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Sperm Longevity and Motility in Ringer's Lactate Solution with Addition of Egg Yolk among Five Phenotypes of Kokok Balenggek Chicken

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ABSTRACT

The application of cryopreservation to preserve germplasm in such specific breed requires preliminary studies, primarily related to the resistance of spermatozoa to low temperatures (4 – 5 °C) as measured by their motility and longevity. In this study, semen taken from five phenotypes of Kokok Balengeek Chicken (KBC) (*Biriang*, *Jalak*, *Kinantan*, *Kuriak*, and *Taduang*) was used to evaluate the effect of Ringer's Lactate-egg yolk diluent on longevity and motility of spermatozoa. The treatments consisted of Ringer's Lactate (RL) solution added with egg yolk at a concentration of 1% (RLKT1), 3% (RLKT3), and 5% (RLKT5). Evaluation of fresh semen showed that the spermatozoa of *Jalak* had the highest motility, namely 75.63 ± 0.5% (P<0.05). Post-dilution longevity and motility observations were carried out at 0, 24, 48, and 72 h, significantly decreasing each time (P<0.05). The lowest range of reduction was found in *Jalak* spermatozoa diluted with RL with longevity of 7.75 ± 0.70 days. Overall, the RL diluent showed the highest motility after 24 h, namely 41.13 ± 2.27%. Adding egg yolks to Ringer's Lactate solution could not maintain the motility of KBC spermatozoa when stored at 4-5°C for 48-72 h.

Keywords: Kokok Balenggek chicken, Semen, Egg yolk, and Ringer's Lactate solution

Introduction

Artificial insemination (AI) is an assisted reproductive technology essential in improving quality and quantity of poultry population. Semen obtained from selected superior cocks should be stored for an extended period before being inseminated into hen reproductive tract. This required the development of appropriate conditions for short-term (liquid semen) and long-term (frozen semen) storage. Dilution and storage methods allow for more accessible transportation to distant sites, fertilize more females, and increase the utilization of semen from superior males (Lake, 1960; Łukaszewicz *et al*., 2004).

The most frequently used technology for storing poultry semen for short periods (hours or days at low temperatures) is chilling technology in which diluents has an important role for maintaining the quality of spermatozoa. It has long been known that the administration of diluent is essential in maintaining the viability of spermatozoa in vitro (Bilgili *et al*., 1987). Diluted poultry semen can be stored for up to 24 h without compromising its viability and fertility (Donoghue and Wishart, 2000). The diluent should contain nutrients that will be used as an energy source for spermatozoa. In addition, the diluent used must be able to maintain a pH level and osmolarity that are similar to the original conditions (Bustani and Baiee, 2021). Research on finding a suitable diluent for storing poultry semen is still being carried out, especially since the biochemical composition in poultry semen plasma was identified for the first time (Zong *et al*., 2023). Several unique buffer solutions can be used as a diluent for chicken semen (Bootwalla and Miles, 1992) with additional ingredients such as skim milk, egg albumin, gentamicin sulfate (Sexton, 1978), caproic acid (Lake and Ravie, 1981), egg yolk and antibiotics (Magfira *et al*., 2017).

The diluent commonly used in chicken semen is Ringer's Lactate solution (Danang *et al*., 2012; Jaswandi *et al*., 2023; Junaedi *et al*., 2016; Solihati *et al*., 2006). Ringer's Lactate solution consists of mineral salts with pH (buffer) and isotonic properties that can support spermatozoa motility for longer. Chicken semen contains electrolyte elements such as sodium chloride, calcium, potassium, sodium, and magnesium. Ringer's Lactate solution has the same sodium chloride content as electrolyte elements from chicken semen plasma, such as sodium, chloride, calcium, and magnesium (Solihati *et al*., 2006). The study's results (Danang *et al*., 2012) showed that chicken semen in Ringer's Lactate solution lasted 18 h after being ejaculated. Adding 5% egg yolk in Ringer's Lactate solution showed better progressive spermatozoa motility than 10%

(Hidayat, 2001). The addition of egg yolk can help sperm to withstand cold shock (Amirat *et al*., 2004). Egg yolk contains a low-density lipoprotein (LDL) fraction (Moussa *et al*., 2002). LDL can interact specifically with semen plasma proteins and has a very high binding capacity (Manjunath and Thérien, 2002).

