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EFFECT OF VARIOUS AVIAN EGG YOLK TYPES ON THE VIABILITY AND LONGEVITY OF POST-THAWED RESIQUIMOD-SEXED SPERMATOZOA IN SAPERA GOATS

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ABSTRACT

Improving the reproductive efficiency of Sapera goats through artificial insemination necessitates optimal quality of sexed frozen semen. This study aimed to determine the effect of various avian egg yolk types on the viability and longevity of post-thawed Resiquimoid-sexed Sapera goats spermatozoa. The research used a Completely Randomized Design (CRD) with four egg yolk types: Commercial Chicken (T1), Native Chicken (T2), Quail (T3), and Duck (T4) as treatments and five replications. Data were analyzed using One-Way ANOVA and Duncan's Multiple Range Test. The results indicated that post-thaw viability ranged from 53.30–64.90% in the upper layer, while longevity ranged from 26.17–29.46 hours in the upper layer and 25.11–28.95 hours in the lower layer, showed no significant differences among treatments. Conversely, the egg yolk type significantly influenced viability in the lower layer ($P < 0.05$). Specifically, Native Chicken (T2), Duck (T4), and Commercial Chicken (T1) egg yolks maintained superior and stable viability (60.90%, 59.80%, and 56.20%), whereas Quail egg yolk (T3) exhibited significantly lower performance (52.40%). These findings suggest that the choice of egg yolk is critical for maintaining the sperm viability of the lower layer in Resiquimod-based sexed semen.

Keywords : *Sapera Goats, Sexing, Egg Yolk, Viability, Longevity.*

Introduction

Enhancing the reproductive efficiency of goats has direct implications for food security and rural economics in Indonesia. With a continuously growing goat population reaching approximately 15,710,055 (BPS, 2024), technological innovation is highly demanded. The Sapera Goat, recognized as a superior dairy breed, is a primary focus in national genetic improvement programs. Sperm sexing technology offers a strategic opportunity to accelerate this genetic progress and increase dairy productivity by selectively directing the production of female offspring with high genetic merit.

The Resiquimod (R848)-based sexing method demonstrates significant potential in enriching the proportion of X-bearing spermatozoa, which is crucial for dairy production. R848 functions as a toll-like receptor 7/8 (TLR7/8) ligand capable of separating X and Y chromosome-bearing sperm. However, the application of this method for Artificial Insemination (AI) requires high-quality frozen semen. The cryopreservation and thawing processes are known to induce severe stress on spermatozoa, causing oxidative stress, internal ice crystal formation, and lipid peroxidation of the cell membrane. These structural damages directly reduce sperm viability and longevity. Therefore, developing an extender formulation capable of mitigating these negative impacts is highly essential.

Tris-Egg Yolk (TEY) extenders are commonly used to protect the sperm cell membrane. The lipoproteins and lecithin contained in the egg yolk stabilize the membrane and reduce cellular damage during cryopreservation. The lipoproteins and lecithin contained in the egg yolk stabilize the membrane and reduce cellular damage during cryopreservation. However, the specific protective lipid profiles vary significantly among avian species. Duck yolk contains a high proportion of lecithin (Quraini et al., 2022) and Native Chicken yolk provides complex phospholipids (Buan et al., 2026). Meanwhile, Quail yolk is characterized by a dense cholesterol profile (Maapola et al., 2023) and standard Commercial Chicken yolk has a lighter lipid composition (Anisah et al., 2024). These distinct macronutrient differences may offer varying degrees of protection during cryopreservation. For

instance, Duck yolk in a Tris-based extender has been reported to yield higher post-thaw viability compared to other formulations (Umami et al., 2015). Although the R848 swim-up method has been successfully applied to separate ram sperm by specifically reducing X-sperm ATP and motility (Setiawan et al., 2024), there is a lack of comprehensive studies evaluating its post-thaw interaction with different avian egg yolk types in Sapera goats. The metabolic suppression induced by R848 during the sexing process may alter sperm sensitivity, necessitating a specific lipid profile for optimal post-thaw protection.

