026.890	3026.890	3026.890	3026.890	3026.890

three layers of layer hens is presented in Table 5 and Figure 3. The body weight of male crossbred chickens was higher than that of female crossbred. This result also happens with the crossing between the Pelung rooster and three strains of layer hens resulting in the body

weight of male crossbred chickens being higher than that of female crossbred chickens (Table 6 and Figure 4).

Table 7 and Figure 5 show us that the body weight of the crossbred from the Pelung rooster was a bit higher than that of the Bangkok rooster; this might be due to the

higher body weight of the Pelung rooster (4.5 kg on average) than of Bangkok rooster (3.25 kg on average).

Table 8, Figures 6 and 7 tell us that the mature Weight of crossbred chicken, for example, crossbred chicken resulted from crosses between the Pelung rooster and Isa brown strain of layer hen which was predicted using Gompertz function ($y = a \cdot \exp(-b \cdot \exp(-c \cdot x))$; $y = 3026.89 \cdot \exp(-5.48 \cdot \exp(-0.23 \cdot x))$) will be 3026.89 g (a parameter) with inflection weight (a/e) of 1113.53 g and age of inflection ((ln(b))/c) at 7.40 weeks. Estimated growth curve parameters for the rest of the crossbred can be seen in Table 8. This study also showed that the mature Weight of a crossbred resulting from crossing between the Pelung rooster and Isa brown layer hen could be reached at about 28 weeks of age with a body weight of around 3000 g (Table 9).

Based on Table 8 and Figure 6, the prediction of body weight of crossbred chicken from age 0-8 weeks was entirely accurate with a coefficient of determination of 0.9932 for crossbred resulting from crossing between Pelung rooster and Isa brown layer hens. The results of this study are not much different from previous studies in that the Gompertz function is very accurate in describing growth curves in various types of poultry (Michalczyk et al. 2016; Eleroğlu et al. 2014; Masoudi & Azarfar 2017; N'dri et al. 2018; Adenaike et al. 2017; Urfa et al. 2017; Anang et al. 2017; Cyril et al. 2021; Nguyen et al. 2023; Brito et al. 2021; Hagan et al. 2022).

CONCLUSION

Based on the results of this study, it can be concluded that the cross between Pelung roosters and layer hen of Isa brown strain produced crossbred chicken with the highest body weight at eight weeks of age. This crossbred chicken would reach an inflection weight of 1113.53 g at an inflection age of 7.40 weeks and a mature Weight of 3026.89 g. Therefore, this crossbred chicken could be an alternative for fulfilling the demand for local chicken in Indonesia because the slaughter age could be shortened from 8 months to 8 weeks.

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Effect of Carbohydrate Type and Phenotype on the Quality of Post-Thawing Frozen Semen of KUB Chicken

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ABSTRAK

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Ayam Kampung Unggul Balitbangtan (KUB) merupakan ayam yang memiliki fenotip yang berbeda-beda. Dilaporkan bahwa fenotip ayam berhubungan dengan kualitas semen. Penelitian ini bertujuan untuk mengetahui karakteristik dan kualitas *post-thawing* semen ayam KUB dengan fenotip berbeda yang dibekukan dalam pengencer ringer laktat kuning telur (RLEY) dengan penambahan fruktosa atau glukosa. Semen dikoleksi dengan metode massase dari 20 ekor ayam KUB dengan fenotip jengger tunggal dan warna bulu hitam atau coklat tua dengan warna leher bulu merah (SCNR), jengger tunggal warna bulu hijau-hitam dengan warna leher bulu putih (SCNW), jengger *pea* dan hitam atau gelap warna bulu coklat dengan warna leher bulu merah (PCNR), dan jengger *pea* warna bulu hijau-hitam dengan warna leher bulu putih (PCNW). Semen dari setiap fenotip ayam dibagi menjadi tiga bagian, atau dibekukan dalam tiga jenis pengencer yaitu RLEY, RLEY+fruktosa (RLEYF), dan RLEY+glukosa (RLEYG). Motilitas sperma paling tinggi ditemukan paling tinggi pada pengencer dengan penambahan glukosa pada fenotip SCNR dan PCNW ($P<0.05$). Viabilitas sperma tertinggi ditunjukkan pada pengencer RLEYG pada fenotip PCNW ($P<0.05$). Abnormalitas tertinggi ditemukan pada pengencer RLEY dan RLEYF pada kelompok SCNW, PCNR, dan PCNW, sedangkan pada kelompok RLEYG hanya ditemukan pada kelompok PCNR. Recovery rate (RR) tertinggi ditunjukkan oleh kelompok RLEYG pada fenotip SCNR. Dari hasil penelitian ini dapat disimpulkan bahwa kualitas semen pasca thawing dipengaruhi oleh jenis glukosa dan fenotip ayam dan yang terbaik ditemukan pada pengencer dengan penambahan glukosa dalam fenotip SCNR.

Kata Kunci: Semen Ayam, Jengger, Pengencer, Suplementasi Media, Pembekuan

ABSTRACT

Magfira, Karja NWK, Arifiantini RI, Sartika T. 2023. Effect of carbohydrate type and phenotype on the quality of post-thawing frozen semen of KUB chicken. JITV 28(1):56-68. DOI:<http://dx.doi.org/10.14334/jitv.v28i1.3067>.

The superior Balitbangtan Kampung Chicken (KUB) chickens have different phenotypes. It was reported that the chicken phenotype was related to semen quality. This study aimed to determine the post-thawing characteristics and quality of KUB chicken semen with different phenotypes frozen in Ringer's lactate egg yolk (RLEY) diluent with the addition of fructose or glucose. Semen was collected using the massaging method from 20 KUB chickens with a single comb phenotype and black or dark brown feather color with a red feather neck (SCNR), green-black single comb with white feather neck collar (SCNW), pea comb and black feathers or dark brown fur with a red neck (PCNR), and a green-black pea comb with a white neck (PCNW). Semen from each chicken phenotype was divided into three parts or frozen in three types of diluents: RLEY, RLEY+fructose (RLEYF), and RLEY+glucose (RLEYG). The highest sperm motility was shown in the diluent with the addition of glucose in the SCNR and PCNW phenotypes ($P<0.05$). The highest sperm viability was found in the RLEYG diluent in the PCNW phenotype ($P<0.05$). The highest abnormality was found in the RLEY and RLEYF diluents in the SCNW, PCNR, and PCNW groups, whereas in the RLEYG group, it was only found in the PCNR group. From the results of this study, it can be concluded that the type of glucose and chicken phenotype influences the quality of post-thawing semen. The best is found in diluents with glucose attachments in the SCNR phenotype.

Key Words: Chicken Semen, Comb, Extender, Medium Supplementation, Freezing

INTRODUCTION

Superior Balitbangtan chickens (KUB) are local indigenous chickens from West Java developed by researchers at the Indonesian Research Institute for

Animal Production (IRIAP), Bogor (Bakrie et al. 2021). KUB chickens have high egg production (89.10 eggs/6 months), and males can reach a body weight of 1 kg within 2.5 months and are more resistant to disease (Silalahi et al. 2019). This chicken has been distributed

in Indonesia, including North Sumatra (Hasyim et al. 2020), Jawa Tengah (Hidayah et al. 2019), and Central Sulawesi (Wardi, 2019). According to the Indonesian Agricultural Minister No. 274/Kpts/SR.120/2/2014 (Ditjen PKH 2014) that the phenotypic characteristics of KUB chickens are variable; the feathers of KUB chickens are mostly black (64%), yellow to black, the legs are blackish-grey, with a predominantly single comb (71%), and the rest are pea-shaped.

The phenotype of KUB chickens, such as feather color and comb type reported, influenced semen quality. Phenotypic differences between chickens affect semen characteristics, e.g., comb size (Makhafola et al. 2012). Feather color can also indirectly affect semen quality. Feather colorations strongly correlate with carotenoid metabolism in birds (Weaver et al. 2018). Carotenoids contained in avian plasma can protect cell membrane proteins from oxidative damage (Lucas et al. 2014). Roosters with pale chest feathers had higher levels of oxidative stress, indicated by high levels of malondialdehyde (MDA). MDA was indicated as an indicator of lipid peroxidation, which is believed to cause low sperm motility (Pintus & Ros-Santaella, 2021) and reduce sperm fertility (Thelie et al. 2019). Combs on chickens function as thermoregulation to suppress the incidence of heat stress in chickens. According to (Adedeji et al. 2015), the heat stress index in chickens decreased with the increase in comb size. Chickens with heat stress conditions have more MDA levels (Mujahid et al. 2007), which causes a decrease in motility (Harsha et al. 2021).

Chicken is an animal with a short life span; therefore, the superior genetic material of the KUB chicken must be preserved. According to (Sartika 2016), KUB chickens are selected based on egg production. Therefore the information on semen quality is limited. Genetic material from superior roosters can be conserved to preserve germplasm and genetic diversity in chickens and to be used after the death of the animals (Telnoni, 2016). However, the success of cryopreservation in poultry semen is still low compared to Ruminants. The motility of thawed sperm from cocks varies between 34–40% (Pranay et al. 2018), 27–34% (Mosca et al. 2019), and 40–54% (Askarianzadeh et al. 2018). The morphometry of poultry sperm is a relatively small area. Therefore, the addition of semen diluent may affect the osmotic pressure of the sperm, resulting in damage to the cell membrane (Long 2006).

Biological and biophysical factors affect sperm resistance to freezing, such as membrane permeability, lipid composition, and membrane fluidity (Blesbois 2007; Blesbois et al. 2007). The lipid components in the membrane are divided into three classes, namely phospholipids, cholesterol, and glycolipids (sugary lipids) (Albert et al. 2002). This study used ringer lactate egg yolk as a base extender. Ringer's lactate contains salt, which has a buffering and isotonic effect that maintains

sperm motility (Iswati et al. 2018). Egg yolk has been widely used as a poultry semen thinner (Saleh et al. 2022) because it contains low-density lipoprotein that protects sperm (Alkali et al. 2022). The addition of sugar can modulate the fluidity of goat sperm membranes through interactions with the phospholipid bilayer (Aboagla & Terada 2003). The use of sugars (fructose and glucose) in chicken semen diluent for cryopreservation is still limited. Fructose had a better effect than glucose in maintaining the motility of dog sperm which was preserved at five °C (Ponglowhapan et al. 2004). Using fructose has been reported to maintain the motility of chicken spermatozoa at four °C for 12 hours, with motility of 63.3%. (Getachew et al. 2015). However, the production of lactic acid from the byproduct of the anaerobic metabolism of fructose is higher than glucose (Li et al. 2017), where the abundance of lactic acid in chicken semen diluent has a toxic effect that causes a decrease in sperm motility (Rochmi & Sofyan 2019).

The success of cryopreservation must be supported by using a cryoprotectant that matches the composition of the semen. In this study, dimethylsulfoxide (DMSO) was used as an intracellular cryoprotectant to prevent the formation of ice crystals in cells. In addition, dimethylsulfoxide has hydrophobic properties that can diffuse through the plasma membrane and replace the phospholipid portion damaged by cryopreservation (Rakha et al. 2016). Although DMSO has benefits in semen freezing, the use of DMSO in chicken semen freezing is also limited. According to research results (Junaedi et al. 2016), using 7% DMSO in chicken semen diluent resulted in the highest quality of PTM and reported (Rakha et al. 2016) the best dose of DMSO for poultry semen diluent is 8%. In this study, the quality of fresh cock semen and the effect of monosaccharides (glucose and fructose) in Ringer lactate egg yolk (RLKT) diluent on the quality of frozen KUB cock semen with different phenotypes are investigated.

MATERIALS AND METHODS

All procedures in this study were approved by the Balitbangtan Animal Ethics Board (KKHB) under No: Balitbangtan/Balitnak/A/03/2021.

Animals

The chickens used were 20 KUB cocks aged ± 1.5 years divided into four groups with different phenotypes: Single comb and black or dark brown feather color with feather neck red color (SCNR), Single comb and greenish-black feather color with white feather neck color (SCNW), Pea comb and based black or dark brown feather color with feather neck red color (PCNR) and Pea comb and greenish-black feather color with white feather

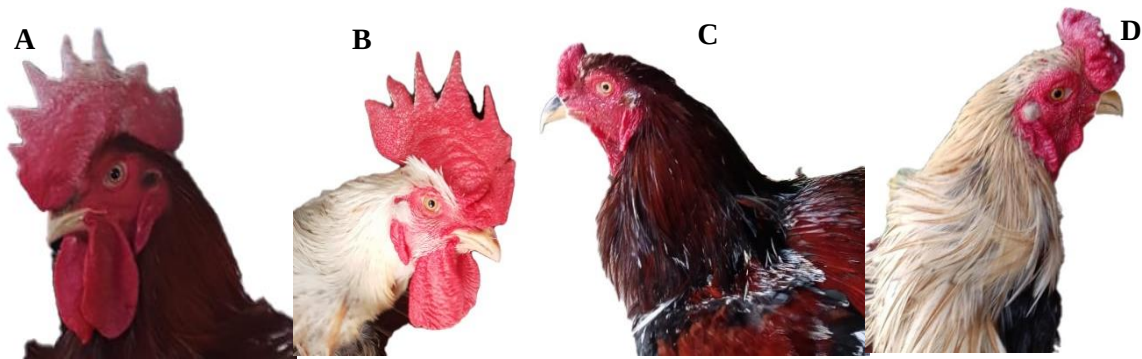


Figure 1. A) Single comb and black or dark brown feather color with feather neck red color (SCNR), B) Single comb and greenish-black feather color with white feather neck color (SCNW), C) Pea comb and based black or dark brown feather color with feather neck red color (PCNR), D) Pea comb and greenish-black feather color with white feather neck color (PCNW)

neck color (PCNW) (Figure 1). The chickens were reared in a cage of 45 cm × 35 cm × 45 cm, received 120 g/head/day and were given water ad libitum.

Diluent preparation

Ringer lactate egg yolk (RLEY) diluent is prepared from 83% Ringer lactate (PT. Widatra Bhakti), 10% egg yolk, and 7% Dimethyl Sulfoxide (DMSO), according to (Junaedi et al. 2016). First, all the ingredients were mixed and homogenized. Then 1000 IU/mL penicillin and 1 mg/mL streptomycin were added to the diluents. The carbohydrates used in this study were 0.6% (w/v) of glucose (RLEY-glucose) and 0.6% (w/v) of fructose (RLEY-F) (Octa et al. 2014).

Semen collection and evaluation

Semen is collected weekly using the abdominal massage method using a 1.5 mL microtube. The massage method is performed continuously on the back of the chicken up to the base of the tail until the chicken shows symptoms of erection (Tarif 2013). Semen from each group was pooled according to the phenotypic group and examined for its quality.

Semen quality was examined macroscopically and microscopically. Macroscopic examinations such as semen volume, pH, color, and consistency were evaluated. Microscopic evaluations included mass movement, sperm motility, viability, abnormality, and sperm concentration. The mass movement was evaluated by dripping semen on the object glass and observed under a light microscope. Assessment is determined using the scoring method (+++/3). Sperm motility was observed by dripping one drop of semen on a glass object, adding 8-10 drops of Ringer's lactate, and then homogenizing. The mixture was then observed under a microscope for five fields of view and expressed in percentages. Observations of viability and abnormalities

were carried out simultaneously, mixing one drop of semen and 8-10 eosin nigrosin on a glass object and then preparing a smear. The preparations were then dried on a heated table and observed under a microscope for ten fields of view. Sperm concentration was calculated using a Neubauer chamber with a dilution of 500 times.

The evaluation procedure refers to Arifiantini (2012), adapted for poultry semen. Sperm motility was assessed by diluting semen with lactated Ringer's solution, homogenized, and viewing it under the microscope. Sperm motility was assessed using five fields, and the values were expressed as a percentage. In addition, abnormal sperm and viability were stained with eosin nigrosin. The Neubauer chamber was used to calculate the concentration of sperm—all microscopic evaluations were performed with a binocular microscope (Olympus CX 23).

Semen from each individual per group is pulled and analyzed for motility. Semen with sperm motility of >70% were used in this study and divided into three aliquots diluted with RLEY, RLEYG, and RLEYF, respectively, to achieve a concentration of 200×10^6 per mL divided into four straw. The diluted semen was then filled into 0.25 ml mini straws (Minitube, Germany) and kept at equilibrium at 5°C for 2 hours (Junaedi et al. 2016). After equilibration, the straws were placed 6 cm above the surface of the liquid nitrogen for 10 minutes and then stored in a liquid nitrogen container for further evaluation. The frozen semen was analyzed 24 hours after freezing. The method refers to Khaeruddin et al. (2020) with slight modification. Before evaluation, the semen was thawed at 37°C for 30 seconds (Khaeruddin et al. 2020). The evaluation of frozen semen quality was based on sperm motility, viability, morphology, recovery rate, decreased viability and increased abnormality. The sperm motility, viability, and morphology tests were similar to those performed on fresh semen with slight modification. Recovery rate decreased viability and increased abnormality were calculated by comparing the motility of post-thawing sperm and fresh semen.

Data analysis

This study used a factorial, completely randomized design with two factors: the first factor is a chicken phenotype, and the second factor is semen diluent. All procedures were repeated five times. Sperm motility, viability, and morphology data are reported as mean $\pm$ SEM. The research data were analyzed using IBM SPSS Statistics 25.