Kokok Balenggek chicken (KBC) is a genetic resource for "singing" chickens in West Sumatra (Rusfidra *et al*., 2012). KBC has a melodious crowing sound and is layered (can reach 24 syllables) ("*Balenggek"*: Minang language). KBC's melodious and unique crowing sound is thought to be the only chicken nation with the cawing type of *"Balenggek"* in the world (Rusfidra, 2004). That is why KBC has a high position for the Minangkabau people (Fumihito *et al*., 1996). Through letter number 2919/Kpts/OT.140/6/2011, the Minister of Agriculture decided that the KBC has been designated as a national livestock family.

Based on color, there are at least five KBC phenotypes that are most common in West Sumatra, namely *Biriang* with a red neck, back, and loin color; *Jalak* with yellow shank and beak, chest, wings, and tail feathers are black, and tail feathers and waist are yellowish; *Kinantan* with white fur, legs, beak, eyes, wings, tail and neck; *Kuriak* with spotted plumage, black eyes, legs, and beak; and *Taduang* with black legs, beak and eyes (Muryanto and Pramono, 2014). Phenotypic differences between males allegedly contributed to differences in spermatozoa quality (Gee *et al*., 2004), including differences in storability (longevity) at low temperatures (Siudzińska and Łukaszewicz, 2008). This difference causes the diluent to have different conditions to maintain its viability (Lake, 1983). Therefore, it is necessary to study the effect of Ringer's Lactate solution added with egg yolk on the longevity and motility of KBC spermatozoa.

Materials and Methods

Extender preparation

The sperm diluent was made from Ringer's Lactate solution (PT. Widatra Bhakti), egg yolk, penicillin, and streptomycin (Junaedi *et al*., 2016) with the following details (Table 1). The solution was then homogenized and centrifuged for 15 min at 3000 rpm. After centrifugation, the supernatant was taken and used as a semen diluent.

Tabel 1. Composition of extenders

Composition	Extenders			
	RL	RLKT1	RLKT3	RLKT5
Ringer's lactate (ml)	10	9,9	9,7	9,5
Egg yolk (ml)	0	0,1	0,3	0,5
Penicillin (IU/ml)	1000	1000	1000	1000
Streptomycin (mg/ml)	1,0	1,0	1,0	1,0

RL = Ringer's Lactate solution; RLKT1 = Ringer's Lactate solution + Egg Yolk 1%; RLKT3 = Ringer's Lactate solution + Egg Yolk 3%; RLKT5 = Ringer's Lactate solution + Egg Yolk 5%.

KBC fresh semen collection and evaluation

Semen collection was carried out on five KBC phenotypes (*Biriang*, *Jalak*, *Kinantan*, *Kuriak*, and *Taduang*) of two individuals aged ±1.5–2 years. Semen collection was carried out every other day at 08.00 – 09.00 WIB using the dorsal massage technique and collected using a 1.5 mL microtube. The massage method is carried out continuously on the back of the chicken to the base of the tail until the chicken shows symptoms of an erection (Arifiantini, 2012; Suprijatna *et al*., 2005). Semen from each group was pooled according to the phenotypic group and evaluated for quality. Semen quality includes macroscopic; volume, pH, color, and consistency, and microscopic quality; mass movement, spermatozoa motility, viability, morphology, and concentration of spermatozoa. The evaluation procedure refers to Arifiantini (2012), adapted for poultry semen. Spermatozoa motility was evaluated by diluting semen with Ringer's Lactate solution, homogenized, and viewed under a microscope lens with 400x magnification. Sperm motility was assessed from five visual fields, and values were expressed in percent. Sperm morphology and viability were stained with eosin-nigrosin. Spermatozoa concentration was calculated using a Neubauer chamber. Microscopic evaluation using an Olympus microscope (CX 23).

Semen dilution

After being evaluated, the semen was diluted according to the treatment with a ratio of 125 µL of fresh semen plus 375 µL of diluent. After diluting, the semen was re-evaluated microscopically before being put into a tube to be stored in the refrigerator (4–5°C) until spermatozoa motility reached 0%. Semen is evaluated every 24 h to see changes in motility while in the refrigerator.