Therefore, this study aimed to determine the comparative effect of various avian egg yolk types (Commercial Chicken, Native Chicken, Quail, and Duck) in Tris extenders on the viability and longevity of post-thawed R848-sexed Sapera Goat spermatozoa. Finding the most compatible egg yolk type is expected to optimize post-thaw quality and maximize the success rate of AI in Sapera Goats.

Materials and Methods

Extender Preparation and Media Formulation

The basic Tris-citrate buffer was prepared by dissolving 0.03 M Tris(hydroxymethyl) aminomethane, 0.0095 M Citric acid monohydrate, and 0.0028 M Glucose into double-distilled water, followed by sterilization. The medium was then supplemented with 20% (v/v) of avian egg yolk sourced from four different species (Commercial Chicken, Native Chicken, Quail, and Duck), representing the four experimental treatments. Penicillin and Streptomycin were added to the mixture to prevent bacterial contamination. Additionally, glycerol (6% v/v) was incorporated as the permeating cryoprotectant. The final extender was then homogenized using a magnetic stirrer for 30 minutes.

Semen Collection and R848-Based Sexing Procedure

Fresh semen was collected twice a week using an artificial vagina from a 3-year-old Sapera Buck (maintained on a daily diet of concentrate, fresh forage and corn silage). Due to limited semen volume per ejaculate, evaluating all treatments required pooling samples across two separate collection sessions to form one complete replicate. Thus, a total of 10 ejaculates were systematically grouped into 5 replications. Only ejaculates that met the minimum quality standards (mass movement ++, progressive motility ≥ 70%, and high concentration) were used for the experiment. To perform sexing, the semen was washed via centrifugation to remove seminal plasma. The sperm pellet was diluted in the Tris-citrate-glucose buffer containing 0.1 μM of Resiquimod (R848) to selectively suppress the motility of X-bearing spermatozoa. The mixture was incubated in a water bath at 37°C for 40 minutes at a 45° angle to allow the highly motile Y-bearing spermatozoa to swim up. Following incubation, the top fraction (Y-enriched) and the bottom fraction (X-enriched) were carefully collected into separate tubes.

Cryopreservation and Thawing Process

Both separated fractions were centrifuged, and the resulting pellets were diluted with the four respective Tris-avian egg yolk extenders. The diluted semen was equilibrated in a refrigerator at 5°C for 4 hours to prevent cold shock. Subsequently, the semen was packaged into 0.25 mL mini straws and sealed using PVA powder. Pre-freezing was conducted by suspending the straws 10 cm above liquid nitrogen (approximately –110°C) for 10 minutes, before plunging them directly into liquid nitrogen (–196°C) for long-term storage. For post-thaw evaluation, the straws were thawed using a digital thawing device at a constant 37°C for 15–30 seconds.

Evaluation of Sperm Quality

Viability

Sperm viability was assessed by placing a drop of thawed semen on a glass slide, mixed with a drop 2% eosin stain, and smeared. The slide was fixed over a Bunsen flame and observed under a microscope at 400× magnification. Spermatozoa that absorbed the stain (pink/red) were considered dead, while unstained ones were recorded as live. A total of 200 spermatozoa were counted per slide. The viability percentage was calculated using the following formula (WHO, 2010):

$$Viabilitas\ (\%) = \frac{Number\ of\ live\ spermatozoa}{Total\ observed\ spermatozoa} \times 100$$

Longevity

Longevity was determined by storing the thawed semen at room temperature and observing the progressive motility periodically until it reached 0%, up to a maximum of 24 hours. The time elapsed until sperm motility ceased completely was estimated using a linear regression equation (Sugiyono, 2007):

$$Y = bX + a$$

Where:

Y : Dependent variable, representing sperm motility (%).

X : Independent variable, representing incubation time (hours).

b : Slope/Gradient, representing the rate of motility degradation over time.

a : Intercept, representing the initial sperm motility.