RESULTS AND DISCUSSION

Quality of fresh KUB semen quality with different phenotype

The characteristics of fresh semen of KUB chickens based on the phenotype are shown in table 1. The semen volume of the SCNR group was lower than that of the SCNW and PCNR groups ($P < 0.05$) but not different from PCNW ($P > 0.05$). The characteristics of fresh semen of KUB chickens based on the phenotype are shown in table 1. The semen volume of the SCNR group was lower than that of the SCNW and PCNR groups ($P < 0.05$) but not different from PCNW ($P > 0.05$). Microscopic quality only sperm motility and ejaculate concentration showed the difference. Sperm motility in the SCNR group was lower than SCNW and PCNR ($P < 0.05$) but not different from PCNW ($P < 0.05$). The concentration of sperm per ejaculate in the SCNR group showed the lowest number among all groups ($P < 0.05$),

but among the other three groups, there was no significant difference ($P > 0.05$).

Table 2 shows that sperm with diluents RLEY and RLEYF did not differ in each phenotype ($P > 0.05$), while semen with added glucose (RLEYG) showed differences where the SCNR phenotype showed the highest motility and did not differ from PCNW ($P > 0.05$) but differed from that of PCNW ($P > 0.05$). SCNW and PCNR ($P < 0.05$). Semen in the SCNR and PCNW phenotype groups were different in each diluent ($P < 0.05$), while the group only with glucose added (RLEYG) showed the highest motility ($P < 0.05$), but in the PCNR group, the semen in RLEY diluent was lower than RLEYF and RLEYG ($P < 0.05$). Overall, sperm quality after freezing in this study was generally lower than in previous studies. For example, KUB chicken semen diluted in RLEY+ genistein 10 M showed sperm motility of 43% after freezing (Arif 2020). It was also reported by Junaedi et al. (2016) that free-range chicken semen cryopreserved using RLEY showed a post-thaw motility (PTM) value of 37.22% with a viability of 54.47%. Sentul chicken semen in RLEY+7% glycerol provides 30.18% PTM and 47.07% sperm viability. In Telnoni (2016), it was reported that the PTM of Sentul Kampung (SK) Kedu chicken in RLEY+10% DMSO was about 40.83%. The differences between this result and previous studies showed differences in the resistance of chicken sperm to freezing and the compatibility between the semen composition of individual chickens and the diluent. Our preliminary studies using dimethylformamide (DMF) 7% as a cryoprotectant in

Table 1. Fresh KUB rooster semen quality

Variable	Phenotypic			
	SCNR	SCNW	PCNR	PCNW
Macroscopic				
Semen volume (mL)	0.48 $\pm$ 0.02 ^a	0.61 $\pm$ 0.02 ^b	0.66 $\pm$ 0.04 ^b	0.54 $\pm$ 0.10 ^{ab}
pH	6.76 $\pm$ 0.03 ^a	6.80 $\pm$ 0.03 ^b	6.71 $\pm$ 0.01 ^a	6.70 $\pm$ 0.00 ^a
Consistency	thick	thick	thick	thick
Color	Milky white	Milky white	Milky white	Milky white
Microscopic				
Mass movement	2.40 $\pm$ 0.15	2.30 $\pm$ 0.12	2.70 $\pm$ 0.10	2.45 $\pm$ 0.13
Sperm Motility (%)	75.00 $\pm$ 2.29 ^a	79.50 $\pm$ 0.88 ^b	80.50 $\pm$ 0.71 ^b	76.00 $\pm$ 1.57 ^{ab}
Sperm Viability (%)	88.46 $\pm$ 2.63	89.43 $\pm$ 1.95	88.96 $\pm$ 2.02	89.72 $\pm$ 1.89
Sperm abnormalities (%)	14.59 $\pm$ 2.29	16.40 $\pm$ 1.81	16.18 $\pm$ 2.58	16.66 $\pm$ 2.00
Sperm concentration ($\times 10^6$ /Ejaculate)	737.54 $\pm$ 90.53 ^a	903.23 $\pm$ 101.27 ^b	880.19 $\pm$ 77.24 ^b	923.70 $\pm$ 70.23 ^b

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. ^{a-c} superscripts on the same column show a significant difference extender, and ^{A-C} superscripts on the same line show a significant difference phenotype in $P < 0.05$

RLEY diluent were also less suitable for KUB chicken semen. KUB chicken results from six generations of kampung chicken whose egg production was selected according to specific criteria and is probably not compatible with RLEY diluent; therefore, it is necessary to use it with other types of diluents.

The lack of success in chicken semen cryopreservation is also caused by the small cytoplasm, which causes a lack of cryoprotectant movement (Svoradova et al. 2021). The freezing process can reduce sperm quality caused by the formation of ice crystals, cold shock, and lipid peroxidation. Therefore, freezing can reduce the percentage of live sperm and increase abnormal sperm (Siudzińska & Ukaszewicz 2008); this can be seen in Tables 6 and 7, where the decrease in post-thawing viability reached 43-60%, and an increase in abnormality reached 19-32%. The freezing process can also cause the formation of ROS, which can disrupt the stability of the sperm membrane. Poultry sperm are susceptible to forming reactive oxygen species (ROS) due to freezing (Rezaie et al. 2021). The production of ROS in sperm takes place in the membrane and mitochondria. An increase in dysfunctional sperm correlates with ROS production, decreasing mitochondrial function (Dutta et al. 2019). High ROS production can damage the plasma and acrosome membranes, leading to infertile sperm (Khan et al. 2021). Reactive oxygen species can be suppressed in the presence of antioxidants, either from seminal plasma or in addition to diluents.

The percentage of viability is shown in table 3, where the difference in viability is only shown in the RLEY diluent, namely the SCNR phenotype was not different from that of SCNW ($P<0.05$) and was higher than PCNR and PCNW ($P>0.05$). The phenotype of SCNW was also not different from that of PCNW ($P>0.05$) but higher than that of PCNR ($P<0.05$). The SCNR group did not show any difference in viability in each diluent. Semen from the RLEY group had the lowest viability than RLEYF and REYG in the SCNW and PCNR groups ($P<0.05$), but there was no difference in viability in the group with the addition of carbohydrates ($P>0.05$). The PCNW group showed differences in the viability of each diluent group, where sperm in the RLEY diluent had the lowest viability and RLEYG had the highest viability ($P<0.05$). The decrease in viability can be seen in table 6. Table 6 shows that only the RLEY diluent showed significant differences in each phenotype. The percentage decrease in viability in the SCNR phenotype was lower than that of PCNR and PCNW but not different from SCNW ($P<0.05$). SCNW phenotype had a percentage decrease in viability that was not different from PCNW ($P>0.05$) but different from PCNR, while PCNW did not differ from PCNR. Differences in decreased viability between diluents in phenotype were not shown in the SCNR phenotype ($P>0.05$). The PCNW phenotype group showed differences in the decrease in viability of each diluent, where the RLEYG diluent had the lowest percentage, followed by RLEYF, and the highest was RLEY ($P<0.05$). Decreased viability is also

Table 2. The motility of KUB Frozen-thawed semen in three different diluent and different phenotype

Extender	Phenotype $\pm$ (SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	11.60 $\pm$ 1.33 ^a	8.96 $\pm$ 1.50 ^a	10.56 $\pm$ 1.56 ^a	10.64 $\pm$ 1.79 ^a
RLEYF	16.88 $\pm$ 1.72 ^b	14.00 $\pm$ 1.58 ^a	18.72 $\pm$ 1.54 ^b	16.60 $\pm$ 1.72 ^b
RLEYG	29.80 $\pm$ 1.24 ^{Ac}	20.80 $\pm$ 2.21 ^{Bb}	22.20 $\pm$ 1.75 ^{Bb}	25.40 $\pm$ 1.11 ^{ABc}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. a-c superscripts on the same column show a significant difference extender. A-C superscripts on the same line show a significant difference phenotype in $P<0.05$

Table 3. Viability of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype $\pm$ (SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	38.74 $\pm$ 2.71 ^A	34.33 $\pm$ 2.54 ^{ABa}	25.45 $\pm$ 1.78 ^{Ca}	29.31 $\pm$ 2.70 ^{Ba}
RLEYF	39.56 $\pm$ 2.90	39.25 $\pm$ 2.38 ^{ab}	37.48 $\pm$ 2.41 ^b	36.55 $\pm$ 2.41 ^b
RLEYG	44.94 $\pm$ 2.01	42.58 $\pm$ 2.41 ^b	41.87 $\pm$ 2.27 ^b	46.30 $\pm$ 1.89 ^c

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. a-c superscripts on the same column show a significant difference extender, and A-C superscripts on the same line show a significant difference phenotype in $P<0.05$

Table 4. The recovery rate of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	15.47±1.78 ^a	13.28±1.96 ^a	11.13±1.86 ^a	14.00±2.36 ^a
RLEYF	22.51±2.30 ^b	23.55±1.94 ^b	17.39±1.96 ^a	21.84±2.27 ^b
RLEYG	39.73±1.65 ^{Ac}	27.93±2.21 ^{Bb}	25.84±2.75 ^{Bb}	33.41±1.47 ^{Bc}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. ^{a-c} superscripts on the same column show a significant difference extender, and ^{A-C} superscripts on the same line show a significant difference phenotype in $P<0.05$

Table 5. Abnormality of KUB frozen-thawed semen in different extender and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	34.23±2.91 ^A	47.78±4.73 ^{Ba}	44.31±4.30 ^{A Ba}	43.71±2.02 ^{AB}
RLEYF	40.43±1.56 ^A	41.75±1.99 ^{A ab}	48.49±2.01 ^{Ba}	46.49±2.66 ^{AB}
RLEYG	39.89±1.63 ^A	35.54±1.57 ^{A b}	34.24±2.91 ^{A b}	47.98±1.36 ^B

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. ^{a-c} superscripts on the same column show a significant difference extender, and ^{A-C} superscripts on the same line show a significant difference phenotype in $P<0.05$

defined as an increase in the number of dead sperm, which decreases motility. Dead sperm produce lactic acid, increasing the degree of acidity in the media (Iswati et al. 2018). Lactic acid triggers cytoplasmic acidity, which affects the immotile flagellum (Matsuzaki et al. 2015).

The freezing capability of semen derived from each phenotype diluted using RLEY, RLEYF, and RLEYG is shown in table 4. The difference in the percentage recovery rate (RR) of the diluent group in phenotype was only shown in the group with added glucose (RLEYG), where SCNR had the highest RR compared to SCNW, PCNR, and PCNW. However, the three phenotypes were not different ($P>0.05$). The recovery rate compares post-thawing motility to fresh semen motility and describes the cryodamage index (Buffone et al. 2012). Damage during cryopreservation is caused by dramatic changes in temperature, osmotic pressure, toxic effects of cryoprotectant, and generation of ROS due to oxidative stress (Kumar et al. 2021). Therefore cryopreservation also causes cell death caused by the formation of intracellular ice crystals that can penetrate the membrane and affect cell function (Oberoi et al. 2014).

Abnormalities can cause decreasing motility, and their increase in the treatment is also high. The diluent did not affect abnormalities in the SCNR and PCNW phenotype groups ($P>0.05$). Meanwhile, semen with added glucose (RLEYG) had the lowest abnormality in SCNW and PCNR phenotypes ($P<0.05$). Sperm without added carbohydrates (RLEY) had the lowest abnormality

in the SCNR phenotype, although not different with PCNR and PCNW ($P>0.05$) but different with SCNW ($P<0.05$). Sperm abnormalities with added glucose (RLEYG) in the SCNR, SCNW, and PCNR groups did not show a different percentage ($P>0.05$) but were lower than in the PCNW group ($P<0.05$). Sperm abnormality is an essential factor in determining sperm fertility, one of which is the excessive production of ROS (Agarwal et al. 2014). Cryopreservation causes an increase in the production of ROS, which stimulates lipid peroxidation, thereby reducing the integrity of the membrane so that the deformation of cell organelles and protein denaturation cannot be avoided (Partyka et al. 2012). Therefore, in the semen freezing process, it is necessary to add cryoprotectants to minimize damage to sperm. Cryoprotectant is required for the cryopreservation of sperm. There are two types of cryoprotectants: extracellular and intracellular. Extracellular cryoprotectants protect the cell membrane from the osmotic pressure created by freezing; one of these agents is sugar (Darsini et al. 2019). Osmotic stress can cause structural changes in sperm, altering sperm morphology (Yeste et al. 2017). After thawing, the quality of the frozen semen decreases significantly in three different diluents. On the other hand, after sperm disposition at the female genital tract, the avian sperm is stored in the sperm storage tubule (SST) before fertilization.

Decreased sperm viability (DV) showed in Table 6, where the percentage decrease in viability in the SCNR phenotype showed no significant difference in each

diluent. The percentage of sperm viability in the RLEY diluent group showed the highest decrease in sperm viability in each phenotype ($P < 0.05$), while the RLEYG diluent only had the lowest percentage in the PCNW phenotype group ($P < 0.05$). The different decrease in viability in each phenotypic group was only shown by the RLEY diluent, where the SCNR phenotype had the lowest DV compared to the other phenotypes ($P < 0.05$). In contrast, the two diluents with added sugar showed no difference in each phenotypic group ($P > 0.05$). The RLEYG diluent showed the lowest DV percentage compared to the other two diluents, indicating that sugar can more maintain sperm viability than fructose or without sugar.

Sperm motility and viability are closely related to mitochondrial activity. High mitochondrial activity is positively correlated with the rate of cell metabolism and leads to a high survival rate (Choi et al. 2013). The opening of the mitochondrial pore by freezing causes the release of pro-apoptotic in the cytoplasm and thus promotes cell degradation (Fang et al. 2020), which affects sperm motility (Feyzi et al. 2018). According to (Treulen et al. 2018). Reactive oxygen species that cause pathological calcium excess in somatic cell mitochondria set off the mitochondrial permeability transition (MPT), resulting in mitochondrial malfunction and cell death, directly involved in sperm cell destruction. Sperm motility in chickens correlates with glucose uptake used for sperm motility. These results are based on the study reported by (McLean et al. 1997) that single-combed chickens absorb glucose better than rosy-combed chickens. According to McGary et al. (2002), broad-combed chickens have better fertility than small-combed chickens. Table 7 shows the abnormality (IA) increase in each diluent in each phenotypic group. The increase in abnormality based on the diluents was only seen differently in the SCNW, and PCNR phenotypes, where the RLEY diluent showed the highest percentage and RLEYG had the lowest percentage ($P < 0.05$). The highest percentage of IA in the RLEY diluent was from the SCNW phenotype, and the lowest was from the SCNR phenotype ($P < 0.05$). The increase in abnormalities in the RLEYF diluent of the PCNR phenotype was not significantly different from that of the PCNW phenotype but was higher than the SCNR and SCNW groups ($P < 0.05$). The percentage increase in abnormalities in sperm diluted using RLEYG showed no difference with the SCNW and PCNW groups ($P > 0.05$) but was higher than the PCNR groups ($P < 0.05$). Overall, the percentage of IA in the SCNR phenotype group was only around 19-25%. It has been described above that single-combed chickens can absorb glucose better, so this may be one of the reasons the results in the SCNR group are better than the other phenotypes.

In this study, the chickens of the SCNR phenotype showed better sperm motility than sperm from other phenotypic groups. SCNR is thought to contain

carotenoids that act as antioxidants against free radicals during freezing. Carotenoids strongly correlate with plumage coloration in birds (Weaver et al. 2018). Carotenoids are non-enzymatic antioxidants that protect sperm from oxidative stress (Triques et al. 2019). Carotenoids are found in the inner membrane of the mitochondria, which have closely related to the function of the mitochondria as a site for aerobic cell respiration (Hill et al. 2019). The carotenoid content, which is thought to be present in the SCNR phenotype, may protect sperm from free radical damage. It has the lowest abnormality compared to the other three phenotypes. Low sperm abnormalities were negatively correlated with PTM and sperm viability. This correlation indicates low abnormal morphology leading to PTM and high viability in sperm.

Morphological defects or types of abnormalities in this study refer to (Feyisa et al. 2018), who reported that there were 11 types of abnormalities in chicken sperm, namely coiled head, bent head, broken head, knotted head, detached head, broken midpiece, bent tail, knotted tail, broken tail, coiled tail, detached tail. The results showed that a broken midpiece (BM) was the most common morphological defect. The percentage of sperm BM of each diluent in the phenotype showed that only the RLEYG diluent had differences. The SCNW phenotype group had the lowest percentage that differed from PCNW ($P < 0.05$) but did not differ from SCNR and PCNR ($P > 0.05$). Broken midpiece sperm of each phenotype in the diluents showed that the SCNW group had different percentages in each diluent. The sperm group in the REG diluent had the lowest percentage compared to RLEY and RLEYF ($P < 0.05$), while in both diluents, there was no significant difference ($P > 0.05$). It is well known that the mitochondria, which are the site of ATP metabolism, are located in the midpiece of the sperm. Therefore, damage to the midpiece area is believed to decrease sperm motility. The results of ATP hydrolysis in the mitochondria can diffuse into the flagella by activating the dynein arm on the microtubules so that there is a movement of the flagella (Gwo & Arnold 1992), which explains the reason PTM in this study is very low.

The results showed that semen in RLEYG diluent was better than RLEYF and RLEY. Sperm requires energy to move and reach the egg in the female reproductive tract; this process involves ATP to maintain flagellar movement through glucose metabolism or glycolysis. In semen diluents, glucose has two primary functions: energy supply and cryoprotectant agent. Glucose could directly decompose to give energy to sperm, but fructose had to be turned into glucose before it could supply energy (Tang et al. 2021). The glycolysis process is supported by a transporter protein that transports glucose into the sperm membrane in the midpiece called glucose transport (GLUT) (Setiawan et al. 2020).