Design experiment and data analysis

The study was conducted using a randomized block design (RBD) and one-way analysis of variance (ANOVA) for fresh semen evaluation data and two ways for longevity and motility data. If there was a difference, a Duncan test was carried out with a 95% significance level.

Results and Discussion

Evaluation of fresh semen on five KBC phenotypes

The evaluation results of fresh semen on five KBC phenotypes (Table 2) showed that fresh semen of KBC produced a volume ranging from 0.2-0.3 mL/ejaculate, with the *Kinantan* phenotype KBC having the highest average total semen volume (0.36±0.16 mL/ejaculate), the pH of semen ranges from 6.8–7.7 with a thick consistency and a milky white color. The mass movement of spermatozoa ranged from 1.8–2.6 (++/+++), with the *Kinantan* phenotype having the highest average of spermatozoa mass movement (2.6±0.15), and sperm motility ranged between 66%-75% which was the highest one among those treatments. The sperm viability was ranged between 72%-89%, with the highest viability being in the *Taduang* phenotype (89±0.74%), sperm abnormalities were 10.5%-26% with the lowest

abnormalities was found in the *Jalak* phenotype (10.5±1.75%), and the concentration of spermatozoa was 539–1180 million/ejaculate where the highest concentration of spermatozoa was found in the *Kinantan* phenotype (1180.6±112 million/ejaculate).

Furthermore, the observations in this study found that the color and consistency of semen in KBC were classified as usual, namely white and thick. The consistency and color of KBC semen in this study tended to be the same when compared to Pelung chickens (Junaedi and Husnaeni, 2019) but slightly different from the Green Jungle chicken, whereas Andaruisworo and Yuniati (2021) reported that the semen of the Green Jungle chicken was white to cloudy white. Color and consistency illustrate the concentration of spermatozoa (Almahdi *et al*., 2014). This study found that the average concentration of spermatozoa in KBC was 817.2±102.3 million /ejaculate. This result was lower when compared to the concentration of spermatozoa in Pelung chickens, which is 5,043.33±51 million/mL or 1,160±11.73 /ejaculate (Junaedi and Husnaeni, 2019) and higher when compared to the Green Forest chicken, which is 898±4, 24 million/mL or 61.96±0.29 million/ejaculate (Bebas and Laksmi, 2013). The concentration of spermatozoa plays an essential role in the reproductive success of poultry. Wishart and Staines (1999) said that less than 2% of the spermatozoa enter the sperm storage (SST) in the uterovaginal junction canal, while the rest will be pressed out of the vagina so that high concentrations will increase the number of spermatozoa that will enter the SST. Meanwhile, Arifiantini (2012) explained that the success of AI is closely related to the fertility of spermatozoa, one of which is influenced by the concentration of spermatozoa.

The pH value of KBC obtained in this study tends to be the same, namely 7.2±0.07 when compared to the Pelung chicken, pH 7.2 (Kusuma *et al*., 2018) and the Green Forest chicken, which is pH 7 (Andaruisworo and Yuniati, 2021). However, there are variations in pH seen in each KBC phenotype, where *Kuriak* phenotype semen has a lower degree of acidity, namely pH 6.8±0.05, and *Taduang* phenotype has a higher degree of acidity, pH 7.7±0.05 ($p<0.05$). The degree of acidity (pH) is essential to know because it will affect the viability of spermatozoa, where the viability of spermatozoa will decrease as the pH decreases. Differences in the degree of acidity can be influenced by individual differences, motility, and metabolism of spermatozoa (Mphaphathi *et al*., 2016). The average motility of KBC spermatozoa shown in this study was 70.63±1.36%. This result is lower when compared to the results reported by Junaedi *et al*. (2016) in Pelung chickens, namely 84.69±1.12, and Kusuma *et al*. (2018), namely 86%. However, it is higher when compared to the Green Forest chicken, which is 45% (Andaruisworo and Yuniati, 2021). Spermatozoa motility in this study found that the *Jalak* phenotype had higher motility, namely 75.63±0.5%, compared to the other phenotypic AKBs, and the *Taduang* phenotype had the lowest motility, namely 66.25±1.06% ($p<0.05$). It is essential to know the motility of spermatozoa considering that motility will significantly affect the speed of spermatozoa in the female reproductive tract so that it can increase fertilization rates (Danang *et al*., 2012). The viability of fresh semen of KBC in this study was 79.4±1.04 %. This result is lower when compared to the Pelung chicken, which is 89.17±1.23% (Junaedi and Husnaeni, 2019) and higher when compared to the Green Forest chicken, which is 30% (Andaruisworo and Yuniati, 2021). Meanwhile, the *Jalak* phenotype had a higher viability of 85±0.82% among the five KBC phenotypes ($p<0.05$). Furthermore, the abnormality of *Jalak* phenotype spermatozoa was the lowest (10.5±1.75 %), and the abnormality of *Biriang* phenotype spermatozoa was the highest (26±1.05 %) among the five KBC phenotypes ($p<0.05$). Viability and abnormality are needed in assessing the quality of spermatozoa because of their role in the success of fertilization, especially in determining the success of insemination. Solihati *et al*. (2006) said that the minimum standard required for viability is ≥ 45%, with motility ≥ 40% to support the success of insemination in chickens.