To determine the absolute longevity, defined as the estimated time when sperm motility completely ceases (where Y = 0), the formula was derived as follows:

$$Y = \frac{a}{b}$$

Experimental Design and Statistical Analysis

The study was conducted using a Completely Randomized Design (CRD) with four treatments: Commercial Chicken (T1), Native Chicken (T2), Quail (T3), and Duck (T4) egg yolks at a constant 20% level with five replications. The data for viability and longevity of both top and bottom fractions were statistically analyzed using a One-Way Analysis of Variance (ANOVA) at a 95% confidence level. Significant differences between treatment means ($P < 0.05$) were further analyzed using Duncan's Multiple Range Test (DMRT) with the following formula (Steel & Torrie, 1991):

$$LSR = SSR \times S_{\bar{y}}$$

$$S_{\bar{y}} = \sqrt{\frac{MSE}{r}}$$

Where:

LSR : Least Significant Range,

SSR : Studentized Significant Range value,

$S_{\bar{y}}$: Standard Error,

MSE : Mean Square Error from ANOVA,

r : Number of Replications.

Results and Discussion

Fresh Semen Quality Assessment

Fresh semen quality assessment is a crucial step to ensure the eligibility of spermatozoa before undergoing sexing and cryopreservation. The average semen volume reached 1.19 mL per ejaculate, which is higher than the findings of Hidayati et al. (2018) who reported an average volume of 0.95 mL. The semen color was cream with a viscous consistency and a typical odor, indicating the absence of contamination during collection (Achmad et al., 2024). The pH level was recorded at 6.15. These characteristics indicate that the buck's health and rearing management are excellent, meeting the general eligibility criteria for fresh semen.

Table 1. Fresh Semen Assessment Results of Sapera Goats (n = 10 ejaculates)

Parameters	Averages
Volume (mL)	1.19 ± 0.32
Color	Cream
Odor	Typical
Consistency	Viscous
pH	6.15 ± 0.32
Mass Movement	+++
Sperm Concentration Total (10^7 cells/mL)	404.80 ± 113.40
Motility (%)	92.50 ± 3.17
Viability (%)	91.85 ± 4.72

Microscopic evaluation also demonstrated highly superior spermatozoa quality for further processing. The mass movement was recorded as very active (+++), exhibiting large and dense waves. The average percentage of individual motility and viability were exceptionally high at 92.50% and 91.85%, which are superior to the findings in previous studies on Sapera Goats (Hidayati et al., 2018). With a concentration reaching 404.80×10^7 cells/mL, the overall fresh semen quality exceeded the minimum standards required for sexing and cryopreservation procedures, which necessitate a minimum mass movement of (++) and progressive motility of ≥ 70% (Wurlina et al., 2020).

Effect of Avian Egg Yolk Types on Post-Thaw Sperm Viability

The observation of post-thaw goat sperm viability following Resiquimod (R848)-based sexing was conducted using Tris extender combined with various avian egg yolk types at a constant 20% level. To accurately map the physiological changes, the evaluation was separated into two distinct fractions, namely the upper and lower layers. This separated observation is highly crucial to determine the specific protective efficacy of different egg yolk lipids on the sexed sperm subpopulations.