Table 6. Decreased viability (DV) of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	49.72±2.70 ^A	55.09±2.54 ^{ABa}	63.50±1.78 ^{Ca}	60.41±2.69 ^{BCa}
RLEYF	48.90±2.90	50.17±2.38 ^{ab}	51.47±2.35 ^b	53.17±2.41 ^b
RLEYG	43.51±2.01	46.84±2.41 ^b	47.09±2.27 ^b	43.42±1.89 ^c

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color and PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. ^{a-c} superscripts on the same column show a significant difference extender, and ^{A-C} superscripts on the same line show a significant difference phenotype in P<0.05

Table 7. Increased abnormality of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	19.65±2.92 ^A	31.38±4.73 ^{Ba}	28.14±4.30 ^{ABa}	27.05±2.02 ^{AB}
RLEYF	25.84±1.56 ^A	25.34±1.10 ^{A ab}	32.31±2.02 ^{Ba}	29.83±2.66 ^{AB}
RLEYG	25.30±1.62 ^{AC}	19.14±1.57 ^{ABb}	18.06±2.91 ^{Bb}	31.33±2.37 ^C

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color and PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. ^{a-c} superscripts on the same column show a significant difference extender, and ^{A-C} superscripts on the same line show a significant difference phenotype in P<0.05

Table 8. Broken midpiece in KUB Post Thawing chicken sperm in different diluents and phenotypes.

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	37.75±3.28	37.03±3.27 ^a	34.87±1.95	31.24±3.04
RLEYF	34.90±2.99	36.31±1.54 ^a	39.19±2.24	35.71±2.03
RLEYG	32.43±2.14 ^{AB}	28.23±2.53 ^{Ab}	34.15±1.94 ^{AB}	37.81±2.28 ^A

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color and PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. ^{a-c} superscripts on the same column show a significant difference extender, and ^{A-C} superscripts on the same line show a significant difference phenotype in P<0.05

Glucose is transported into the cell by GLUT, and protons will leave the cell. The release of protons from the cell leads to intracellular alkalinization and promotes flagella movement (Mannowetz et al. 2012). In addition, glucose has an excellent cryoprotective effect on sperm by interacting with the plasma membranes of sperm, as reported in wild boar sperm (Gómez-Fernández et al. 2012).

Comb growth is controlled by androgens, which are known to regulate spermatogenesis (Yamamoto, 2016). Comb growth is closely related to testosterone and aggressiveness in poultry (Mukhtar & Khan2012). The stimulation of intracellular fluid generation by fibroblasts in the comb and dewlap by testosterone and dihydrotestosterone is known to affect the growth of these tissues (Leão et al. 2017). The comb is located on the head and functions for thermoregulation (Iyasere et

al. 2021), so a large comb provides better temperature regulation than smaller combs. However, chickens do not have sweat glands, so their heat dissipation system is not optimal.

For this reason, chickens are more susceptible to heat stress. The optimal body temperature of chickens is between 39.9 - 41°C. A rise in body temperature can lead to death in chickens (Ramadiani et al. 2021). In addition, birds exposed to temperatures above their thermoneutral zone develop respiratory alkalosis, which stimulates the hypothalamic-pituitary-adrenal axis to produce more cortisol, corticosterone, catecholamines, glucocorticoids, and adrenocorticotropin, which harms their physiology, intestinal function, and reproductive function (Kumar et al. 2021). Heat stress also reduces carbohydrate absorption by decreasing glucose transporter concentrations and amino acid absorption (Goel et al. 2021), resulting in

weight loss and reproductive mechanisms, which explains that single comb and black or dark brown feather color with red feather neck color (SCNR) has better-post-thawing sperm quality compared to other groups.

As described above, sugar can be an extracellular cryoprotectant that can protect spermatozoa from damage caused by cryopreservation. The plasma membrane is covered by glycocalyx sugars, which can bind with lipids and proteins that protect sperm from damage (Herdis et al. 2019). In addition, sugar can be immune-protective, preventing antigenicity during transport in SST (Peláez et al. 2011). Therefore, adding sugar to the semen diluent can replace the carbohydrate component in the plasma membrane, which is damaged by freezing. Moreover, glucose can inhibit the performance of 2-deoxyglucose (DOG), which can hinder the glycolysis process, while the presence of fructose is insufficient to prevent the performance of 2-deoxyglucose (DOG). 2-deoxyglucose is a glucose analog that can inhibit the role of hexokinase, one of the enzymes regulating glycolysis (Pasupuleti 2007).

Sperm abnormalities after thawing showed a significant effect of freezing on chicken sperm. Cryopreservation can increase osmotic pressure so that the plasma membranes of the cell can be damaged (Miranda et al. 2018). Poultry semen is very sensitive to freezing due to its different morphology from mammalian sperm. The smaller surface of the sperm makes the poultry sperm vulnerable to osmotic pressure and freezing (Long 2006). Single-comb chicken sperm can absorb glucose better than pea-comb chicken. This result could explain the lowest sperm abnormality in the RLEYG diluent. Normal sperm have more opportunities to fertilize the egg. Therefore morphology is an essential factor when evaluating sperm. Freezing can cause changes in sperm morphology. These changes are caused by changes in the ultrastructural of sperm due to drastic temperature changes, osmotic pressure, and the formation of crystal ice in sperm (Sharma & Sharma 2020).

Consequently, the crystal ice formed by cryopreservation can lead to membrane structure, integrity, and morphological changes (Darsini et al. 2019). Apart from crystal ice formation, swelling and shrinkage of cells due to water changes and cryoprotectants are also unavoidable in the early stages of freezing, which most cell organelles cannot tolerate (Medeiros et al. 2002; Ozkavukcu et al. 2008). It has been described above that the small surface of the sperm at the time of freezing is susceptible to osmotic pressure. The low results of PTM semen in this study could have several causes. The first possibility is that the diluent used in this study is incompatible with KUB chicken semen. Some of the diluents commonly used in chicken semen include Beltsville poultry semen extender (BPSE) (Telnoni 2016), Tris-Skimmed milk (Bustani & Baiee

2021), Ringer's Lactate Low-Density lipoprotein for chicken or quail (Magfira et al. 2017), and a combination of coconut water, egg yolk, and fructose (Rochmi & Sofyan 2019). Another possibility is that KUB chickens have low freezing capacity. In addition, KUB chickens result from six generations of selection which can lead to changes in semen quality after freezing. Semen quality in F1 to F5 from KUB chicken has not been reported, so there is no comparison of semen quality. However, (Ansah & Buckland 1983) found that the fertile period of frozen semen in selected hens increased in generations F3 to F7 and decreased in generation F8. This result suggests a selection effect on the quality of frozen chicken semen. Factors influencing freezing ability have been widely reported, such as genetics and individual variation within a pedigree (Tesfay et al. 2020); it was reported that genetic differences reflected in the semen volume, pH, motility, viability, and abnormalities of sperm (Furthermore, it was reported that pH was correlated with sperm abnormalities (Natarajamani et al. 2014). These differences in the fresh semen quality allow the quality of frozen semen to be influenced by pedigree or genetics.

CONCLUSIONS

The best motility and recovery were found in the SCNR phenotype group, diluted using added glucose. The PCNW group had high abnormalities in all diluent groups. In this study, abnormalities significantly affect the decrease in the motility of chicken semen. Further research is needed to evaluate the seminal plasma semen content of KUB chicken and also to find the more suitable freezing diluents.

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Effect of Dietary with Different Energy and Protein Levels on Laying Quails Performance

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ABSTRAK

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Performa burung puyuh merupakan fungsi dari beberapa faktor produksi, salah satunya adalah pakan. Akan tetapi, kandungan energi termetabolis dan protein kasar yang optimal untuk performa burung puyuh petelur hingga saat ini masih kontradiktif. Tujuan dari penelitian ini adalah untuk mengevaluasi pengaruh pemberian pakan dengan aras energi termetabolis dan protein kasar yang berbeda terhadap performa puyuh petelur. Sebanyak 540 puyuh petelur berumur delapan minggu dialokasikan secara acak pada sembilan perlakuan pakan. Setiap perlakuan terdiri dari empat ulangan dengan 15 ekor puyuh petelur per ulangan. Perlakuan pakan terdiri dari tiga aras energi termetabolis (2.700, 2.800, dan 2.900 kkal/kg) dan tiga aras protein kasar (17, 19, dan 21%) dalam rancangan percobaan faktorial. Setelah satu minggu periode adaptasi, perlakuan pakan dilakukan selama lima minggu (umur 9-13 minggu). Terdapat adanya interaksi antara energi termetabolis dan protein kasar pada konsumsi pakan ($P < 0,05$). Konsumsi pakan menunjukkan penurunan seiring dengan peningkatan aras energi termetabolis ketika aras protein kasar 17 dan 19%. Akan tetapi, konsumsi pakan cenderung meningkat ketika energi termetabolis tinggi dikombinasikan dengan protein kasar tinggi (2.900 kkal/kg dan 21%). Interaksi antara energi termetabolis dan protein kasar tidak terlihat pada variabel lain (produksi telur, berat telur, massa telur, dan rasio konversi pakan) ($P > 0,05$). Pemberian pakan dengan aras energi metabolis yang berbeda berpengaruh nyata terhadap rasio konversi pakan ($P < 0,05$), tetapi tidak berpengaruh nyata terhadap produksi telur, berat telur, dan massa telur puyuh petelur ($P > 0,05$). Protein kasar sebagai faktor tunggal berpengaruh nyata terhadap produksi telur, berat telur, massa telur, dan rasio konversi pakan puyuh petelur ($P < 0,05$). Analisis regresi linier menunjukkan bahwa konsumsi energi termetabolis secara linier meningkatkan produksi telur, berat telur, massa telur, dan rasio konversi pakan. Selain itu, konsumsi protein kasar juga secara linier meningkatkan produksi telur, berat telur, massa telur, dan rasio konversi pakan. Dapat disimpulkan bahwa untuk memberikan performa puyuh petelur yang optimal diperlukan pakan yang mengandung energi termetabolis 2900 kkal/kg dan protein kasar 21%.

Kata Kunci: Puyuh Petelur, Energi Termetabolis, Protein Kasar, Rasio Konversi Pakan, Produksi Telur

ABSTRACT

Edi DN, Andri F. 2023. Effect of dietary with different energy and protein levels on laying quails performance. JITV 28(1): 69-75. DOI: <http://dx.doi.org/10/14334/jitv.v28.i1.3061>.

The performance of quail is a function of several production factors, one of which is feed. However, the optimum metabolizable energy and crude protein for the performance of laying quails currently needs to be revised. This study aimed to evaluate the effects of feeding diets with different metabolizable energy and crude protein levels on the performances of laying quails. Five hundred forty (540) laying quails aged eight weeks were randomly allocated into nine dietary treatments. Each treatment consisted of four replicates with 15 laying quails per replicate. Dietary treatments comprised three metabolizable energy levels (2700, 2800, and 2900 kcal/kg) and three crude protein levels (17, 19, and 21%) in a factorial research methodology arrangement. After one week of the adaptation period, the feeding trial was conducted for five weeks (9-13 weeks old). Interaction between metabolizable energy and crude protein levels was recorded on feed intake ($P < 0.05$). Feed intake was reduced along with the increase in the metabolizable energy when crude protein levels were 17 and 19%. However, the feed intake tended to be improved when high metabolizable energy was combined with high crude protein contents (2900 kcal/kg and 21%, respectively). No interaction was recorded on the other variables (egg production, egg weight, egg mass, and feed conversion ratio) ($P > 0.05$). Feeding diets with different metabolizable energy levels had a significant effect on feed conversion ratio ($P < 0.05$) but without any significant impact on egg production, egg weight, and egg mass of laying quails ($P > 0.05$). Crude protein as a single factor significantly affects egg production, weight, mass, and feed conversion ratio of laying quails ($P < 0.05$). Linear regression analysis indicated that metabolizable energy intake linearly improved egg production, weight, mass, and feed conversion ratio. Moreover, crude protein intake linearly improved egg production, weight, mass, and feed conversion ratio. Therefore, feeding diets containing 2900 kcal/kg metabolizable energy and 21% crude protein are required to provide optimum performance of laying quails.

Key Words: Laying Quail, Metabolizable Energy, Crude Protein, Feed Conversion Ratio, Egg Production

INTRODUCTION

Quail is one of the potential livestock in Indonesia. They have several advantages, such as fast growth, high egg production, lesser space and feed requirements, and cheaper capital investment (Agboola et al. 2016). For that reason, quail is suited for both industrial and small-scale farming to produce eggs and meat. According to DGLAH (2021), the quail population in 2017 was 14.6 million birds, while in 2021, the population increased to about 15.2 million birds. Furthermore, in terms of productivity, the quail egg production in 2017 was 25.0 thousand tons, while in 2021, was increased to about 25.3 thousand tons. These data prove that quail farming is essential to fulfilling animal product demand in Indonesia.

The performance of quail is a function of several production factors, one of which is feed. In Indonesia, protein sources are still imported, such as soybean meal, corn gluten meal, distillers' grain, solubles, and meat bone meal (Rakhmawati et al. 2022). The primary source of dietary energy for quail comes from corn. Edi (2021) stated that 70% of the energy requirement for poultry comes from corn, and the rest comes from protein and other nutrient sources. Prices of maize and imported feedstuffs tend to fluctuate and have an upward trend so that they will be correlated with the feed price.

Commercial quail feed has a higher nutrient content than the Indonesian National Standard SNI 01-3907-2006 for laying quail feed. The crude protein content of commercial quail is around 19-22%, while the standard in SNI is at least 17%, and the metabolizable energy is 2700 kcal/kg. Protein is one of the essential nutrients in poultry, and the availability of energy supports chicken activities and production goals (Saraswati et al. 2017; Andri et al. 2020). In addition, protein content functions for growth and production (Silodae & Polakitan 2018). Excess and lack of protein will have a negative impact biologically and economically.

One of the determinations of feed ingredients is more focused on the cost per unit spent to get the cost per unit product (meat, eggs) (Samadi 2012), meaning the comparison of production with costs incurred per unit of production. Therefore, efforts to increase feed efficiency can be made, including by making feed formulations with the right nutrient balance as needed. Macro-nutrients that can be used as indicators of nutrient balance in poultry are crude protein and metabolizable energy. However, to date, the optimum nutrient content for the performance of laying quails still needs to be revised. For that reason, the effects of feeding diets with different metabolizable energy and crude protein levels on the performances of laying quails were evaluated in this study.

MATERIALS AND METHODS

Birds and diets

A total of 540 laying quails aged eight weeks were involved in this study. The laying quails were acclimatized for one week before experimental treatments. At this period, the initial egg mass of laying quails was recorded at 7.29 ± 0.72 g (coefficient of variation was 9.88%). The laying quails were randomly assigned to nine dietary treatments. Each treatment consists of four replicates with 15 laying quails per replicate. Dietary treatments consisted of three dietary ME (metabolizable energy) levels (2700, 2800, and 2900 kcal/kg) and three CP (crude protein) levels (17, 19, and 21%) in a factorial arrangement. The ingredients and nutrient composition of the dietary treatments are shown in Table 1. Diets were offered in mash form, with the amount of 30 g/bird/day. Drinking water was provided ad libitum throughout the experimental period. The feeding trial was conducted for five weeks.

Laying performance

Feed intake (g/bird/day) was calculated by a formula = offered feed - refused feed (Habsari et al. 2018). Egg production (%) was calculated by a formula = (number of egg/number of laying quails) $\times$ 100% (Sartika & Iskandar, 2019). A digital balance determines egg weight (g) (Edi et al. 2018). Egg mass (g/bird/day) was calculated by a formula = egg production $\times$ egg weight (Dijkslag et al. 2021). Finally, the feed conversion ratio was calculated by a formula = feed intake/egg mass (Leke et al. 2022).

Statistical analyses

Data were presented as means followed by the standard error of means. Data were analyzed using univariate variance analysis, with ME and CP levels regarded as fixed factors. When significant differences were found ($P < 0.05$), Duncan's tests were performed to separate means. Statistical analyses were performed by using IBM SPSS Statistics Version 22.

RESULTS AND DISCUSSION

As can be seen in Table 2, interaction ($P < 0.05$) between metabolizable energy and crude protein levels was recorded on feed intake. Feed intake was reduced along with the increase in the metabolizable energy when crude protein levels were 17 and 19%. However, the feed intake tended to be improved when high metabolizable energy was combined with high crude protein contents (2900 kcal/kg and 21%, respectively)..