Spermatozoa longevity of Kokok Balenggek chicken in four different extenders

The life span of spermatozoa (longevity) was measured by observing how long it took to remain motile, and in this study, it was observed at 4–5°C. The average longevity of KBC spermatozoa in the four types of extenders (Table 3) did not show any difference ($P>0.05$). However, if one

Tabel 2. Semen quality of Kokok Balenggek chicken

Parameter	Phenotyps					Average
	Biriang	*Jalak*	*Kinantan*	*Kuriak*	*Taduang*	
Macroscopic						
Volume (mL)	0.21±0.12	0.24±0.19	0.36±0.16	0.28±0.16	0.31±0.15	0.28±0.16
pH	7.4±0.06^{bc}	7.4±0.06^{bc}	7.1±0.05^{b}	6.8±0.05^{a}	7.7±0.05^{c}	7.2±0.07
Color	Milky White	Milky White	Milky White	Milky White	Milky White	Milky White
Consistency	Thick	Thick	Thick	Thick	Thick	Thick
Microscopic						
Mass Movement	2.3±0.19	1.8±0.12	2.6±0.15	1.6±0.11	1.8±0.10	2.0±0.10
Concentration (10^6/ejaculate)	804.1±78.5^{ab}	874.3±73^{ab}	1180.6±112^{b}	687.3±97.1^{ab}	539.7±105.4^{a}	817.2±102.3
Motility (%)	70±0.76^{ab}	75.63±0.5^{b}	68.75±0.99^{ab}	72.5±0.71^{ab}	66.25±1.06^{a}	70.63±1.36
Viability (%)	72.4±1.37^{a}	85±0.82b^{c}	72.4±0.41^{a}	78.2±0.48^{ab}	89±0.74^{c}	79.4±1.04
Abnormality (%)	26±1.05^{c}	10.5±1.75^{a}	18.1±1.32^{abc}	21.2±1.18^{bc}	15.4±1.40^{ab}	18.3±1.94

^{a,b,c} Different letters on the same line indicates statistical difference ($p<0.05$) among phenotypes.

looks at each phenotype, there is a difference (P<0.05) where the spermatozoa of *Jalak* phenotype in RL solution have the most extended longevity (7.75±0.70 d) and *Kuriak* phenotype spermatozoa in RLKT3 solution have the lowest longevity (3.25±0.20 d). The longevity of spermatozoa in local chickens has not been widely reported. Several studies regarding longevity in local chickens have been reported in Kampung chickens with a longevity of more than 4.5 d (Indrawati *et al*., 2013) and 6.7 d(Hardiyanti and Kurniawan, 2020), and in Merawang chickens with a longevity of 4.43–5.93 d (Magfira *et al*., 2017). The longevity of KBC spermatozoa in this study was also not much different from previous reports, with a longevity of 5.03–5.93 d. Research on the longevity of spermatozoa has previously been reported in other animals, such as dogs with 3.5 d (Wicaksono and Arifiantini, 2009) and horses with a longevity of 3-5 d (Bozkurt *et al*., 2007). In their report, Bozkurt *et al*. (2007) said that the longevity of spermatozoa depends on the concentration of spermatozoa and the extender used. Furthermore, Helfenstein *et al*. (2010) added that longevity is also affected by the tail length of spermatozoa, where spermatozoa with a long tail usually show high motility with short longevity. In contrast, spermatozoa with short tails will have extended longevity despite low motility.