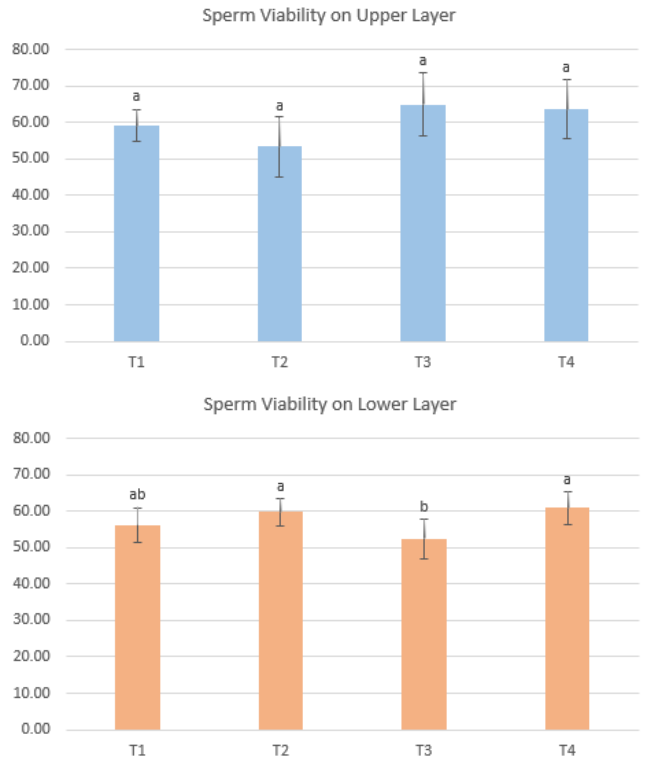

Figure 1. Sperm Viability

Notes:
T1 = Commercial Chicken Egg Yolk,
T2 = Native Chicken Egg Yolk,
T3 = Quail Egg Yolk,
T4 = Duck Egg Yolk

Based on the analysis of variance (ANOVA), there was no significant difference in the viability of the upper layer across the different types of egg yolk (Figure 1). This uniform response is closely related to the physiological characteristics of the spermatozoa post-sexing. Spermatozoa in the upper layer possess smaller and lighter heads, which leads to much faster movement and higher energy consumption (Rasad et al., 2020). Consequently, they demand a massive supply of extracellular nutrients to maintain their high metabolism (Esmail et al., 2023; Saifudin et al., 2018). The high 20% level effectively provided sufficient basic protection and energy substrates across all treatments, aligning with the fact that macronutrient compositions among avian eggs share general functional similarities despite having different absorption efficiencies (Bashir et al., 2015).

In contrast, the lower layer exhibited a highly specific protective response, where Duck (T4) and Native Chicken (T2) maintained significantly higher viability (60.90% and 59.80%, respectively) compared to Quail egg yolk (T3) which performed poorly (52.40%). Meanwhile, Commercial Chicken (T1) demonstrated an intermediate viability (56.20%) that was not statistically different from the other treatments. X-bearing spermatozoa in this lower layer undergo metabolic suppression and significant ATP depletion due to the R848 compound binding to TLR7/8 receptors during the sexing process (Setiawan et al., 2024; Umehara et al., 2019). This cellular stress makes their membrane defense highly dependent on the quality of extracellular protectants. Duck yolk, which is reported to have a high lecithin proportion (Quraini et al., 2022), and Native Chicken yolk, presumably containing complex phospholipids (Buan et al., 2026), likely provided superior membrane fluidity to prevent cell tearing. Conversely, Quail egg yolk has been associated with a high phosphatidylcholine ratio (Maapola et al., 2023) and a dense cholesterol profile (Anisah et al., 2024). At a 20% level, this lipid density presumably increases the media viscosity, which may physically hinder the paralyzed lower-layer spermatozoa from clearing their cellular metabolic waste (Sofa et al., 2023). This blockage could trigger rapid lactic acid accumulation and lower the media pH, ultimately accelerating the cell death of the suppressed spermatozoa in this layer (Rasad et al., 2020).

Effect of Avian Egg Yolk Types on Post-Thaw Sperm Longevity

The observation of post-thaw goat sperm longevity following Resiquimod (R848)-based sexing was conducted using Tris extender combined with various avian egg yolk types at a constant 20% level. To accurately map the physiological changes, the

evaluation was separated into two distinct fractions, namely the upper and lower layers. This separated observation is highly crucial to determine the specific protective efficacy of different egg yolk lipids on the sexed sperm subpopulations.