Table 1. Ingredient and nutrient composition of the experimental diets

Item	2700 kcal/kg ME			2800 kcal/kg ME			2900 kcal/kg ME		
	17% CP	19% CP	21% CP	17% CP	19% CP	21% CP	17% CP	19% CP	21% CP
Ingredient (%)									
Corn	49.26	44.68	42.03	47.77	48.00	42.87	55.79	51.37	48.21
Rice bran	18.00	16.29	13.13	17.41	11.81	10.52	8.19	6.73	3.71
Soybean meal	16.43	22.44	28.00	16.47	21.84	27.52	17.27	22.41	28.00
Meat bone meal	7.61	7.73	8.32	7.90	8.93	9.28	8.53	9.30	9.90
Crude palm oil	0.20	0.47	0.31	2.00	1.24	1.65	1.77	2.00	2.00
Limestone	3.73	3.70	3.61	3.70	3.60	3.60	3.70	3.60	3.60
Grit	3.73	3.70	3.61	3.70	3.60	3.60	3.70	3.60	3.60
Sodium	0.08	0.07	0.07	0.07	0.07	0.06	0.09	0.07	0.07
Toxinil	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Salt	0.11	0.11	0.11	0.11	0.10	0.10	0.11	0.10	0.10
Choline	0.04	0.02	0.02	0.04	0.02	0.02	0.04	0.03	0.02
L-Lysine	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
DL-Methionine	0.13	0.11	0.10	0.14	0.11	0.10	0.14	0.11	0.10
Premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Nutritional content based on calculations (%)									
ME (kcal/kg)	2700	2700	2700	2800	2800	2800	2900	2900	2900
CP	17.00	19.00	21.00	17.00	19.00	21.00	17.00	19.00	21.00
Calcium	3.60	3.60	3.60	3.60	3.63	3.67	3.65	3.66	3.72
Phosphorus	0.90	0.90	0.90	0.90	0.88	0.90	0.80	0.83	0.83
Lysine	0.89	1.03	1.15	0.89	1.02	1.15	0.89	1.02	1.15
Methionine	0.40	0.40	0.42	0.40	0.40	0.41	0.40	0.40	0.41

ME= metabolizable energy, CP= crude protein

No interaction ($P>0.05$) was recorded on the other variables (egg production, egg weight, egg mass, and feed conversion ratio). As a single factor, different metabolizable energy levels had a significant effect ($P<0.05$) on the feed conversion ratio of laying quails. Diets containing 2900 kcal/kg metabolizable energy had better results ($P<0.05$) on feed conversion ratio than those fed diets containing 2,700 kcal/kg metabolizable energy. However, it was observed that different metabolizable energy levels did not significantly affect ($P>0.05$) egg production, weight, and mass of laying quails.

In line with the current findings, de Freitas et al. (2005) also found that the increase in energy level caused a linear reduction in feed intake. It was also reported that diets with varying metabolizable energy levels also had a significant effect on the feed intake of quails, in which the diet with high metabolizable energy

was associated with reduced feed intake (Jahanian & Edriss 2015). In a study by Ratriyanto et al. (2017), high metabolizable energy also reduced the feed intake of laying quails. The reduction of feed intake due to the higher metabolizable energy content in the feed is probably related to the fact that the feeding activity of birds was aimed at fulfilling the energy requirement. Therefore, when the energy requirement is fulfilled, the birds will stop feeding activity so that the feed intake is adjusted according to the energy content in the feed (Fonseca et al. 2021). The reduction of feed intake due to the increment of dietary energy is also reported in other poultry, such as laying hens (Kang et al. 2018; Granghelli et al. 2019) and ducks (Xuang et al. 2018; Awad et al. 2022).

In this study, egg production, weight, and mass did not differ among varying metabolizable energy levels. This finding confirmed the previous study by Agboola

Table 2. Effects of feeding diets with different metabolizable energy and crude protein levels on performances of laying Japanese quails

Treatments	FI (g/b/d)	EP (%)	EW (g)	EM (g/b/d)	FCR
ME (kcal/kg)					
2700	25.32 ^b	77.08	10.63	8.20	3.17 ^b
2800	24.37 ^a	79.36	10.85	8.23	3.03 ^{ab}
2900	24.37 ^a	79.39	10.50	8.36	2.99 ^a
CP (%)					
17	23.49 ^c	72.33 ^c	10.20 ^c	7.18 ^c	3.33 ^e
19	24.65 ^d	78.17 ^d	10.85 ^d	8.28 ^d	3.03 ^d
21	25.93 ^e	85.32 ^e	10.94 ^e	9.33 ^e	2.82 ^c
ME x CP					
2700 x 17	23.83 ^{gh}	70.97	10.21	7.20	3.38
2800 x 17	23.49 ^{fg}	76.08	10.24	7.23	3.30
2900 x 17	23.14 ^f	69.95	10.15	7.09	3.33
2700 x 19	26.14 ^j	77.05	10.70	8.25	3.23
2800 x 19	24.09 ^h	77.15	11.35	8.15	3.00
2900 x 19	23.72 ^{gh}	80.33	10.51	8.45	2.85
2700 x 21	26.00 ^j	83.23	10.98	9.15	2.90
2800 x 21	25.53 ⁱ	84.84	10.97	9.31	2.79
2900 x 21	26.25 ^j	87.90	10.86	9.54	2.78
Pooled SEM	0.21	1.32	0.11	0.16	0.05
P-value					
ME	<0.001	0.575	0.328	0.620	0.036
CP	<0.001	<0.001	0.006	<0.001	<0.001
ME x CP	<0.001	0.544	0.629	0.726	0.424

ME= metabolizable energy, CP= crude protein, FI= feed intake, EP= egg production, EW= egg weight, EM= egg mass, FCR= feed conversion ratio. ^{a,b} different superscripts within a column indicate significant differences ($P < 0.05$); ^{c,d,e} different superscripts within a column indicate significant differences ($P < 0.05$); ^{f, g, h, i, j} different superscripts within a column indicate significant differences ($P < 0.05$)

et al. (2016), who also found that diets with varying metabolizable energy had no significant effect on hen day production and egg weight of laying quails. Similarly, Hijab & Albaddy (2022) also found that diets with varying metabolizable energy did not significantly alter laying quails' hen day production and egg weight. In another study, Ratriyanto et al. (2018) also found that the diet's high and low metabolizable energy contents provide equal egg mass in laying quails.

This study found that the high metabolizable energy provides a better feed conversion ratio of laying quails than the low metabolizable energy. In line with this study, Ratriyanto et al. (2018) also observed that feeding high metabolizable energy diets significantly A

study by Fonseca et al. (2021) also reported that improved feed efficiency compared to feeding low metabolizable energy diets.

feeding diets with high energy content resulted in a better feed conversion ratio than feeding diets with low energy content. Therefore, the better feed conversion ratios reflect the lower feed intake due to the high metabolizable energy content in the diets.

Table 2 shows that the crude protein as a single factor significantly affects ($P < 0.05$) egg production, egg weight, egg mass, and feed conversion ratio of laying quails. The increase in crude protein levels from 17, 19, to 21% gradually improved ($P < 0.05$) egg production, weight, and mass but reduced feed conversion ratio.

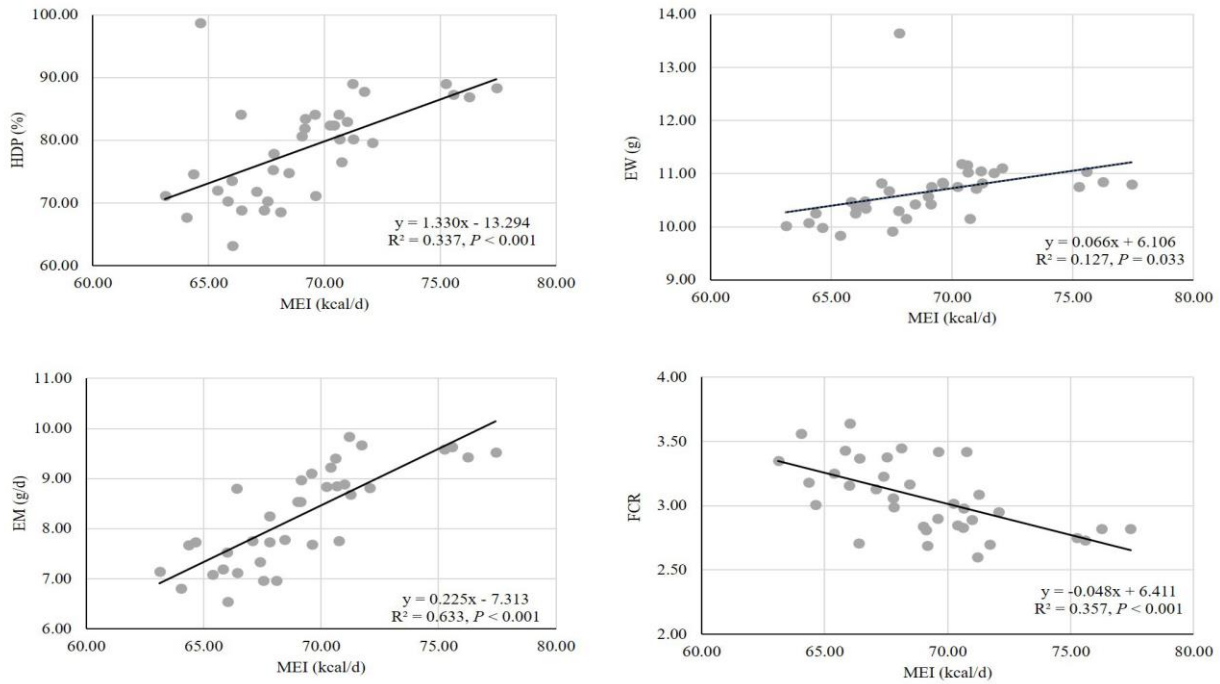


Figure 1. Linear regression of metabolizable energy intake (MEI) on hen day production (HDP), egg weight (EW), egg mass (EM), and feed conversion ratio (FCR)

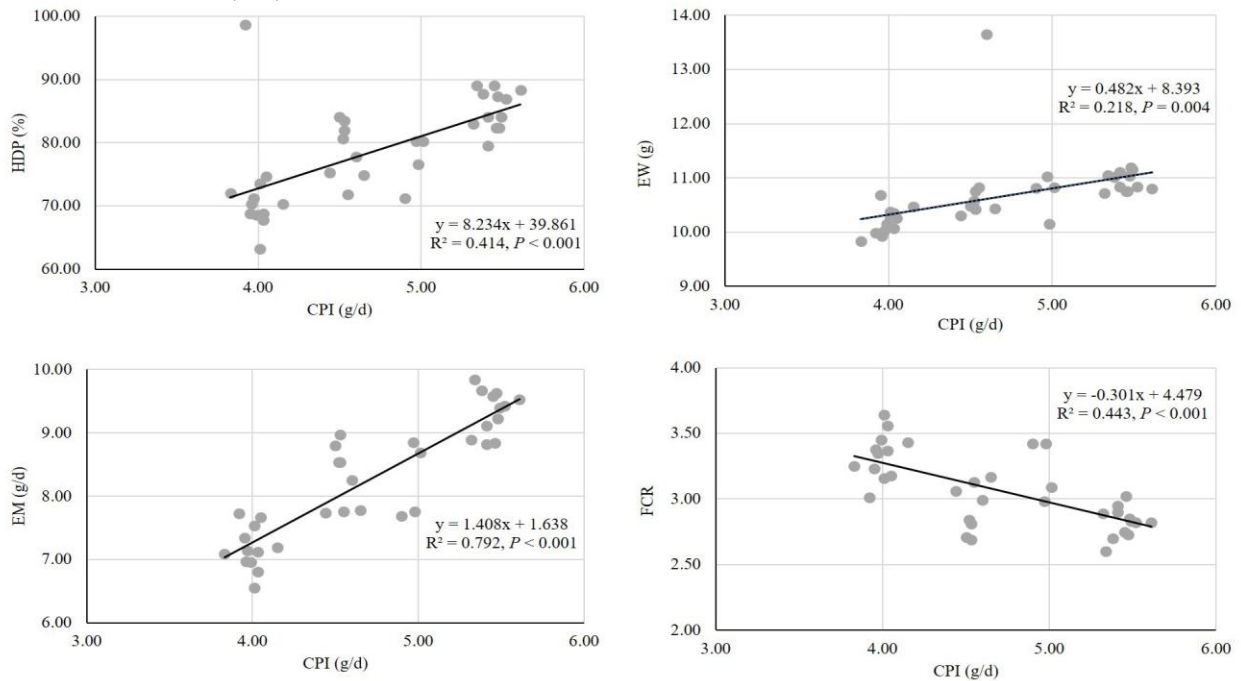


Figure 2. Linear regression of crude protein intake (CPI) on hen day production (HDP), egg weight (EW), egg mass (EM), and feed conversion ratio (FCR)

In line with this finding, El-Hindawy et al. (2021) also reported that the feeding diets with high crude protein contents resulted in higher egg production, egg weight, egg mass, and feed conversion ratio of laying quails. Jesuyon et al. (2021) also found that increasing crude protein levels in the diets increased the egg weight of laying quails. In another study, Salih et al. (2021) also

noted that the higher crude protein contents in the diets resulted in higher egg production, egg mass, and feed conversion ratio. The protein supply from the diets is essential for egg formation synthesis (Macelline et al. 2021; Mnisi et al. 2022); the higher crude protein contents could provide better performance of laying quails.

Regression analyses between metabolizable energy and crude protein intakes on egg production, egg weight, egg mass, and feed conversion ratio of laying quails were also undertaken. The results showed that metabolizable energy intake linearly improved egg production, weight, mass, and feed conversion ratio (Figure 1). Similarly, crude protein intake linearly improved egg production, weight, mass, and feed conversion ratio (Figure 2). Previously, Bouvarel et al. (2010) also showed that a higher energy intake could improve laying performance. In addition, Gunawardana et al. (2008) also observed that increased crude protein intakes could improve laying performance in another study. This finding indicated that metabolizable energy and crude protein were critically important in supporting the production performance of laying quails.

In this study, a diet containing 2900 kcal/kg metabolizable energy and 21% crude protein provides the optimum performance of laying quails. However, the high energy content in the feed could reduce feed intake (Fonseca et al. 2021), and consequently, the reduction of other nutrient intakes will follow it. Therefore, high crude protein should be followed by high-energy diets to fulfill the protein requirement for optimum performance. In addition, feeding high-energy and protein diets could support estrogen synthesis, promoting egg formation and thus improving the production performance of laying quails (Lotfi et al. 2018).

CONCLUSION

According to the results of the current study, feeding diets containing 2900 kcal/kg metabolizable energy and 21% crude protein are required to provide optimum performance of laying quails.

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Quality of Physical and Sensory of Super-native Chicken Breast Marinated with Herbs and Spices with Different Levels of Marination Concentration

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ABSTRAK

Oktafa H, Prayitno AH, Handayani HT. 2023. Kualitas fisik dan sensori daging dada ayam kampung super yang dimarinasi dengan konsentrasi marinasi yang berbeda. JITV 28(1):76-85. DOI: <http://dx.doi.org/10.14334/jitv.v28i1.3092>.

Ayam kampung super adalah ayam hasil persilangan antara ayam kampung jantan dan ayam petelur. Diversifikasi produk olahan daging ayam kampung super dapat dikembangkan menjadi produk *ready to cook* dengan cara dimarinasi bumbu dan rempah lokal. Penelitian ini bertujuan untuk mengetahui pengaruh konsentrasi marinasi bumbu dan rempah lokal yang berbeda terhadap kualitas fisik dan sensori daging ayam kampung super. Materi penelitian terdiri atas daging dada ayam kampung super, bubuk cabai, gula, garam, lada, bawang putih, kayu manis, minyak kelapa sawit, air jeruk nipis, monosodium glutamat, dan jahe. Perlakuan level konsentrasi marinasi yaitu 10, 20, dan 30% dari bobot daging. Parameter kualitas fisik yang diuji yaitu warna, nilai pH, kadar air, susut masak, daya ikat air, rendemen, dan keempukan. Setiap perlakuan terdiri dari 3 ulangan. Kualitas sensori diuji dengan uji mutu hedonik oleh 40 panelis tidak terlatih. Parameter mutu hedonik yang diuji yaitu warna, aroma, rasa, tekstur, keempukan, dan *juiciness*. Data hasil uji kualitas fisik dianalisis dengan analisis variansi pola searah dan hasil uji mutu hedonik dianalisis dengan analisis non parametrik melalui uji Hedonic Kruskal-Wallis dan jika terdapat perbedaan yang nyata ($P < 0,05$) diuji lanjut dengan uji Duncan's New Multiple Range Test. Hasil penelitian menunjukkan bahwa level konsentrasi marinasi bumbu dan rempah lokal yang berbeda dapat mempengaruhi kualitas fisik (*redness*, nilai pH, dan kadar air) dan sensori (warna dan tekstur) daging dada ayam kampung super. Konsentrasi marinasi bumbu dan rempah lokal level 20% merupakan perlakuan terbaik berdasarkan mutu hedonik menurut penilaian panelis karena memiliki tekstur daging yang halus dengan skor 3.98.

Kata Kunci: Rempah Lokal, Daging Dada, Kualitas Fisik, Marinasi, Sensori

ABSTRACT

Oktafa H, Prayitno AH, Handayani HT. 2023. The physical and sensory quality of super-native chicken breast marinated with herbs and spices with different levels of marination concentration. JITV 28(1):76-85. DOI: <http://dx.doi.org/10.14334/jitv.v28i1.3092>.

Super-native chickens result from crosses between male native chickens and laying hens. The diversification of processed products from super-native chicken can be developed into ready-to-cook products by marinating them with local herbs and spices. This research was conducted on the effect of marination concentrations of different local herbs and spices on the hedonic quality of super-native chicken breast. The research material consisted of super-native chicken breast, chili powder, sugar, salt, pepper, garlic, cinnamon, palm oil, lime juice, monosodium glutamate, and ginger. The treatment levels of marination concentration were 10, 20, and 30% of the weight of the meat. Physical quality parameters tested were color, pH value, moisture, cooking loss, water-holding capacity, yield, and hardness. Each treatment consisted of 3 replications. In addition, sensory quality was tested by a hedonic quality test by 40 untrained panelists. The hedonic quality parameters tested were color, flavor, taste, texture, tenderness, and juiciness. The data from the physical quality test were analyzed by analysis of variance. The hedonic quality test results were analyzed by non-parametric analysis through the Kruskal-Wallis hedonic test. If there was a significant difference ($P < 0.05$), the test was continued with Duncan's New Multiple Range Test. The results showed that the concentration level of different local herbs and spices marination could affect the physical quality (*redness*, pH value, and moisture) and sensory (color and texture) of super-native chicken breast. The marination concentration of local herbs and spices at 20% level was the best treatment based on hedonic quality with a smooth meat texture and a score of 3.98.