Spermatozoa motility of Kokok Balenggek chicken in four different extenders

The results showed that the motility of KBC spermatozoa in Ringer's Lactate solution added with chicken egg yolks and stored at 4–5°C showed a decrease in line with storage time. The decrease percentage every 24 h of storage ranges from 9% to 30%. At 24 h, it was seen that the motility of KBC spermatozoa in each extender decreased. However, treatment with a single Ringer's Lactate solution showed the highest motility (41.13±2.27%). It was feasible to proceed to the artificial insemination stage, where the motility standard for insemination was at least 40% (Arifiantini, 2012). Motility continued to decrease with observation time (48 and 72 h), with motility below 40% and unsuitable for insemination (Table 4).

Decreasing the storage temperature is one of the most commonly used cryopreservation methods. Low temperatures decrease metabolic activity, which in turn can extend the life span of spermatozoa (Barbas and Mascarenhas, 2009). The temperature used in this study was 4–5°C, where at this temperature, the spermatozoa could still carry out metabolic activity even though it was low. In addition, drastic temperature changes can trigger stress on spermatozoa, where there is a change in the lipid structure of the spermatozoa cell membrane (Watson, 2000), so the selection of an extender is essential in the preservation process in order to maintain the longevity and motility of spermatozoa until it is used for artificial insemination.

In this study, the egg yolk used could not maintain the motility of KBC spermatozoa. The egg yolks used in this study were purebred chicken egg yolks. Widiastuti *et al*. (2018) reported that the spermatozoa motility of Pelung chicken diluted using purebred chicken egg yolk was lower than using quail egg yolk and duck egg yolk. So the type of egg yolk used in this study is thought to affect the motility of KBC spermatozoa. In a study conducted by Widiastuti *et al*. (2018), the egg yolk concentration used was 10%, while the highest concentration in this study was only 5%. So it is necessary to conduct further research by increasing the concentration of egg yolk and the type of egg yolk used to study the characteristics of KBC liquid semen.

In general, the protein content in chicken spermatozoa membranes is as much as 50%, which besides acting as an energy source, this protein consists of integral (intrinsic) proteins and peripheral (extrinsic) proteins, which also play a role in regulating inter-cell communication (Tarvis, 2013). The presence of phospholipid-H_2O influences cell membrane transport, while recognition between cells is influenced by

Tabel 3. Longevity of spermatozoa (days) in various extenders in different KBC phenotypes

Extenders	Phenotypes					Average
	Biriang	*Jalak*	*Kinantan*	*Kuriak*	*Taduang*	
RL	4.88±0.56^{bc}	7.75±0.70^{bc}	6.88±0.27^{c}	4.38±0.21^{a}	5.75±0.23^{ab}	5.93±1.25
RLKT1	7.38±0.53^{bc}	6.63±0.28^{bc}	7.13±0.29^{c}	4.75±0.21^{a}	3.75±0.10^{ab}	5.93±1.42
RLKT3	5.63±0.50^{bc}	5.38±0.23^{bc}	6.63±0.38^{c}	3.25±0.20^{a}	4.25±0.19^{ab}	5.03±1.17
RLKT5	6.50±0.29^{bc}	5.25±0.16^{bc}	5.88±0.40^{c}	4.13±0.21^{a}	5.13±0.30^{ab}	5.38±0.79

RL = Ringer's Lactate solution; RLKT1 = Ringer's Lactate solution + Egg Yolk 1%; RLKT3 = Ringer's Lactate solution + Egg Yolk 3%; RLKT5 = Ringer's Lactate solution + Egg Yolk 5%.
^{a,b} Different letters on the same line indicates statistical difference (p<0.05) among phenotypes.