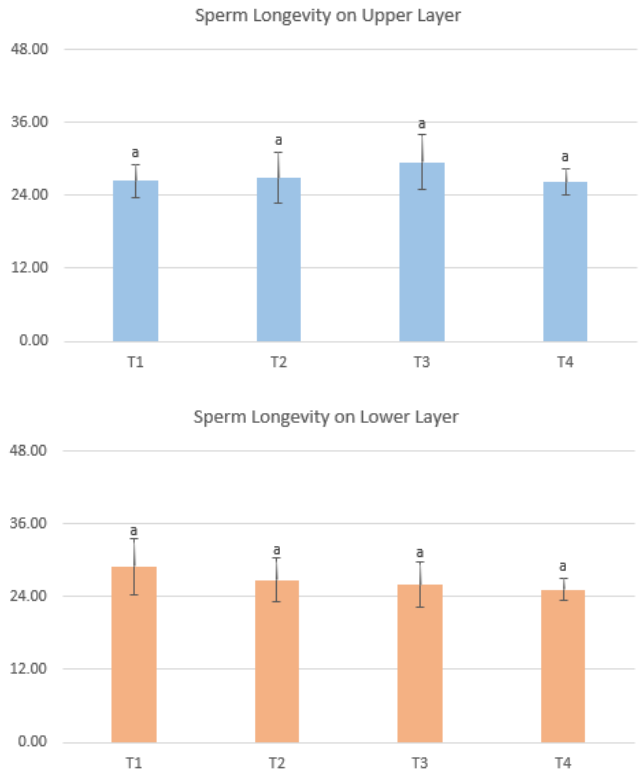

Figure 2. Sperm Longevity on Upper Layer

Notes:
T1 = Commercial Chicken Egg Yolk,
T2 = Native Chicken Egg Yolk,
T3 = Quail Egg Yolk,
T4 = Duck Egg Yolk

Based on the analysis of variance (ANOVA), there was no significant difference in the longevity of the upper layer across the different types of egg yolk (Figure 2). Hyperactive Y spermatozoa in this fraction have massive metabolic demands and high lactic acid production. However, the 20% level proved capable of providing abundant buffering agents to neutralize the acidic environment toxicity regardless of the egg yolk source (Rochmi & Sofyan, 2019). This uniform significance confirms that the similar proportion of basic energy-providing nutrients across avian species guarantees a highly adequate duration of protection for Y spermatozoa at this maximum level (Bashir et al., 2015).

A similarly uniform significance pattern was observed in the lower layer, where all egg yolk types showed no significant differences in maintaining longevity. Spermatozoa X in this layer experience metabolic suppression by the R848 compound, which minimizes early-phase energy usage (Umehara et al., 2019). Nevertheless, this dormancy phase still requires a continuous intake of protective molecules to prevent membrane lysis over time. Although Commercial Chicken yolk is reported to possess a lighter and less dense lipid characteristic compared to other avian eggs (Anisah et al., 2024; Buan et al., 2026), the maximum 20% level successfully compensated for this limitation by providing a sustainable and concentrated macronutrient supply. Consequently, all tested avian egg yolks demonstrated an equally excellent capacity to supply basal energy and preserve the lifespan of post-thaw X spermatozoa (Câmara et al., 2019).

Limitations of the Study

This study utilized semen from a single Sapera Buck. Thus, the five replicates represent repeated measures rather than independent biological replicates. Consequently, the observed protective efficacies of the egg yolks may be partially influenced by this individual's specific physiological traits. Future studies involving multiple bucks are necessary to ensure the generalizability of these findings across the Sapera Goats population.

Conclusion

In conclusion, the use of various avian egg yolk types at a 20% level provides an equally effective protective capacity for the viability of upper-layer spermatozoa, as well as the longevity of spermatozoa in both layers. However, regarding the viability of lower-layer spermatozoa, Duck (T4) and Native Chicken (T2) egg yolks demonstrated superior protection compared to Quail egg yolk (T3), while Commercial Chicken (T1) showed an intermediate capacity. Therefore, Duck and Native Chicken egg yolks at a 20% level are recommended as the most optimal extenders for the cryopreservation of R848-sexed goat semen, as they comprehensively accommodate the viability and longevity requirements of both spermatozoa layers.

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