Key Words: Local Spices, Breast Meat, Physical Quality, Marination, Sensory

INTRODUCTION

Super-native chickens result from crosses between male native chickens and laying hens (Pakaya et al.

2019). Super-native chickens are already known by the Indonesian people as a meat producer with a slaughter weight of around 1.2 kg (Jacob et al. 2019). Therefore, maintaining super-native chickens is relatively more

manageable, and the nutritional requirements do not have to be high-cost (Aziz 2019). Super-native chicken is trendy for consumption by the people of Indonesia because it is known to be tastier and lower in fat and cholesterol. Super-native chicken has a high nutritional content and a delicious taste resembling native chicken (Lestari et al. 2022). In addition, super-native chickens require a shorter maintenance time when compared to native chickens, which is around 60-65 days to get a weight of about 1 kg (Aziz 2019).

The percentage of the super-native chicken carcass is around 68.40-70.08% (Tahalele et al. 2018). Super-native chicken meat as a source of Indonesian animal germplasm continues to be developed into ready-to-cook products. The demand for super-native chicken products in a ready-to-cook state continues to increase (Jacob et al. 2019; Lestari et al. 2022) due to the Covid-19 pandemic. Ready-to-cook (RTC) products that are currently trending are marinated chicken products. Ready-to-cook products have become popular with the Indonesian people because of restrictions during the Covid-19 pandemic, making it easier for the cooking process.

Chicken meat and its products are widely liked and consumed by the community because they are rich in the nutrients they need (Zelpina et al. 2020). The meat industry has for decades been applying marinade technology (O'Neill et al. 2019). Marinating technology is soaking meat in a seasoned liquid called marinade (Augustyńska-Prejsnar et al. 2023). A marinade is a seasoned solution that functions as a meat marinade, usually used to improve the taste, impression of juice, and tenderness of meat after cooking (Sengun et al. 2021). The role and perception of the marinade have changed from being a flavoring and tenderizing agent to increasing meat yield and quality. A marinade is also applied to meat products as a preservative and to enhance color (O'Neill et al. 2019). In addition, marinating meat can increase its tenderness of meat (Latoch 2020).

Marinade ingredients that can be used are local herbs and spices, widely available in Indonesia as a source of local potential. Local herbs and spices such as chili powder, sugar, salt, pepper, garlic, cinnamon, palm oil, lime juice, and ginger have been extensively studied separately for marinades and flavorings in meat processing. Marinating technology can also increase food safety and add value to the product (Nurwantoro et al. 2012). The principle of marinating technology is soaking meat in a marinade containing certain ingredients so that slow passive transport of the marinade material into the meat by osmosis occurs (Sengun et al. 2021). The increase in the taste and tenderness of the meat due to the marinade process is caused by the rise in the water-holding capacity of the meat (Patriani & Hafid 2021).

Applying marinating technology to meat can improve the panelists' taste, tenderness, and acceptability (Gómez et al. 2020). Local herbs and spices have the potential to be used simultaneously as ingredients for marinating super-native chicken. Breast meat is one of the most preferred parts of meat by some consumers (Devatkal et al. 2019). Chicken breast meat is usually marinated to increase the tenderness and juiciness of the meat (Singh & Deshpande 2019). So far, no one has evaluated the application of local herbs and spices to the hedonic quality of super-native chicken breast. The hedonic quality test of a food ingredient is an indicator that can show consumer acceptance of the product (Harahap 2017). Therefore, this study was conducted to know the effect of marinating local herbs and spices with different concentrations on the physical quality and sensory of super-native chicken breast.

MATERIALS AND METHODS

Materials

The material used in this study consisted of super-native chicken breast, chili powder, sugar, salt, pepper, garlic, cinnamon, palm oil, lime juice, ginger, and monosodium glutamate (MSG). The tools used in this study consisted of a pH meter, color reader, oven, water bath, and texture analyzer.

Methods

This research consists of several stages. First, this research started with marinade preparation, meat preparation, marinating process, cooking, physical quality, and sensory evaluation.

Preparation of meat

A total of 20 kg of super-native chicken breast meat used in this study came from super-native chicken (unisex) obtained from local farms in Jember.

Preparation of marinade

The marinade formulation was prepared according to Oktafa et al. (2022). The marinade formulation used in this study used herbs and spices, which included: 12% chili powder, 12.25% sugar, 8% salt, 1% pepper, 17% garlic, 0.25% cinnamon, 25% palm oil, 10% lime juice, 12% MSG, and 2.5% ginger.

Table 1. Hedonic quality assessment score of chicken breast marinated with local herbs and spices with different concentrations.

Score	Variable					
	Color	Flavor	Taste	Texture	Tenderness	Juiciness
1	White	Very unscented	Not very tasty	Very rough	Not very tender	Very dry
2	Pale	Doesn't smell good	Not tasty	Rough	Not tender	Dry
3	Pink	Slightly delicious	Slightly tasty	Slightly smooth	Slightly tender	Slightly juicy
4	Bright red	Delicious	Tasty	Smooth	Tender	Juicy
5	Dark red	Very delicious	Very tasty	Very smooth	Very tender	So juicy

Marinating process

The marinating process was prepared according to Oktafa et al. (2021). Super-native chicken breast mixed with marinade with concentration levels: 10, 20, and 30% of the total weight of the meat. Marinate the chicken breast until it is evenly distributed. Vacuum-packed chicken breast, then marinated for 60 minutes.

Cooking process

The cooking process was made according to Oktafa et al. (2021). The marinated chicken breast was steamed for 45 minutes, and after the meat was cooked, it was cooled at room temperature before the panelists evaluated the hedonic quality.

Physical quality test

Physical quality parameters tested were color (Park et al. 2021), pH value, moisture (Zhang et al. 2018), cooking loss, water-holding capacity (Luckose & Pandey 2014), cooking yield (Niu et al. 2020), and hardness (Pöldvere et al. 2014).

Sensory quality test

The cooked, super-native chicken breast marinated was evaluated for hedonic quality by 40 untrained panelists, according to Sujarwanta et al. (2019). The hedonic quality parameters evaluated by the panelists were: color, flavor, taste, texture, tenderness, and juiciness (O'Neill et al. 2019). The hedonic quality assessment scores of chicken breast marinated with local herbs and spices with different concentrations were presented in Table 1.

Statistic analysis

The physical quality test was analyzed by analysis of variance. The hedonic quality test was analyzed by

analysis non-parametric test of Hedonic Kruskal-Wallis, and if there was a significant difference ($P < 0.05$), then further tested with Duncan's New Multiple Range Test (Zhang et al. 2018).

RESULTS AND DISCUSSION

Physical quality

Physical quality is one of the important factors that must be considered in producing marinated chicken meat. The physical qualities of marinated meat that need to be considered include color stability, pH value, water-holding capacity, and meat tenderness (Li et al. 2020). In addition, the moisture, cooking loss, and cooking yield of super-native chicken breast marinated were tested in this study. The data on the physical quality test results of super-native chicken breast marinated with local herbs and spices with different concentration levels are presented in Table 2.

Color

One of the qualities of marinated chicken meat is determined by the color of the meat. The results showed that the concentration level of marinade of different local herbs and spices had no significant effect ($P > 0.05$) on L^* (lightness) of marinated chicken meat. The L^* value indicates the lightness of the color of the marinated chicken meat. The L^* value of chicken meat marinated with local herbs and spices ranged from 48.59-50.53. The concentration level of marinade of different local herbs and spices resulted in marinated chicken meat with almost the same lightness of meat color.

The concentration level of marinade of different local herbs and spices significantly ($P < 0.05$) on the a^* (redness) of marinated chicken meat. The a^* value indicates the redness color of the marinated chicken meat. The a^* value of marinated chicken meat ranged from 12.75-15.24. The a^* value of marinated chicken increased with the higher concentration of local spices

and herbs. The highest a^* value was obtained from marinated chicken meat with a concentration level of 30% local spices and herbs marinade. This red color could be due to the chili powder's 30% marinade concentration level, which gave more red color than the 10% and 20% marinade concentration levels. Berry et al. (2019) stated that the color of chili powder is red. The carotenoid content influences the red color of chili powder.

The concentration level of marinade of different local herbs and spices had no significant effect ($P>0.05$) on the b^* (yellowness) of marinated chicken meat. The b^* value indicates the yellowish color of the marinated chicken meat. The b^* value of chicken meat marinated with local herbs and spices ranges from 17.56-18.73. Different levels of concentration of local spices and herbs marinade produce marinated chicken meat with almost the same yellow color as the meat.

Value of pH

The pH value is one factor that needs to be evaluated in processing processed meat products (Imran et al. 2016). The results showed that the concentration level of marinade of different local spices and herbs had a significant effect ($P<0.05$) on the pH value of chicken meat marinated with local spices and herbs. The pH value of chicken meat marinated with local herbs and spices ranges from 5.08-5.24. The pH value of marinated chicken meat is still following previous studies. The results of Ünal et al. (2020) reported that the pH value of marinated chicken meat was around 3.53-5.66.

The pH value is an essential criterion for changing the quality of marinated meat. The lowest pH value was obtained from chicken meat with a concentration level of 30% local marinade and spices. This low pH value could be due to the acidity of local herbs and spices at a marinade concentration of 30% lower than the marinade concentration level of 10 and 20%. Ünal et al. (2020) reported that the difference in the pH value of marinated chicken meat was due to the difference in the pH value of the marinade used. Vlahova-Vangelova & Dragoev (2014) stated that the herbs and spices used as marinades are slightly acidic because they contain polyphenolic compounds.

Moisture

The moisture contained in meat has a significant role in the quality and has economic value for the meat processing industry. The concentration level of marinade of different local spices and herbs had a significant effect ($P<0.05$) on the moisture of marinated chicken meat. The value of moisture of chicken meat

marinated with local herbs and spices ranges from 65.25-71.28%. The value of the moisture of marinated chicken meat is still following previous studies. The results of Gök & Bor (2016) reported that the moisture of marinated chicken meat ranged from 58.85-70.51%. The value of the moisture of the marinated chicken meat decreases as the pH value decreases.

The lowest moisture value was obtained from chicken meat with a concentration level of 30% local marinade and spices. This low moisture could be due to the low pH value of the marinated chicken meat. Ünal et al. (2020) reported that a low pH value causes the meat structure to open, generating more moisture. Therefore, the lower pH value of marinated chicken meat contributes to insufficient water-holding capacity and more water loss.

Water-holding capacity

The water-holding capacity of meat is essential because many physical properties of meat are related to water-holding capacity, such as color and tenderness (Ketnawa & Rawdkuen 2011). The results showed that the concentration level of marinade of different local spices and herbs did not affect the water-holding capacity of marinated chicken meat. The water-holding value of chicken meat marinated with local herbs and spices ranges from 33.06-36.97%. Therefore, the water-holding capacity of marinated chicken meat is higher than in previous studies. The results of Ünal et al. (2020) reported that the water-holding capacity of marinated chicken meat was around 15.14-28.67%.

The concentration level of marinade of different local herbs and spices resulted in marinated chicken meat with almost the same water-holding capacity. Increasing the level of marinade concentration did not change the water-holding capacity of the marinated chicken. The water-holding capacity relates to pH value (Prayitno et al. 2022). The results showed that the water-holding capacity of chicken meat marinated with local herbs and spices decreased as the pH value decreased (Table 2). However, the decrease in the water-holding capacity with different concentration levels of local herbs and spices has the same effect.

Cooking loss

Cooking loss describes the loss of liquid and solutes from meat during cooking and is an essential factor in the meat processing industry (Pathare & Roskilly 2016). The concentration level of different local herbs and spices marinade did not affect the cooking loss of marinated chicken meat. The cooking loss value of marinated chicken meat with local herbs and spices ranges from 28.09-32.30%. The cooking loss value of

marinated chicken meat is still following previous research. The results of Ünal et al. (2020) reported that the cooking loss of marinated chicken meat ranged from 15.19-37.81%. The cooking loss value of marinated chicken meat increased with decreased water-holding capacity (Table 2). However, the increase in cooking loss with different concentration levels of local herbs and spices has the same effect.

The highest cooking loss value was obtained from chicken meat with a concentration level of 30% local marinade and spices. On the other hand, the lowest was from chicken meat with a 10% local spice and marinade concentration. However, the value of cooking loss differs from the concentration level of different local herbs and spices marinade. However, it has the same effect.

Cooking yield

The cooking yield describes the change in the weight of cooked chicken meat due to water loss during the cooking process. The concentration level of marinade of different local spices and herbs did not affect the cooking yield of marinated chicken. The cooking yield value of chicken meat marinated with local herbs and spices ranges from 67.70-71.91%. The cooking yield of marinated chicken can be affected by the cooking loss value. The cooking yield value of marinated chicken meat decreases along with the increasing cooking loss value (Table 2). However, the reduced cooking yield value of marinated chicken meat with different local herbs and spices concentration levels has the same effect.

The highest cooking yield value was obtained from chicken meat with a concentration level of 10% local marinade and spices. On the other hand, the lowest cooking yield value was obtained from chicken meat with a 30% local spice and marinade concentration level. However, the value of cooking yield differs from the concentration level of different local herbs and spices marinade. However, it has the same effect.

Hardness

Hardness is one of the texture properties of meat which is considered an essential attribute of meat consumption (Pathare & Roskilly 2016). The concentration level of marinade of different local spices and herbs did not affect the hardness of the marinated chicken meat. The hardness value of marinated chicken meat and local spices ranges from 79.87-83.58 N. Hardness of marinated chicken meat can be influenced by the value of water-holding capacity. The hardness value of the marinated chicken meat increased along with the decreasing value of the binding capacity (Table 2). However, the increased hardness value of marinated

chicken meat with different concentration levels of local herbs and spices has the same effect.

The hardness value of marinated chicken meat is still following previous research. The results of Ünal et al. (2020) reported that the hardness value of marinated chicken ranged from 60.28-134.18 N. The highest hardness value was obtained from chicken meat with a concentration level of 30% local marinade and spices. The lowest hardness value was obtained from chicken meat with a local marinade and spices concentration level of 10%. However, the hardness value has differences with the concentration level of marinade of different local herbs and spices. However, it has the same effect.

Sensory quality

The hedonic quality test was one of the sensory characteristics of meat products mainly carried out to determine the level of acceptance from panelists of the meat products produced (Prasetyo & Prayitno 2021). The results of the hedonic quality test that have been evaluated by the panelists on super-native chicken breast cooked with local herbs and spices with different concentration levels are presented in Table 3.

Color

Meat color is one of the quality parameters of meat and its processed products (Thangavelu et al. 2019). Color also affects consumer acceptance of the product, where color is the first parameter seen (Saputro & Susanto 2016). The results showed that the concentration level of marinade of different local spices and herbs had a significant effect ($P < 0.05$) on the color of the marinated breast meat. The hedonic quality score of the color of the marinated breast meat ranged from 3.28-3.85, from pink to bright red. The color of the breast meat that had been marinated with local spices and herbs at the marinade concentration level of 10 and 20% did not change significantly, while at the 30% marinade concentration level it had different color changes according to the results of the evaluation by the panelists. This color change could be because, at the 30% marinade concentration level, the chili powder level, which gives a red color, was more than the 10 and 20% marinade concentration levels.

In this study, panelists could significantly distinguish the color of breast meat marinated with local herbs and spices with different levels of marinade concentration visually. The color of the super-native chicken breast can be influenced by the spices used as a marinade, namely red chili powder. Berry et al. (2019) stated that the color of chili powder is red. In addition, the carotenoid content influenced the color of chili powder. Perception of meat color can influence p'anelists'

Table 2. The physical quality of super-native chicken breast marinated with herbs and spices with different levels of marinade concentrations

Variable	Marinade concentration level		
	10%	20%	30%
Color			
L^* ^{ns}	50.45±0.02	48.59±1.44	50.53±1.75
a^*	13.68±0.29 ^{ab}	12.75±1.61 ^a	15.24±0.44 ^b
b^* ^{ns}	17.56±2.33	18.73±0.14	18.32±0.96
Value of pH	5.24±0.03 ^a	5.11±0.05 ^b	5.08±0.06 ^b
Moisture (%)	71.28±0.11 ^c	69.11±0.49 ^b	65.25±1.28 ^a
Water-holding capacity (%) ^{ns}	36.97±2.44	34.47±0.85	33.06±2.56
Cooking loss (%) ^{ns}	28.09±2.97	29.24±4.07	32.30±6.47
Cooking yield (%) ^{ns}	71.91±2.97	70.76±4.07	67.70±6.48
Hardness (N) ^{ns}	79.87±2.96	78.12±3.07	83.58±4.98

^{ns}= Not significant, ^{a-d}, different superscripts at the same row indicate significant differences (P<0.05)

Table 3. The hedonic quality of super-native chicken breast marinated with herbs and spices with different levels of marinade concentrations

Variable	Marinade concentration level		
	10%	20%	30%
Color	3.28±1.13 ^a	3.30±1.13 ^a	3.85±1.05 ^b
Flavor ^{ns}	3.73±0.67	3.78±1.12	3.65±0.76
Taste ^{ns}	3.70±0.82	3.90±0.77	3.78±0.99
Texture	3.53±0.50 ^a	3.98±0.47 ^b	3.73±0.67 ^a
Tenderness	3.70±0.46	3.70±0.82	3.70±0.64
Juiciness ^{ns}	3.38±0.58	3.53±0.71	3.60±0.74

^{ns}= Not significant, ^{a-d}= Different superscripts at the same row indicate significant differences (P<0.05)

panelists' decisions in choosing meat and its processed products (Torrico et al. 2018).

