

Comparative evaluation of different semen extenders on reproductive performance in Nunukan chickens

Fikri Ardhani*, I Made U. Raharja, Bryta Mbincar, Jusyenti Manullang, Julinda R. Manullang, Novemia Fatmarischa

Animal Science Department, Agriculture Faculty, Mulawarman University, Jl. Paser Balengkong Kampus Gunung Kelua Samarinda, 75123, Kalimantan Timur, Indonesia.

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*Correspondence:

Corresponding author: Fikri Ardhani
E-mail address: fikri_ardhani@faperta.unmul.ac.id

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ABSTRACT

This study evaluated the effectiveness of five different semen extenders on sperm quality and reproductive performance in Nunukan chickens, an indigenous breed from North Kalimantan, Indonesia, to support conservation through artificial insemination protocols. Semen was collected from adult Nunukan roosters (10-12 months) and diluted with five extenders: NaCl 0.9% (T1), Ringer's solution (T2), NaCl + 15% egg yolk (T3), Ringer's solution + 15% egg yolk (T4), and Beltsville Poultry Semen Extender (BPSE) (T5). Sperm motility and viability were assessed every 30 minutes for 180 minutes during storage at 4°C. Artificial insemination was performed on 25 hens (5 per treatment) using 0.1 mL diluted semen containing 1×10^8 active spermatozoa after 1-hour storage when motility and viability remained high, and fertility and hatchability rates were evaluated. BPSE demonstrated superior sperm preservation, maintaining 73% motility and 82% viability after 180 minutes, while egg yolk-supplemented extenders showed intermediate performance (NaCl+EY: 35% motility, 32% viability; Ringer's+EY: 38% motility, 35% viability) and basic extenders performed poorly (NaCl: 12% motility, 18% viability; Ringer's: 15% motility, 22% viability). Fertility rates ranged from 74-83% across treatments with no significant differences ($p > 0.05$), while hatchability rates varied from 61-74%, with BPSE achieving 73% comparable to natural mating (74%). All extenders preserved reasonable reproductive performance for short-term storage, particularly BPSE, which provided optimal preservation, while egg yolk-supplemented formulations served as practicable alternatives for artificial insemination in breeding programmes for Nunukan chickens.

Introduction

Nunukan chicken is a typical poultry breed from Kalimantan in Indonesia, which is noted for its adaptability to more tropical environments as well as for its genetic diversity (Alwi *et al.*, 2014). This local breed is facing genetic erosion because of interbreeding with more commercial breeds, which makes it necessary to undertake conservation using reproductive biotechnologies. Artificial insemination (AI) offers a practical solution for genetic preservation while improving breeding efficiency and reducing male-to-female ratios in poultry production (Aklilu, 2025; Long, 2006).

To preserve spermatozoa, appropriate extenders which maintain their viability and motility during storage are necessary. More basic extenders like sodium chloride (NaCl) and Ringer's solution provide osmotic balance and electrolytic as well as ionic supports, while more specialised ones like BPSE offer a more comprehensive protective effect for the sperm (Arif *et al.*, 2025). The addition of egg yolk improves the extender's activity, which can be partially attributed to the ingredient of egg yolk, which is lecithin. It can stabilise the membrane of the spermatozoa and prevent damage during freezing properties (Widiastuti *et al.*, 2018; Łukaszewicz *et al.*, 2023). It has been shown in various studies that egg yolk at a concentration of 15 to 20% significantly enhances the quality of the spermatozoa and other related parameters during storage (Swelum *et al.*, 2018; Widiastuti *et al.*, 2018).

This study focused on Nunukan chicken sperm motility, viability, fertility rate, and hatchability with five extender treatment groups including NaCl, NaCl with egg yolk phosphate, Ringer's solution, Ringer's solution with egg yolk phosphate, and BPSE. This research aimed to evaluate the impact of various extenders on the sperm motility and fertility of Nunukan chickens alongside their reproductive efficiency, utilising artificial insemination as a method to biocontrol the genetic materials of indigenous chickens in Indonesia.