Tabel 4. Motility of KBC spermatozoa (%) in various extenders

Extenders	Observation time			
	0h	24h	48h	72h
RL	70.63±0.84^{Aa}	41.13±2.27^{aB}	29.37±1.90^{aC}	19.00±1.52^{aD}
RLKT1	68.5±0.83^{Aab}	39.00±1.84^{aBb}	26.88±1.49^{abC}	15.88±1.12^{abD}
RLKT3	68.25±0.78^{Ac}	34.00±1.74^{Bc}	19.37±1.40^{Cc}	10.5±0.92^{cD}
RLKT5	67.25±0.96^{Abc}	37.75±1.75^{Bbc}	23.25±1.43^{bCc}	14.75±1.07^{bcD}

RL = Ringer's Lactate solution; RLKT1 = Ringer's Lactate solution + Egg Yolk 1%; RLKT3 = Ringer's Lactate solution + Egg Yolk 3%; RLKT5 = Ringer's Lactate solution + Egg Yolk 5%.
^{A,B,C,D} Different letters indicate differences in motility in each hour of observation (p<0.05), and ^{a,b,c} letters indicate differences in motility in each type of extender (p<0.05).

glycocalyx (Hammerstedt and Graham, 1992). Egg yolk added to the diluent should be able to support cells in maintaining their viability and motility. This is because egg yolks contain phospholipids which play a role in replacing cell membrane phospholipids damaged by cold shock. However, in this study, the addition of egg yolk in diluent was not able to maintain the motility of KBC spermatozoa. Al-Ahmad *et al.* (2008) reported that the granules contained in egg yolk could prevent metabolic turnover, and its sustainability would decrease spermatozoa motility. Vera-Munoz *et al.* (2011) added that diluent added with LDL taken from egg yolks was found to be more able to maintain spermatozoa motility than whole egg yolks. This is because the complex chemical content in egg yolks has disappeared and is no longer in contact with spermatozoa. This is reinforced in research conducted by Magfira *et al.* (2017), where administration of LDL isolated from egg yolks is better able to maintain motility than using egg yolks. The failure to maintain spermatozoa motility in this study is also thought to be related to changes in pH during the preservation period. In this study, the storage temperature used was 4–5°C, at which the spermatozoa were still active and metabolizing and would lower the pH of the diluent. Furthermore, the time used in this study is also thought to have contributed to the increase in spermatozoa mortality. Dead spermatozoa will have a toxic effect on surviving spermatozoa and lower the pH of the diluent, so the longer the storage time, the higher the mortality rate of spermatozoa (Solihati *et al.*, 2006).

Spermatozoa of the Jalak phenotype showed lower motility reduction in each diluent than other KBC phenotypes ($p<0.05$). The decrease in spermatozoa motility of *Jalak* phenotype was 7.5–23.75% in every hour of observation, with the lowest decrease in motility occurring in a single Ringer's Lactate solution in a shelf life of 24 48 h. Meanwhile, the highest decrease in motility was found in Ringer's Lactate solution, which was added with 5% egg yolk during a shelf life of 0–24 h. Furthermore, the highest range of decreased spermatozoa motility was seen in the *Kinantan* phenotype, ranging from 4.37 to 49.37% in each hour of observation. The lowest decrease in spermatozoa motility was found in Ringer's Lactate solution added with 5% egg yolk during a shelf life of 24–48 h, while the highest decrease in motility was found in Ringer's Lactate solution added with 3% egg yolk during a shelf life of 0 – 24 h (Figure 1).

The motility of KBC spermatozoa diluted using four different extenders did not show a significant difference during storage ($p>0.05$). It tended to decrease in motility with a relatively high range of decline. Chickens from different phenotype (Figure 2) may show differences in