Visually, the panelists were thought to have almost the same perception of the color of the super-native chicken breast, which has been marinated with local herbs and spices at the marinade concentration level of 10 and 20%, while at the 30% marinade concentration level, it shows a significant color change so that the panelists' assessment of the quality score the color of the chicken meat that has been marinated becomes higher.

Flavor

Meat flavor is one of the critical factors in consumer acceptance. Poultry meat flavor is naturally formed through specific processes, such as heating, where various complex chemical reactions occur between nonvolatile precursors from fat tissue and lean tissue

(Purba 2014). The results showed that the concentration level of different local spices and herbs of marinade did not affect marinated breast meat's flavor. The hedonic quality score for the flavor of the marinated breast meat ranged from 3.65-3.78, from slightly delicious to delicious.

Panelists' assessment of the hedonic quality of the aroma of breast meat marinated with local herbs and spices with different levels of marinade concentration did not experience a significant difference; this could be because the panelists had difficulty distinguishing the aroma of breast meat marinated with local herbs and spices at different levels of marinade concentration by smelling it. The flavor of the meat is produced from a combination of various components that stimulate the olfactory receptors in the nasal passages (Torrico et al. 2018), while the flavor-forming compounds derived from local herbs and spices as a marinade for super-native chicken breast with different concentration levels were thought to have almost the same flavor after being

smelled by the panelists, based on this perception, the panelist's assessment of the hedonic quality score of the marinated breast meat flavor was not different for each treatment.

Taste

The taste of meat is a sensory quality of meat related to the sense of taste of consumers. The main flavor components of processed meat, namely in the form of volatile and nonvolatile components, significantly influence the acceptance of processed meat, especially the taste of meat (Purba 2014). The results showed that the concentration level of different local spices and herbs of marinade did not affect the marinated breast meat's taste. The hedonic quality score of the marinated breast meat ranged from 3.70-3.90, from slightly tasty to tasty; this follows the opinion of Melda et al. (2013) which states that the taste of chicken meat is between slightly-tasty to tasty.

Panelists' assessment of the taste of breast meat that had been marinated with local herbs and spices with different levels of marinade concentration did not experience a significant difference; this could be because the panelists had difficulty distinguishing the taste of the marinated breast meat from the marinade of local spices and herbs at different concentration levels by the way they were felt so that increasing the concentration of the marinade of local herbs and spices did not affect the panelists' taste assessment. In addition, the taste of the meat is produced from a combination of various components that stimulate the taste receptors on the tongue (Torrico et al. 2018), while the specific flavoring compounds derived from local herbs and spices as a marinade for super-native chicken breast with different levels of concentration are thought to have almost the same taste after being felt by the panelists, based on that perception, the panelist's assessment of the hedonic quality score of the taste of the marinated meat did not differ for each treatment.

Texture

The texture is a sensory property of meat related to its smoothness of the meat. The texture is also an important parameter of meat and its products that can affect consumer acceptance (Latoch 2020). The results showed that the concentration level of marinade of different local spices and herbs had a significant effect ($P < 0.05$) on the texture of the marinated breast meat. The hedonic quality score of the marinated breast meat texture ranged from 3.53-3.98, which was from slightly smooth to smooth.

In this study, panelists could significantly distinguish the texture of breast meat marinated with

local herbs and spices with different levels of marinade concentration when the meat was chewed; this is because the panelists were able to distinguish the texture of the breast meat that had been marinated with local herbs and spices at different levels of marinade concentration based on the level of softness when chewing the meat for each treatment. Panelists are thought to have different perceptions of the texture of the meat for each treatment even though they use the same piece of meat, namely the breast, which is known to have a smooth texture (Devatkal et al. 2019) that had been marinated differently for each treatment.

Tenderness

Tenderness is the main parameter in determining the meat quality that can be tested through sensory tests (Hussein et al. 2019). According to Dunshea et al. (2021), meat's tenderness can be sensory by chewing. The results showed that the concentration level of marinade of different local spices and herbs did not affect the tenderness of the marinated breast meat. The hedonic quality score of the tenderness of the marinated breast meat is 3.70, from slightly tender to tender. Therefore, tenderness is essential to meat quality (Lapase et al. 2016). According to Oktafa et al. (2022), the overall tenderness of meat involves three aspects: the ease of initial penetration of the teeth, the ease with which the meat is chewed, and the amount of residue left after mastication.

Panelists' assessment of the tenderness of breast meat marinated with local herbs and spices with different levels of marinade concentration did not experience a significant difference; this could be because the panelists had difficulty distinguishing the tenderness of the breast meat which had been marinated with local herbs and spices at different levels of marinade concentration when biting the meat. Furthermore, panelists are thought to have almost the same perception of the level of tenderness of the meat used in this study, namely using breast meat which is known to be tender (Devatkal et al. 2019); so based on this perception, the panelist's assessment of the hedonic quality score of the tenderness of the super-native chicken breast marinated was not different for each treatment.

Juiciness

Juiciness is a sensory property related to the meat's wetness level (Hussein et al. 2019). The results showed that the concentration level of different local herbs and spices marinade did not affect the marinated breast meat's juiciness. The hedonic quality score of the juiciness of the marinated breast meat ranged from 3.38-3.60, from slightly watery to watery. According to

Zhuang & Savage (2013), juiciness is the amount of water from the meat sample during the first five chews. Escobedo Del Bosque et al. (2020) juiciness is the fluid expelled during the first three chews. Juiciness is also defined as water perceived during mastication (Damaziak et al. 2019). The juiciness of meat can also affect individual taste perception (Liu et al. 2022).

Panelists' assessment of the juiciness of breast meat marinated with local herbs and spices with different levels of marinade concentration did not experience a significant difference; this could be because the panelists had difficulty distinguishing the juiciness level of breast meat that had been marinated with local herbs and spices at different levels of marinade concentration when chewing the meat. Panelists are thought to have almost the same perception of the juiciness level of the meat in this study, so based on this perception, the panelists' assessment of the hedonic quality score of the juiciness of marinated super-native chicken breast was not different for each treatment.

CONCLUSION

This study concludes that the concentration level of marinade of different local herbs and spices could affect the physical quality (redness, pH value, and moisture) and sensory (color and texture) of super-native chicken breast. The marinade concentration of local herbs and spices at 20% level was the best treatment based on hedonic quality with a smooth meat texture with a score of 3.98. Based on physical quality, no more water loss during the marinating and cooking.

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Farmers' Perceptions and Behavior which Affect the Adoption Rate of KUB Chickens

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ABSTRAK

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Ayam KUB sebagai bibit unggul yang dihasilkan Badan Penelitian dan Pengembangan Pertanian (Balitbangtan) menjadi salah satu solusi dalam upaya peningkatan kebutuhan protein hewani. Bibit ayam KUB telah berhasil didiseminasikan ke seluruh provinsi di Indonesia. Dengan demikian tingkat adopsi dan perilaku peternak dalam mempertahankan keberlanjutan pemeliharaan ayam KUB perlu dianalisis karena bibit yang didiseminasikan belum tentu berhasil dikembangkan di suatu wilayah. Tujuan dari penelitian ini adalah menganalisis faktor persepsi dan perilaku peternak yang mempengaruhi tingkat adopsi teknologi ayam KUB di Provinsi Jawa Tengah, Indonesia. Penelitian ini akan menggabungkan antara teori "*Theory of Planned Behavior*" (TPB) dan "*Technology Acceptance Model*" (TAM). Teknik sampling ditentukan dengan *multistage sampling* yaitu *purposive sampling* dan *accidental sampling*. Sebanyak 104 sampel diwawancara dan dianalisis dengan analisis jalur (path) dan regresi pada teknik *Structural Equation Modeling* (SEM). Berdasarkan hasil penelitian dapat disimpulkan bahwa variabel persepsi manfaat (25.3%), kemudahan memelihara ayam KUB (23.9%), sikap (21.2%), norma subjektif (16.7%) dan persepsi atas kontrol perilaku (17.3%) berpengaruh positif pada minat mengadopsi Ayam KUB. Selain itu persepsi manfaat (45.8%) dan persepsi kemudahan memelihara ayam KUB (50.1%) sangat berpengaruh positif terhadap variabel sikap peternak. Orang-orang penting termasuk anggota keluarga memiliki peran penting dalam mendukung pemeliharaan ayam KUB, sedangkan penyuluh belum banyak berkontribusi dalam mendukung peternak untuk mengadopsi ayam KUB. Kolaborasi dari kedua teori memiliki hubungan yang saling mempengaruhi khususnya terhadap sikap dan minat mengadopsi ayam KUB yang berefek positif terhadap tingkat adopsi. Tingkat adopsi ayam KUB adalah 3.32 (66.40%). Ini menunjukkan bahwa tingkat adopsi peternak di daerah Jawa Tengah, Indonesia diklasifikasikan sebagai adopsi tinggi.

Kata Kunci: Ayam KUB, Perilaku Peternak, Tingkat Adopsi, TAM, TPB

ABSTRACT

Syarifah I, Sirajuddin SN, Baba S, Najib M. 2023. Farmers' perceptions and behavior that affect the adoption rate of KUB chickens. JITV 28(1):84-98. DOI: <http://dx.doi.org/10.14334/jitv.v28.i1.3200>.

As a superior breed produced by Indonesian Agency for Agricultural Research and Development (IAARD), KUB chicken becomes one of the solutions to increase the need for animal protein. KUB chicken breeds have been successfully disseminated to all provinces in Indonesia. Thus, breeders' adoption rate and behavior in maintaining the sustainability of KUB chicken rearing needs to be analyzed because the disseminated breeds may need to be successfully developed in a particular area. This research will collaborate with two theories; the "Theory of Planned Behavior" (TPB) and the "Technology Acceptance Model" (TAM). It was conducted in June 2022 in the City of Salatiga, Semarang, and Magelang, Central Java Province. The determination of the research location was based on the consideration that a KUB chicken breeders group already had a KUB chicken breeder association called AnaKUB (KUB Chicken Breeders Association) and a breeding population that met the sampling requirements. This research aimed to analyze the perception factors and breeders' behavior that affect the adoption rate of KUB Chicken Technology in Central Java Province, Indonesia. The combined results of path analysis (path) and regression analysis on the Structural Equation Modeling (SEM) technique, it can be concluded that perceived benefits (25.3%), ease of raising KUB chickens (23.9%), attitudes (21.2%), subjective norms (16.7%) and perceptions of control behavior (17.3%) have a positive effect on the intention to adopt KUB chickens. The perceived benefits (45.8%) and the perceived ease of raising KUB chickens (50.1%) have a very positive effect on the attitude variable of the farmer. Important people, including family members, have an important role in supporting the maintenance of KUB chickens. In contrast, extension workers have yet to contribute much in supporting breeders to adopt KUB chickens. The collaboration of two theories has a mutually influencing relationship, especially towards attitudes and intentions to adopt KUB chickens, which positively affect the adoption rate. The adoption rate of KUB chicken is 3.32 (66.40%); this shows that the adoption rate of breeders in the Central Java region is classified as high adoption.

Key Words: Adoption Rate, Breeder Behaviour, KUB Chicken, TAM, TPB

INTRODUCTION

The adoption of an innovation is a mental process or behavior change in the form of knowledge (cognitive), attitude (affective), and skills (psychomotor) in a person since he knows the innovation (Rogers & Shoemaker 1971; Gebiso 2015). Adopting an invention is an internal process in farmers when they encounter an innovation. A new idea is implemented since it is known or heard until the innovation is implemented (Sirajuddin et al. 2017). Adoption is knowledge, persuasion, decision, implementation, and confirmation. So the speed of the adoption process will depend on the dynamic nature of the target.

The rate of adoption is the relative speed of the adoption of innovation conducted by members of a social system which is generally measured as the number of individuals who adopt the new idea in a certain period (Alomar & de Visscher 2017). The adoption rate is a numerical indicator of the steepness of the adoption curve for an innovation (Emerson 1995 in Rogers 2003). The study of agricultural technology adoption is significant in understanding the factors related to the application of technology (new plants, high superior quality, or new production technologies (Sudrajat 2020). In the history of agriculture, adopting agricultural technology is an essential component of agricultural development (Ghimire et al. 2015; Houeninvo et al. 2020). However, if the conditions of innovation are difficult to be implemented by the breeders and make the innovation hard to be adopted and breeders face various problems in managing their livestock business, which are pretty complex, it can hinder an optimal process of adopting technological innovations (Indraini & Sikombong 2014; Dwi et al. 2016; Baba et al. 2020).

Adopting KUB Chicken business innovations can be accepted quickly depending on the pattern and method of delivering technological innovations and regional situations and conditions. In addition, an essential determining factor is the characteristics of innovation in the KUB Chicken business, which consists of KUB DOC (day-old chicks), feed, cages, medicines, and equipment (Aminawar 2014; Dwi et al. 2016; Astarina 2019).

KUB chicken, a superior breed produced by Indonesian Agency for Agricultural Research and Development (IAARD), is one of the solutions to increase the need for animal protein (Hayanti 2014; Winarti 2018). KUB chicken breeds have been successfully disseminated in all provinces in Indonesia. However, the most disseminated breed is based on cooperation agreements with stakeholders or policyholders. Thus it is necessary to know the level of adoption and the behavior of breeders in maintaining the continuity of the KUB chicken business (Cahyono et al. 2020). Furthermore, it must be analyzed because the disseminated breeds may need to be successfully developed in an area.

In this research, a combination of the Theory of Planned Behavior (TPB) and the Technology Acceptance Model (TAM) is used (Borges et al. 2014; Johnson 2014; Ghimire et al. 2015; Borges et al. 2016; Lalani et al. 2016; Nugroho et al. 2018). Several studies on the factors influencing technology adoption related to perception and behavior refer to the Theory of Planned Behavior (TPB) and the Theory of the Technology Acceptance Model (TAM). For example, using TPB, researchers linked the adoption with attitudes toward behavior, subjective norms, and perceptions of behavior control (Ajzen 2005; Ghifarini 2018; Nugroho et al. 2018; Rodi et al. 2019; Ramadhan et al. 2020). In addition, many researchers have developed TAM theory to determine the effect of ease of usage, utility, satisfaction, perceived suitability, and attitudes toward the use (Nah et al. 2004; Ambodo et al., 2017).

In research on the adoption of KUB chickens, only a few discuss the factors that influence the adoption of KUB chickens (Nugroho et al. 2018; Ramadhan et al., 2020; Syarifah et al. 2021). However, several studies have explained the adoption of KUB chicken from individuals' cognitive, attitude, and psychomotor aspects after receiving innovation (Gebiso 2015; Altandjung 2019). In addition, electronic media can be used to analyze KUB chickens' adoption rate (Wahyuningrum & Gunawan 2016).

Research has never been conducted on factors influencing breeders to adopt KUB chickens in Central Java by combining the TAM and TPB theories (Tambunan Tulus 2001; Rauniar et al. 2014). However, as Lalani et al. (2016) and Iskandar (2018) suggested, the combination of the behavior aspects includes the perception of benefits and ease of raising KUB chickens by considering attitudes towards behavior, subjective norms, and perceptions of behavior control need to be assessed.

This research aimed to analyze the perception and behavior of breeders that affect the adoption rate of KUB chicken technology in Central Java Province, Indonesia.

MATERIALS AND METHODS

This research was conducted in June 2022 in the City of Salatiga (34), Semarang (30), and Magelang (40), Central Java Province, Indonesia, with a total sampling of 104 respondents. The research location was chosen due to the KUB chicken group associated with KUB chicken breeders named AnaKUB (KUB Chicken Breeders Association). The breeding population also met the sampling requirements. The research was conducted by interviewing KUB Chicken Breeders and collecting primary data by completing the questionnaires. In this study, the adoption rate of KUB chicken by breeders was examined using descriptive research and a quantitative approach. In addition, data

analysis techniques were utilized to investigate breeders' perceptions and behavior concerning the study's factors/variables. The variables in this study were KUB chicken groups in Central Java regarding the perceived benefits of raising KUB chickens and the perceived ease of raising KUB chickens using the TAM theory by considering the variables: attitudes toward behavior and subjective norms. On the other hand, the TPB theory was used to determine the intention to adopt the innovation and the perception of behavioral control (Ajzen 2006; Borges et al. 2014).

Data collection techniques

The researchers narrow down the population by calculating the sample number, which is done using the Slovin formula as follows:

$$n = \frac{N}{1 + (N \cdot e^2)}$$

where n is the number of samples, N is the number of populations, and e is prediction error (10%).

The research sample was determined by multistage sampling using two or more methods. The method used is purposive sampling and accidental sampling. Purposive sampling is a sampling technique with specific considerations. The sample was selected based on the characteristics adapted to the research objectives, namely KUB chicken breeders who are members of AnaKUB. Accidental sampling is a sampling technique based on chances or without planning. Any KUB chicken breeders who meet researchers by chance can be used as samples if the breeders are suitable as data sources.