Materials and methods

This study utilised adult Nunukan cocks, 10 to 12 months of age,

with a live body weight of 1.5 to 2.0 kg. The cocks were kept in single battery cages measuring 60 × 50 × 45 cm. The hens were kept under natural lighting conditions. In addition, each cock was given feed formulated to contain 18% crude protein, 2,800 kcal/kg metabolisable energy, and fresh water, both of which were offered ad libitum. Cocks underwent a two-week acclimatisation period prior to the commencement of semen collection.

Semen was collected from individual roosters every three days using the abdominal massage technique. Prior to collection, the cloacal area was cleaned with sterile saline solution. Semen samples were collected in pre-warmed sterile tubes and immediately transported to the laboratory for evaluation and processing. Only ejaculates with volume ≥ 0.2 mL, concentration $\geq 2.5 \times 10^9$ sperm/mL, and initial progressive motility $\geq 80\%$ were used for further processing.

Fresh semen quality was assessed using conventional methods (Arif *et al.*, 2025). Volume was measured using graduated micropipettes and recorded in milliliters. Sperm concentration was determined using a hemocytometer with 1:200 dilution in formal saline solution, calculated and expressed as $\times 10^9$ sperm/mL. Progressive motility was evaluated subjectively under light microscopy (400× magnification) by placing 10 μ L of diluted semen (1:10 with physiological saline) on a pre-warmed glass slide. Progressive motility was assessed as the percentage of spermatozoa showing forward progressive movement. Viability was determined using eosin-nigrosin staining technique where a thin smear of semen was prepared, stained, and examined under light microscopy (1000× magnification). Live spermatozoa (unstained) and dead spermatozoa (pink/red stained) were counted from 200 spermatozoa per sample.

Five extender treatments were prepared and evaluated: T1 (Sodium chloride 0.9%), T2 (Ringer's solution), T3 (NaCl 0.9% + egg yolk phosphate 15%), T4 (Ringer's solution + egg yolk phosphate 15%), and T5 (Beltsville Poultry Semen Extender). Egg yolk was obtained from fresh duck eggs, separated aseptically, and added at 15% (v/v) concentration to the respective base extenders following the method described by Santiago-Moreno *et al.*, (2012) and Widiastuti *et al.* (2018). Fresh semen was diluted 1:4 (v/v) with each extender and stored at 4°C. Semen quality

parameters were evaluated immediately after dilution (0 minute) and at 30-minute intervals for 180 minutes of storage.

Nunukan hens (n = 25, 5 hens per treatment) aged 6-10 months were used as recipients for artificial insemination. Each hen received 0.1 mL of diluted semen containing approximately 1×10^8 active spermatozoa via intravaginal insemination using sterile plastic syringes. Insemination was performed every 5 days for three consecutive cycles. Eggs were collected daily for 10 days following the last insemination and stored at 15-18°C with 75-80% relative humidity before incubation. A total of 125 eggs (25 eggs per treatment) were set in a forced-draft incubator maintained at 37.5°C with 60% relative humidity.

Fertility assessment was conducted by candling eggs on day 7 of incubation to determine fertility status. Fertile eggs showed visible blood vessels and embryonic development, while infertile eggs appeared clear without vascular development. For hatchability assessment, eggs were transferred to hatching trays on day 18 of incubation and humidity was increased to 65%. Hatching was recorded on day 21, and reproductive parameters were calculated as follows:

Fertility Rate (%) = (Number of fertile eggs / Total eggs set) $\times$ 100; and Hatchability (%) = (Number of chicks hatched / Number of fertile eggs) $\times$ 100.

Data were analyzed using analysis of variance (ANOVA) in a completely randomized design with repeated measures for time points. Duncan's multiple range test was used for mean separation when significant differences were detected ($p < 0.05$). All statistical analyses were performed using GraphPad Prism version 9.0 software.