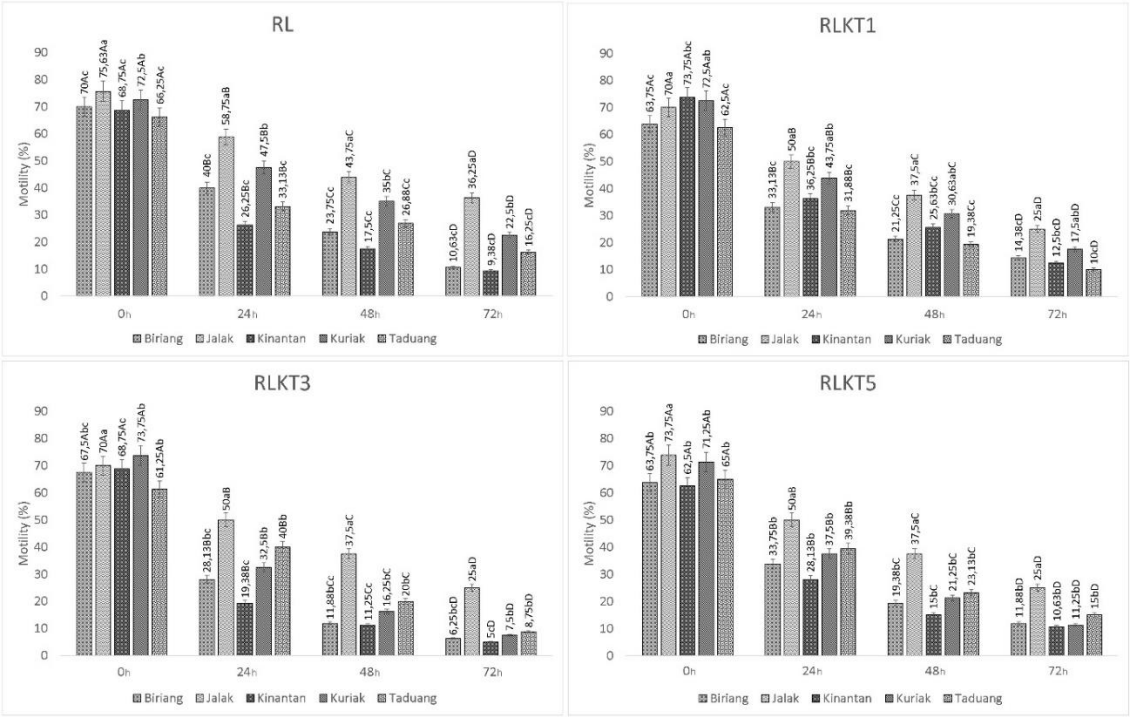

Figure 1. Motility of KBC spermatozoa stored at 4 – 5°C for 72 h using Ringer's Lactate solution without/with egg yolk. Different letters A, B, and C on the same bar indicate differences in motility in each hour of observation ($p<0.05$), and letters a, b, and c indicate differences in motility in each phenotype ($P<0.05$). RL = ringer's lactate solution; RLKT1 = ringer's lactate solution + egg yolk 1%; RLKT3 = ringer's lactate solution + egg yolk 3%; RLKT5 = ringer's lactate solution + egg yolk 5%.

Figure 2. Kokok Balenggek chicken phenotypes: A. *Biriang*; B. *Jalak*; C. *Kinantan*; D. *Kuriak*; E. *Taduang*.

resistance to preservation processes (Siudzińska and Łukaszewicz, 2008). KBC sperm of *Jalak* phenotype had better motility than from other phenotype groups. It is suspected that the *Jalak* phenotype has carotenoids which act as antioxidants to fight free radicals during storage. Yellow, orange, and red pigmentation in most birds results from the production of carotenoids (Koch *et al*., 2016). Carotenoids are non-enzymatic antioxidants that can function to protect sperm from oxidative stress (Triques *et al*., 2019). Carotenoids are located in the inner mitochondrial membrane, which is closely related to the function of mitochondria as a place for aerobic cell respiration (Hill *et al*., 2019). The carotenoid content suspected to be present in the KBC phenotype of the *Jalak* phenotype can protect sperm from damage caused by free radicals so that it has the lowest abnormality compared to the other three phenotypes.

Poultry semen is very sensitive to preservation due to the different morphology of spermatozoa compared to mammalian spermatozoa. The smaller surface of spermatozoa causes avian spermatozoa to be more sensitive to osmotic pressure (Long, 2006). The low motility of spermatozoa in this study could be caused by several things, such as the extender being incompatible with KBC semen. Some extenders that are often used in chicken semen include Bestville Poultry Semen Extender/BPSE (Telnoni *et al*., 2017), Tris-Skim Milk (Bustani and Baiee, 2021), Ringer Lactate-Low Density Lipoprotein chicken or quail (Magfira *et al*., 2017), and a combination of coconut water, egg yolk, and fructose (Rochmi and Sofyan, 2019). Another possibility is that KBC has low freezability. Different types of chickens are reported to produce differences in amino acid content in seminal plasma (Santiago-Moreno *et al*., 2019).

Conclusions

The most extended longevity was found in KBC spermatozoa with the *Jalak* phenotype, which was diluted using single Ringer's Lactate solution. Administration of egg yolk in Ringer's Lactate solution was not able to maintain the motility of KBC spermatozoa when stored at 4–5°C for 48–72 h.

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