Data analysis technique

The data was collected for tabulation, analyzed, and concluded to answer the research objectives. Data analysis techniques were carried out in 3 stages, namely: (1) Questionnaire Feasibility Test, and (2) Descriptive analysis to answer the research objective, namely analyzing the rate of adoption of KUB chicken technology in the study area in the form of (a) group approach and (b) individual approach; (3) SEM (Structural Equation Modeling) to answer the research objective, which is to analyze the factors of perception and behavior of breeders that affect the adoption rate of KUB Chicken technology in Semarang, Salatiga and surrounding districts in the form of (a) Making a Path (b) Evaluation of the measurement model is done in three stages, namely convergent validity test, discriminant validity test and reliability test. (c) Structural evaluation of the model is carried out by looking at the value of the coefficient of determination (R^2) as well as the value of the path coefficient and the structural model equation, (d) Hypothesis Test

Feasibility test of the questionnaire

A validity Test is an index that shows the extent to which a measuring instrument measures what needs to be measured. The validity of a measuring instrument depends on whether or not the measuring device can precisely achieve the desired measurement objectives (Azwar 2003). One of the approaches used to test the validity of items (statements) is to use the Product Moment correlation equation, as follows:

$$r_{XY} = \frac{N(\sum X_i Y_i) - (\sum X_i)(\sum Y_i)}{N(\sum X_i^2) - (\sum X_i)^2}$$

where r_{XY} is the Product Moment correlation coefficient, X is the item score, Y is the total item score, and N is the number of respondents. The result could be concluded If the r_{XY} count $> r$ table ($N-2$; 5%), the measurement results are valid, or if the r_{XY} count > 0.3 , then the item is declared quite valid (Azwar 2003).

Descriptive analysis

Descriptive analysis is a method to answer the research objective: to analyze the adoption rate of KUB chickens and breeders' perceptions. The adoption rate describes the circumstances in which an individual or group member applies a new or recommended technology. This adoption rate measurement uses a weighted value expressed in a percentage. The calculation approach is differentiated according to the target: groups and individuals.

Group approach

The rate of adoption of the group approach is measured by means of a scoring technique based on the value of the score and the percentage of each applied technology component (Santoso 2005):

$$\text{Score value} = \frac{x \cdot VS}{\sum VS}$$

where P is the percentage of farmers who use technology components (%), VS is Value Score, and $\sum VS$ is Total Value Score.

Individual approach

Analyzing the adoption rate for individuals can be done directly by identifying aspects of the technology applied. The formula used is as follows (Hendayana 2014):

$$AR = \frac{FV}{T} \times 100\%$$

where AR is Adoption Rate (%), $F.V.$ is the Factor value from observation of adoption in the field (adoption unit),

Table 1. Variable naming, indicator, and indicator description

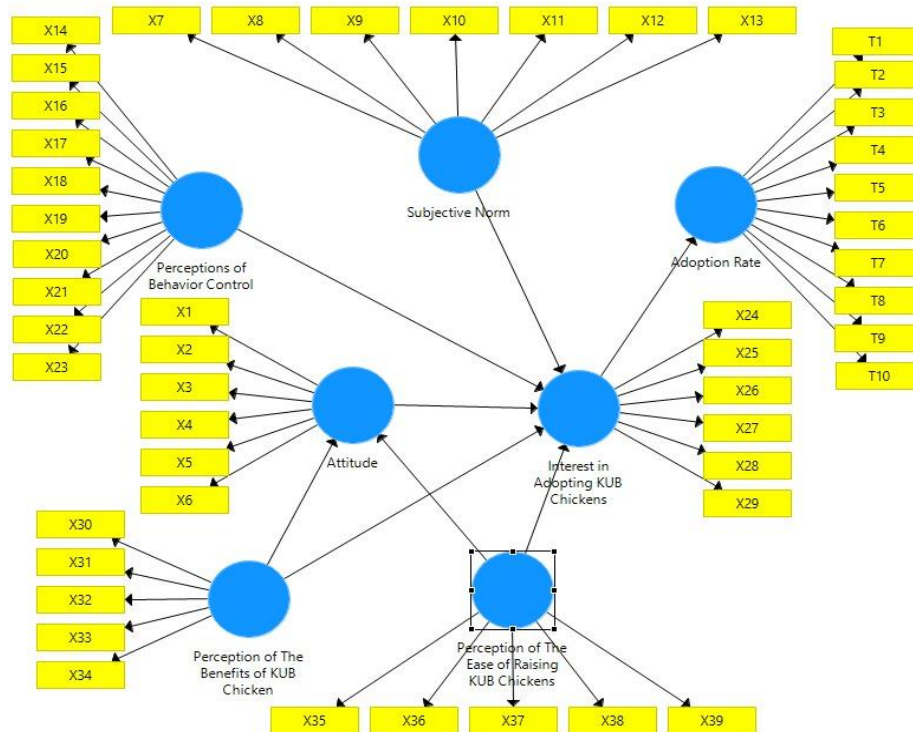
No	Variable	Indicator	Indicator description
1	Attitude	X1-X6	X1= better egg production; X2= interested in seeing successful KUB chicken breeders; X3= KUB chicken adoption is very profitable; X4= Raising KUB chickens will increase income; X5= raising KUB chickens will increase the food supply for the family; X6= employ individuals/family members/wife
2	Subjective norms	X7-X13	X7= important people support; X8= some breeders agree that KUB chickens are good; X9= based on the recommendation of a successful breeder; X10= institution can influence; X11= the extension worker inspired me to become a KUB chicken breeder; X12= anaKUB encouraged me to become a KUB chicken breeder; X13= family members support raising KUB chickens
3	Perceptions of behavioral control	X14-X23	X14= sure to adopt KUB chicken; X15= self-control; X16= has resources; X17= has knowledge and ability; X18= has enough experience to raise KUB chickens; X19= if there is a failure in the KUB chicken, I can handle it; X20= has enough time to raise KUB chickens; X21= KUB chicken feed is easy to get; X22= KUB chicken DOC is easy to get; X23= KUB chicken vaccines and medicines are easy to get
4	Interest in adopting KUB chickens	X24-X29	X24= planning to adopt KUB Chicken; X25= committed to adopting KUB chicken; X26= plans to choose to adopt KUB chickens over other chicken breeds to help the financial needs; X27= making KUB chicken raising as the main livelihood; X28= adopt as recommended by AnaKUB or extension workers; X29= all capital is invested in raising KUB chickens
5	Perceived benefits of KUB Chicken	X30-X34	X30 = increase of income; X31= produce superior breeds production; X32= produce a lot of eggs; X33= family consumption; X34= disease resistant
6	Perceived ease of raising KUB chickens	X35-X39	X35= easy maintenance; X36= easy sales; X37= easy to produce a lot of eggs; X38= easy to get the breeds; X39= easy to get the feed

Table 2. Adoption Rate Variable

Adoption rate variable	Measuring instrument
Type of Livestock (T1)	1= breeding; 2= hatching; 3= raising; 4= breeding; hatching; 5= breeding to raising
DOC source (T2)	1= self-production without clear marriage; 2= other products with unknown origin; 3= production between breeders; 4= production of AnaKUB; 5= BPTP/license of ministry of agriculture
Feed (T3)	1= finished/commercial; 2= family waste; 3= from agricultural waste; 4 = mixture of commercial and waste; 5 = AnaKUB feed
Local wisdom (T4)	1= not; 2= if someone offers; 3= sometimes; 4= partially used; 5= used
Utilization type (T5)	1= bran; 2= corn/oilcake/bran; 3= bran and corn/oilcake; 4= bran, corn, oilcake; 5= various kinds, not only bran, corn, and oilcake
Number of deaths (T6)	1= more than 50; 2= 26-50; 3= 20-25; 4= 11-20; 5= 1-10
Treatment (T7)	1= left alone; 2= self-medication; 3= using traditional medicine; 4= paid medical veterinary/vet treatment; 5= assistance from the local health post/livestock service
Vaccine (T8)	1= no; 2= Vaccines are only given early; 3= if there is assistance from the department only; 4= vaccines but not periodically; 5= periodic vaccines independently
Livestock manure (T9)	1= not managed; 2= Manure is placed in the space provided; 3= Manure treated; 4= manure is used in own garden; 5= sold
Land ownership (T10)	1= no owner; 2= workers; 3= profit sharing, 4= rents; 5= private property

Table 3. The structural model equation

Endogenous variables	Equation
Adoption Rate	$\gamma_{1.1} \text{ adoption interest} + \zeta_1$
Adoption Interest	$\gamma_{2.1} \text{ Attitude} + \gamma_{2.2} \text{ Subjective norm} + \gamma_{2.3} \text{ behavior control} + \gamma_{2.4} \text{ benefit} + \gamma_{2.5} \text{ ease} + \zeta_2$
Attitude	$\gamma_{3.1} \text{ Benefit} + \gamma_{3.2} \text{ ease} + \zeta_3$

**Figure 1.** Research path diagram

T is the Total recommended technology components (units). There are 5 classes of adoption level classification used, namely: (a) 0.00%–20.00% means very low adoption classification; (b) 20.01%–40.00% means low adoption classification; (c) 40.01%–60.00% means moderate adoption classification; (d) 60.01%–80.00% means high adoption classification; and (e) 80.01%–100.00% means very high adoption classification.

Structural Equation Modeling (SEM)

Structural Equation Modeling (SEM) combines path analysis and regression analysis, allowing researchers to simultaneously examine a series of interrelated relationships between measured variables and latent constructs (Latan 2015). SEM is a complex multivariate analysis because it involves many independent and dependent variables that are interconnected to form a model. In SEM it cannot be said that there are independent variables and dependent variables because an independent variable can be a dependent variable in another relationship.

Creating path diagrams

In this study, there were 6 latent variables, namely 1) Attitude, 2) Subjective norms, 3) Perceptions of behavioral control, 4) the benefits of raising KUB chickens, 5) Perceived ease of raising KUB chickens, and 6) Interest in adopting KUB chickens. Each variable has an indicator as a measuring tool to analyze descriptively and hypothetically, as listed in Table 1. In addition, there is 1 adoption rate variable, which has an indicator as a measuring tool to analyze descriptively and hypothetically as listed in Table 2.

Creating this path diagram is in accordance with the hypothesis and research model; latent variables are divided into 2 types: endogenous and exogenous. Endogenous variables where the values are influenced by other variables, while exogenous variables are variables in which other variables do not affect the values. Therefore, exogenous variables are also called independent variables.

Endogenous variables are Attitude Productivity, Interest in Adopting KUB Chicken, and KUB Chicken Adoption Rate. In comparison, the exogenous variables

are subjective norms, the perception of behavior control, the perception of the benefits of KUB chickens, and the perception of the ease of raising KUB chickens. The structural model equation can be seen in Table 3 to form a Path Diagram, as shown in Figure 1.

RESULTS AND DISCUSSION

Descriptive analysis results

The interview results (104 respondents) on the research variables and an empirical description of the data used in the research in a statistically descriptive manner using the minimum value, maximum value, average value, and Deviation Standard of each indicator of every variable can be seen in Table 4. Through this description, the respondents' perceptions of the indicators of each variable in the study will be known to what extent.

Table 4 shows that the variable with the largest average successively is 1) Perception of the Ease of Raising KUB Chickens (4.11); 2) Attitude (4.10); 3) Perception of the benefits of KUB chickens (4.09); 4) Perception of behavior control (4.05); 5) Interest in adopting KUB Chicken (4.01); and 6) Subjective Norms (3.97) (Borges et al. 2016). So the highest average variable is the ease of raising KUB chickens, and the lowest is the subjective norm variable. Meanwhile, the indicator with the highest average is that breeders are interested in seeing successful KUB chicken breeders on the attitude variable. The lowest average is the extension workers inspired me to become a KUB chicken breeder on the subjective norm variable.

The Attitude Variable has an average of 4.10. The indicator that has the highest average is X2, with an average of 4.28, which means that "Breeders are interested in seeing successful KUB chicken breeders ." While the X1 indicator means that " Breeders like to adopt KUB chickens because their egg production is better, " the indicator with the lowest average is 3.99 (Borges et al. 2014; Ambodo et al. 2017). On average, breeders also show that "Raising KUB chickens can increase the food supply for families and family income," with an average value of 4.12. Therefore, it can be assessed that most breeders adopt KUB chickens because they see successful KUB chicken breeders and believe that raising KUB chickens can function as a food provider and can increase family income. But for breeders, KUB chicken egg production cannot guarantee that it is better than other eggs.

The average of the statements in the Subjective Norm Variable is 3.97. The indicator with the highest average is X7, with an average of 4.17, meaning that "Important people support adopting KUB Chickens ." The X13 indicator has a significant average value of 4.11 means that "Family members support the maintenance of KUB

chickens." Meanwhile, the X11 indicator means that "Extensions inspire to become KUB chicken breeders" is the indicator with the lowest average, which is 3.64. It suggested that family members have an important role in supporting the maintenance of KUB chickens. In contrast, extension workers have yet to contribute much in supporting breeders to adopt KUB chickens (Iskandar 2018).

The Perception Variable of Behavior Control averages 4.05 (Makkonen et al. 2016; Rodi et al. 2019). The indicator that has the highest average is X22, with an average of 4.11; which means that "KUB chicken DOC is easy to get." While indicators X18, X19, and X23 of 4.02 represent "having experience in raising KUB chickens, being able to handle KUB chickens when there is a failure, and ease of getting vaccines and medicines", respectively. Thus, in terms of perceptions of behavior control, most breeders stated that DOC breeds were easy to obtain. Still, only a few had experience in raising KUB chickens, including handling if there was a failure in raising KUB chickens. Apart from that, the problem for breeders is challenging to get vaccines and medicine programs so that the maintenance of KUB chickens is raised naturally without the help of complete vaccines.

The KUB Chicken Adoption Interest Variable has an average of 4.01. The indicator with the highest average is X26, with an average of 4.08, which stated, "Plans to choose to adopt KUB chickens over other chickens to help financial needs". While the X29 indicator, which stated that "I will invest all my capital in raising KUB chickens," has the lowest average, 3.90. Another average with a significant value of 4.07 is "Committed to adopt KUB chickens and will adopt KUB chickens as recommended by AnaKUB", meaning that most breeders will choose KUB chickens over other chickens to help family needs according to AnaKUB's recommendations. Still, not all capital will be invested by breeders to raise KUB chickens.

The average of the statements in the Variable Perceived Benefits of KUB Chicken is 4.09. The highest indicator is X33, with an average of 4.14, which states that "Raising KUB chickens is beneficial for family consumption". Another average that has a significant value is 4.09, which states that "The benefits of KUB chickens are increasing income and producing lots of eggs." Meanwhile, the X34 indicator stated that "Raising KUB chickens is disease-resistant" was the indicator with the lowest average, which was 4.05. Thus, the perception of the benefits of KUB chicken for most breeders in Central Java is that it benefits family consumption, increases income, and produces lots of eggs.

The Perceived Variable of Ease of Raising KUB Chickens is 4.11. The highest average indicator is X39, with an average of 4.17, meaning that "KUB chickens are easy to get the feed". Another average value of 4.16 "Easy to get KUB chicken seeds". Meanwhile, the X37

Table 4. Variable descriptive analysis

Indicator Code	Min	Max	Average	Deviation Standard
X1	3	5	3.99	0.45
X2	3	5	4.28	0.51
X3	3	5	4.07	0.32
X4	3	5	4.05	0.40
X5	4	5	4.12	0.32
X6	3	5	4.12	0.40
Att	3.33	5	4.10	0.31
X7	3	5	4.17	0.43
X8	3	5	4.03	0.33
X9	2	5	4.05	0.40
X10	2	5	3.92	0.46
X11	2	5	3.64	0.65
X12	2	5	3.89	0.54
X13	3	5	4.11	0.48
SN	2.43	5	3.97	0.36
X14	4	5	4.10	0.30
X15	3	5	4.04	0.31
X16	3	5	4.06	0.31
X17	3	5	4.05	0.26
X18	3	5	4.02	0.34
X19	3	5	4.02	0.31
X20	3	5	4.09	0.40
X21	3	5	4.06	0.31
X22	4	5	4.11	0.31
X23	3	5	4.02	0.31
PBC	3.50	5	4.05	0.26
X24	3	5	4.01	0.45
X25	3	5	4.07	0.35
X26	3	5	4.08	0.39
X27	3	5	3.93	0.63
X28	3	5	4.07	0.29
X29	3	5	3.90	0.66
IAKC	3.33	5	4.01	0.36
X30	3	5	4.09	0.37
X31	3	5	4.08	0.33
X32	3	5	4.09	0.37
X33	3	5	4.14	0.40

Indicator Code	Min	Max	Average	Deviation Standard
X34	3	5	4.05	0.40
PBKC	3.00	5	4.09	0,32
X35	3	5	4.05	0.32
X36	3	5	4.12	0.47
X37	3	5	4.04	0.46
X38	3	5	4.16	0.40
X39	3	5	4.17	0.45
PERKC	3.40	5	4.11	0.33

Att = Attitude, SN= Subjective Norms, PBC= Perception of Behavior Control, IAKC=Interest in Adopting KUB Chickens, BKC=Perception of the Benefits of KUB Chickens, PERKC=Perception of the Ease of Raising KUB Chickens

Table 5. Variable descriptive analysis of KUB chicken adoption rate

Indicator Code	Min	Maks	Average	Deviation Standard
T1	2	5	3.58	0.97
T2	3	5	4.15	0.68
T3	2	5	3.73	1.14
T4	2	5	3.64	1.08
T5	3	5	4.24	0.94
T6	3	5	4.40	0.76
T7	1	4	2.74	0.85
T8	1	4	1.79	1.18
T9	1	4	2.13	1.44
T10	2	3	2.76	0.43
KUB Chicken Adoption Rate	2.10	4.5	3.32	0.73

indicator, which stated that "Raising KUB chickens produces a lot of quality eggs," has the lowest average, 4.04. Thus the perception of the ease of raising KUB chickens is easy to get the feed and the breeds.