Results and Discussion

The comparative evaluation of different semen extenders on Nunukan chicken spermatozoa quality parameters revealed distinct performance patterns across progressive motility and viability measurements during the 180-minute storage period at 4°C (Figures 1). BPSE demonstrated superior performance as the positive control, maintaining the highest values throughout the storage period with 73% progressive motility and 82% viability at 180 minutes, establishing the benchmark for extender effectiveness. Basic extenders without supplementation exhibited the lowest performance, with NaCl and Ringer's solution retaining only 12% and 15% motility, and 18% and 22% viability respectively at the final time point, indicating rapid deterioration of spermatozoa quality parameters. Egg yolk-supplemented extenders showed intermediate performance, with NaCl_EY maintaining 35% motility and 32% viability, while Ringer's_EY preserved 38% motility and 35% viability after 180 minutes, demonstrating moderate protective effects that significantly improved preservation compared to their basic counterparts while remaining inferior to the specialized BPSE formulation.

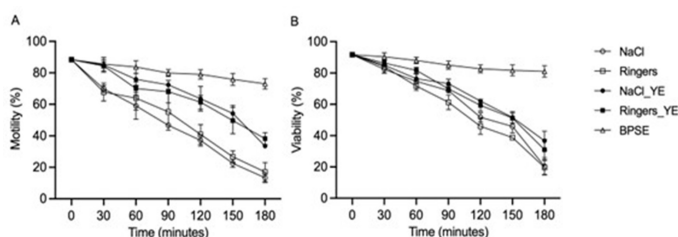


Fig. 1. Temporal Changes in Sperm Motility (A) and Viability (B) of Nunukan Chickens Stored in Five Different Extenders. Data (Mean $\pm$ SEM) are from five independent experiments.

The differential reproductive performance observed among various extender formulations directly reflects their capacity to maintain essential spermatozoa quality parameters critical for successful artificial insemination. At 4°C storage temperature, cellular metabolism is significantly reduced but not completely arrested, creating a critical balance between preserving cellular function and minimizing metabolic stress (Long, 2006;

Blesbois, 2007). Under these hypothermic conditions, spermatozoa face complex metabolic challenges as cellular processes slow down while energy demands for maintaining membrane integrity and basic cellular functions continue, albeit at reduced rates.

The distinct advantages BPSE yielded demonstrate its suitability in comparison to other extenders and as a specialised poultry semen extender. BPSE provides glucose, amino acids, and buffering agents, which strengthen metabolism on multiple levels. BPSE provides glucose for glycolysis, amino acids needed for protein synthesis, and buffering agents for pH balance which fulfils some of the most basic energy substrate needs for spermatozoa during storage (Arif *et al.*, 2025). This is the reason why BPSE is able to retain over 70% of both motility and viability parameters. Nutrients are supplied to essential cell metabolism, even in a quiescent state at 4°C. Apart from membrane stabilisers, BPSE also contains antioxidants which help with oxidative stress damage that builds over storage time, and membrane stabilisers that maintain cell structure during the storage period.

The moderate performance of extenders supplemented with egg yolk is considerably better than basic formulations due to membrane-protective components and minimal energy substrates. Egg yolk is rich in low-density lipoproteins (LDL) and lecithin, which offer cryoprotective stabilising membrane properties vital in preserving the structural integrity of spermatozoa under hypothermic storage (Swelum *et al.*, 2018; Widiastuti *et al.*, 2018). Moreover, egg yolk participates in supplying energy substrates in the form of phosphatidylcholine and other phospholipids which may sustain some cellular metabolism at 4°C. The 15% egg yolk concentration in this study was optimal in thermodynamic protective effects while balancing osmotic stress, which is evidenced by the improved outcomes of both NaCl_EY and Ringer's_EY treatments compared to their basic counterparts. However, the gradual decline observed in these treatments suggests that some level of metabolism and comprehensive metabolic support systems, membrane stabilisation, and limited energy source will provide only moderate preservation capacity for prolonged storage (Łukaszewicz *et al.*, 2023).