Table 5 shows that the average in the KUB Chicken Adoption Rate Variable is 3.32 means that the adoption rate of breeders in the Central Java region reveals a high adoption classification (66.40%). On the other hand, the indicator with the highest average is T6 (4.40), with the average number of livestock deaths reaching only 11-20 heads. Meanwhile, the T8 indicator, which is about the vaccination program carried out by breeders in controlling diseases, which was only carried out at the initial purchase of DOC that the seller had vaccinated, was an indicator with the lowest average (1.79). As a general description, most livestock types developed are raised by taking DOC sources from the AnaKUB livestock association. The feed is primarily agricultural waste and commercial feed mixtures. Although in terms of utilization of local wisdom, some still use it occasionally, the feed used is bran, corn, and oilcake.

Regarding treatment for KUB chickens, they usually use traditional medicine if they get sick. Most manure needs to be appropriately managed; it is only placed in the storage area. In terms of land ownership, most farmers use private land or sharecropping.

Evaluation of the measurement model

Evaluation of the measurement model is carried out in three stages, namely (1) convergent validity test, (2) discriminant validity test, and (3) reliability test.

Convergent validity evaluation

Convergent validity is related to the principle that the manifest variables of a construct should be highly correlated (Kartika et al. 2022); convergent validity is assessed based on the loading factor and the Average Variance Extracted (AVE) value. The rule of thumb in the convergent validity test is that the loading factor

Table 6. Loading Factor Analysis

Indicator Code	AR	Att	SN	PBC	IAKC	PBKC	PERKC
T1	0.797	0.407	0.238	0.296	0.400	0.438	0.391
T2	0.761	0.285	0.199	0.229	0.293	0.337	0.358
T3	0.739	0.209	0.148	0.143	0.278	0.292	0.248
T4	0.775	0.257	0.109	0.160	0.262	0.297	0.314
T5	0.780	0.309	0.134	0.216	0.283	0.318	0.319
T6	0.741	0.320	0.270	0.205	0.345	0.356	0.331
T7	0.791	0.264	0.187	0.208	0.277	0.293	0.301
T8	0.821	0.456	0.332	0.350	0.435	0.425	0.525
T9	0.738	0.217	0.092	0.153	0.216	0.241	0.308
T10	0.735	0.193	0.142	0.087	0.221	0.254	0.177
X1	0.346	0.719	0.495	0.584	0.602	0.636	0.653
X2	0.550	0.714	0.570	0.465	0.642	0.659	0.658
X3	0.177	0.815	0.580	0.766	0.742	0.630	0.671
X4	0.142	0.753	0.566	0.703	0.675	0.568	0.546
X5	0.292	0.848	0.672	0.688	0.768	0.726	0.760
X6	0.373	0.831	0.586	0.578	0.728	0.691	0.666
X7	0.301	0.735	0.776	0.549	0.668	0.611	0.611
X8	0.199	0.614	0.773	0.714	0.607	0.536	0.529
X9	0.202	0.592	0.819	0.581	0.602	0.479	0.482
X10	0.173	0.518	0.817	0.476	0.501	0.415	0.386
X11	-0.014	0.346	0.705	0.412	0.442	0.279	0.324
X12	0.113	0.437	0.711	0.456	0.482	0.293	0.346
X13	0.326	0.649	0.780	0.513	0.654	0.583	0.550
X14	0.306	0.647	0.588	0.801	0.702	0.562	0.657
X15	0.254	0.666	0.545	0.872	0.727	0.648	0.596
X16	0.216	0.658	0.567	0.893	0.709	0.558	0.618
X17	0.248	0.689	0.617	0.884	0.716	0.651	0.642
X18	0.195	0.591	0.530	0.793	0.604	0.534	0.629
X19	0.134	0.663	0.518	0.795	0.621	0.621	0.653
X20	0.270	0.687	0.713	0.708	0.654	0.563	0.557
X21	0.178	0.619	0.545	0.813	0.624	0.555	0.579
X22	0.285	0.784	0.605	0.811	0.761	0.672	0.695
X23	0.221	0.590	0.462	0.819	0.621	0.621	0.561
X24	0.477	0.608	0.524	0.614	0.761	0.657	0.595
X25	0.422	0.669	0.514	0.705	0.786	0.747	0.620
X26	0.216	0.798	0.645	0.652	0.791	0.662	0.681
X27	0.254	0.622	0.570	0.515	0.715	0.511	0.625
X28	0.249	0.737	0.621	0.769	0.790	0.711	0.692

Indicator Code	AR	Att	SN	PBC	IAKC	PBKC	PERKC
X29	0.250	0.660	0.578	0.519	0.761	0.547	0.678
X30	0.312	0.780	0.533	0.695	0.766	0.862	0.671
X31	0.361	0.646	0.494	0.682	0.697	0.854	0.554
X32	0.372	0.752	0.576	0.623	0.761	0.886	0.685
X33	0.425	0.745	0.577	0.549	0.717	0.827	0.681
X34	0.384	0.542	0.358	0.499	0.543	0.757	0.550
X35	0.250	0.609	0.525	0.718	0.692	0.625	0.777
X36	0.354	0.686	0.461	0.526	0.656	0.530	0.776
X37	0.413	0.687	0.483	0.612	0.671	0.707	0.724
X38	0.348	0.695	0.571	0.582	0.669	0.570	0.817
X39	0.377	0.639	0.371	0.525	0.614	0.521	0.831

AR = Adoption Rate, At = Attitude, SN = Subjective Norms;PBC= Perception of Behavior Control; IAKC=Interest in Adopting KUB Chickens, PBKC= Perception of the Benefits of KUB Chickens, PERKC=Perception of the Ease of Raising KUB Chickens

Table 7. AVE Value Research Model

Variable	AVE
Interest in adopting KUB chickens	0.590
Subjective norm	0.593
Perceptions of behavior control	0.673
Perception of ease of raising KUB chickens	0.618
Perception of benefits of KUB chicken	0.703
Attitude	0.611
Adoption rate	0.590

AVE= Average Variance Extracted

Table 8. Cronbach's Alpha Value and composite reliability research model

Variable	CA	CR
Interest in adopting KUB chickens	0.861	0.896
Subjective norm	0.886	0.910
Perceptions of behavioral control	0.946	0.954
Perception of ease of raising KUB chickens	0.844	0.890
Perception of benefits of KUB chicken	0.894	0.922
Attitude	0.871	0.904
Adoption rate	0.923	0.935

CA= Cronbach's Alpha, CR= Composite Reliability

value is more significant than 0.7, and the AVE value is greater than 0.5 (Smith 2003; Latan 2015; Sons 2016; Ma et al. 2020); indicating shows that all indicators in the research model have a loading factor that meets the criteria. So the next step is to evaluate the AVE value for each latent variable. Table 7 shows that all latent variables in the research model already have an AVE value >0.5. The variable with the smallest AVE value is the Interest in Adopting KUB Chicken and the Adoption

Rate (0.590). In contrast, the variable with the highest AVE value is the Perception of the Benefits of KUB Chicken (0.703).

Reliability evaluation

Reliability evaluation was carried out using Cronbach's alpha and composite reliability values. According to (Ghozali 2012), a latent variable must have

a Cronbach's alpha value greater than 0.7 or composite reliability greater than 0.7. Cronbach's alpha (C.A.) value and composite reliability (C.R.) research model can be seen in Table 8.

Table 8 shows that all latent variables in the research model already have Cronbach's alpha values and composite reliability greater than 0.7. The variable with the lowest value is the Perceived Ease of Raising KUB Chickens, with a Cronbach's alpha value of 0.844 and a composite reliability of 0.890. In comparison, the variable with the most significant value is the Perception of Behavior Control, with a Cronbach's alpha value of 0.946 and a composite reliability of 0.954. It means the variables in the questionnaire are reliable.

Discriminant validity evaluation

The discriminant validity test relates to the principle that different construct measurements (manifest variables) should not be highly correlated. Correlation testing between latent variables was carried out using the Fornel Lacker Criterion. A construct is valid by comparing the root value of the AVE (diagonal Fornell-Larcker Criterion) with the correlation value between latent variables. The AVE root value must be greater than the correlation between latent variables. Following are the results of the Fornel Lacker Criterion for the discriminant validity test of the research model following are the results of the Fornel Lacker Criterion for the discriminant validity test of the research model.

The Fornel-Lacker Criterion is shown in Table 9. The test results show that all the roots of the AVE (Fornell-Larcker Criterion) for each construct are more significant than their correlation with other variables. Discriminant validity can also be assessed based on cross-loading. The rule of thumb used in the discriminant validity test with a cross-loading value greater than 0.7 (Latan 2015). Furthermore, the Cross Loading value for the research model shows that each indicator with its latent variables is more fantastic than those with other latent variables. So it is concluded that Discriminant Validity met the requirements.

Evaluation of structural models

The structural model is evaluated by looking at the coefficient of determination (R^2), the value of the path coefficient, and the structural model equation.

Coefficient of determination

The results of calculating R^2 for each endogenous latent variable are in Table 10. According to (Sarstedt 2014), the range of R^2 values is from 0–1, with a higher rate indicating better predictive accuracy. R^2 is

considered weak, moderate, and vital if it shows a value of 0.19, 0.33, and 0.67 (Chin 1998). Table 10 indicates that the Adoption Rate Variable is weak because it is below 0.19. At the same time, the Interest in Adopting KUB Chicken and Attitude variables are classified as vital because it is above 0.67. The meaning of this value is that the exogenous variable that affects the Interest in Adopting KUB Chicken in the tested model represents a 0.870 probability of interest in adopting KUB chicken, which influences attitude in the tested model, representing a 0.806 probability of attitude. Meanwhile, at the Adoption Rate, it seems that many other variables can affect it because the R^2 is only 0.168.

Path Coefficient (β) and Structural Model Equations

The path coefficient value (path coefficient) shows how strong the influence of a variable is on other variables (Wong 2013). The higher the path coefficient value, the stronger the effect is. The path coefficient value (β) is standardized from -1 to +1. A coefficient closer to +1 indicates a strong and positive relationship. While the coefficient is close to -1, it shows a strong negative relationship (Sarstedt 2014).

Calculating the path coefficient in the research model in Table 11 shows that all latent variables have positive coefficient values. Based on the results of calculating the path coefficient value, the structural model equation is in Table 12. ζ_1 , ζ_2 , and ζ_3 Variables represent variables not included in the study. The path coefficient difference can also be used to sequence variables based on their most substantial influence, not only to know which variables influence the dependent variable. The values in Table 12 show that the variable with the most impact on Adoption Interest is the Perceived Benefits of KUB Chicken. While the variable that has the most influence on attitude is the perceived ease of raising KUB chickens (Ammar 2016; Tan 2016; Putri et al. 2021).

Hypothesis test

This stage was carried out after the structural model evaluation stage was established and purposed to determine whether the research hypothesis proposed in this model is accepted or rejected. The hypothesis is accepted if the t-statistics are above 1.96 and the path coefficient is above 0.1 (Ghozali 2012). The results of the hypothesis test can be seen in Table 13.

The estimated standard coefficients are presented in Table 13. shows the direct effect of each variable attitude, subjective norm, perceptions of behavioral control, perceived benefits of KUB chickens, perceived ease of raising KUB chickens, interest in adopting KUB chickens, and adoption rate of KUB chickens (Yazdanpanah et al. 2015). It is proved that the variable perception of the benefits of KUB chicken has a positive

Table 9. Research Model Fornier-Lacker test results

	IAKC	SN	PBC	PERKC	PBKC	Att	AR
IACK	0.768						
SN	0.748	0.770					
PBC	0.826	0.696	0.821				
PERKC	0.843	0.616	0.756	0.786			
PBKC	0.839	0.614	0.731	0.754	0.838		
Att	0.889	0.742	0.807	0.846	0.835	0.782	
AR	0.410	0.259	0.284	0.445	0.439	0.400	0.768

AR= Adoption Rate, At= Attitude , SN= Subjective Norms, PBC= Perception of Behavior Control, IAKC=Interest in Adopting KUB Chickens, PBKC=Perception of the Benefits of KUB Chickens PERKC=Perception of the Ease of Raising KUB Chickens

Table 10. R2 value of research model

Endogenous Variables	R-Square
Adoption Rate	0.168
Interest in Adopting KUB Chickens	0.870
Attitude	0.806

Table 11. Path coefficient value

Path	β
Benefits -> Attitude	0.458
Ease -> Attitude	0.501
Attitude -> Adoption Interest	0.212
Subjective Norm -> Adoption Interest	0.167
Behavior Control -> Adoption Interest	0.173
Benefits -> Adoption Interest	0.253
Ease -> Interest in Adoption	0.239
Adoption Interest -> Adoption Rate	0.410

Table 12. Structural Model Equation

Endogenous Variables	Equation
Adoption rate	$0.410 * \text{Adoption interest} + \zeta_1$
Adoption interest	$0.212 * \text{Attitude} + 0.167 * \text{Subjective Norm} + 0.173 * \text{Behavior Control} + \text{Ease} + \zeta_2$
Behavior control	$0.458 * \text{Benefit} + 0.501 * \text{Ease} + \zeta_3$

and significant effect on farmer attitudes ($\beta = 0.458$, P-Value < 0.000). Thus the H1 hypothesis in this study states that an increase in the perception of the benefits of KUB Chicken can increase the farmer's attitude by 45.8% (Burhansyah 2013; Afolami et al. 2015). The perceived ease of raising KUB chickens positively and significantly affects farmer attitudes ($\beta = 0.501$, P-Value < 0.000). Thus the H2 hypothesis in this study states that increased perceived ease of raising KUB chickens can increase attitudes by 50.1%. The farmer's attitude

variable positively and significantly affects the interest in adopting KUB chickens ($\beta = 0.212$, P-Value < 0.049). Hypothesis H3 in this study shows that an increase in attitude can increase the interest in adopting KUB chickens by 21.2%. The subjective norm positively and significantly affects the interest in adopting KUB chickens ($\beta = 0.167$, P-Value < 0.011). So the H4 hypothesis in this study states that an increase in subjective norms can increase the interest in adopting KUB chickens by 16.7%.

**Table 13.** Hypothesis test

Hipotesis	Path	β	T-statistics	P-value	Remark
H1	Benefits -> Attitude	0.458	3.643	0.000	Significant
H2	Ease -> Attitude	0.501	3.917	0.000	Significant
H3	Attitude -> Adoption Interest	0.212	1.973	0.049	Significant
H4	Subjective Norm -> Adoption Interest	0.167	2.557	0.011	Significant
H5	Behavior Control -> Adoption Interest	0.173	2.504	0.013	Significant
H6	Benefits -> Adoption Interest	0.253	2.502	0.013	Significant
H7	Ease -> Adoption Interest	0.239	3.607	0.000	Significant
H8	Adoption Interest -> Adoption Rate	0.410	5.203	0.000	Significant

β = path coefficient

Further results show that the variable perception of behavioral control has a positive and significant effect on the intention to adopt KUB chickens ($\beta = 0.173$, P-Value < 0.013) (Nugroho et al. 2018; Mahardika et al. 2020). Hypothesis H5 in this study shows that increased perceptions of behavior control can increase the interest in adopting KUB chickens by 17.3%. Furthermore, the perceived benefits of KUB chicken have a positive and significant effect on the intention to adopt KUB chicken ($\beta = 0.253$, P-Value < 0.013). Thus, the H6 hypothesis in this study states that the perception of the benefits of

KUB chicken has a positive and significant effect on the interest in accepting KUB chicken. Therefore, it can conclude that the perception of the benefits of KUB chickens can increase the interest in adopting KUB chickens by 25.3%. Furthermore, the perceived ease of raising KUB chickens positively and significantly affects the interest in adopting KUB chickens ($\beta = 0.239$, P-Value < 0.000).

Thus the H7 hypothesis in this study states that the perceived ease of raising KUB chickens has a positive and significant effect on the interest in adopting KUB

chickens being accepted, meaning an increase in the perceived ease of raising KUB chickens can increase the interest in adopting KUB chickens by 23.9%. Furthermore, in the coefficient variable, interest in adopting KUB chicken has a positive and significant effect on the adoption rate of KUB chicken ($\beta = 0.410$, P-Value < 0.000). Thus the H8 hypothesis in this study states that an increase in interest in adopting KUB chickens can increase the adoption rate of KUB chickens by 41.0% (Sirajuddin et al. 2017; Astarina 2019).

CONCLUSION

The adoption rate of breeders in Central Java, Indonesia, is classified as high to adopt KUB Chicken, which is influenced by the intention and attitude of adopting KUB chickens. Based on the results of this descriptive study, the variable with the most significant influence is the perceived ease of keeping KUB Chicken. In comparison, the lowest variable is the subjective norm variable. The combined results of path analysis (path) and regression analysis on the Structural Equation Modeling (SEM) technique suggested that the TAM and TPB theories both have variables of a positive effect on the interest in adopting KUB chickens. In addition, the perceived benefits and ease of raising KUB chickens on the TAM theory positively affect the attitude variable on the TPB theory. Important people, including family members, have an important role in supporting the maintenance of KUB chickens. In contrast, extension workers have yet to contribute much in supporting breeders to adopt KUB chickens.

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

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