Basic extenders showed insufficient preservation of reproductive parameters, underscoring the importance of energetics and metabolic support in sustaining sperm quality. NaCl and Ringer's solutions provide osmotic support, but they do not fulfil the essential metabolic needs of the spermatozoa (Long, 2006). At storage temperatures below 4°C, spermatozoa within these basic extenders must depend entirely on their scant endogenous energy reserves, which consist mainly of intracellular ATP and glycogen. As storage continues, further cellular energy metabolism will cease to sustain vital cellular functions, including the maintenance of cellular membrane potential, functioning of ion pumps, and structural protein complex stability (Arif *et al.*, 2025). The swift deterioration of motility and viability in these treatments illustrates how the depletion of energy substrates leads to mounting membrane instability and cellular dysfunction which ultimately results in a loss of fertilisation capacity.

The interdependence in the tendencies of progressive motility and viability suggests the cohesive relationship between them as markers in the assessment of spermatozoa quality and their reproductive potential (Surai *et al.*, 2000). This correlation reflects the fundamental biological processes that underlie the fertilisation potential in Nunukan chickens since semen extenders preserve sperm by maintaining its structural characteristics, motility and viability, as well as membranes and acrosomes (Bustani & Baiee, 2021). For successful outcomes in artificial insemination, optimum values for both motility and viability are crucial, as the former allows for the movement of spermatozoa, and the latter, cellular integrity necessary for fertilisation (Donoghue & Wishart, 2000).

Effective artificial insemination relies on properly choosing semen extenders, which has a major impact on the preservation of reproductive parameters. Specialized extenders, such as BPSE, seem to be crucial for preserving fertilisation potential because of their comprehensive metabolic support. Bustani & Baiee (2021) described BPSE's support as favour-

able pH, ATP, and anti-cooling and freeze shock and antioxidant activity. BPSE also showed high fertility rates of 88% in white leghorn chickens (Telnoni *et al.*, 2016). Some formulations that are supplemented with egg yolk seem to have moderate effectiveness. Telnoni *et al.* (2016) noted that these egg yolk-supplemented formulations are intermediate because they offer some membrane protection, albeit limited energy substrate. The poor performance of basic extenders confirms these extenders' unsuitability for practical reproductive use and their lack of adequate support for cellular metabolism under hypothermic storage conditions. Saint Jalme *et al.* (2003) noted that the sperm cell membrane is quite sensitive to any deviations from isotonic conditions. These findings offer critical insights toward the formulation of artificial insemination protocols, showing that extender selection greatly determines the different reproductive parameters preservation and the mechanisms of metabolic and membrane support used. The comparative analysis demonstrates the necessity for suitable extender composition with the spermatozoa's metabolic demands during the storage period which determines the success of the breeding programmes.

The comparative evaluation of different semen extenders on reproductive performance in Nunukan chickens demonstrated consistent results across all treatment groups. Fertilization rates (Figure 2A) showed relatively uniform performance among the extender treatments, with values ranging from 74% to 83%. Natural mating achieved the highest fertilization rate at 83%, followed by BPSE at 81%, Ringers_YE at 76%, NaCl_YE and Ringers both at approximately 75%, and NaCl at 74%. Hatchability rates (Figure 2B) demonstrated greater variation among treatments, ranging from 61% to 74%. Natural mating achieved the highest hatchability rate at 74%, followed by BPSE at 73%, NaCl_YE at 67%, Ringers_YE at 64%, NaCl at 62%, and Ringers at 61%. Statistical analysis revealed no significant differences ($P > 0.05$) between treatment groups for both fertilization and hatchability parameters, indicating that all tested extenders maintained adequate sperm quality during the 60-minute storage period at 4°C, with sperm motility remaining at or above 70% and viability at or above 80% throughout the storage duration.

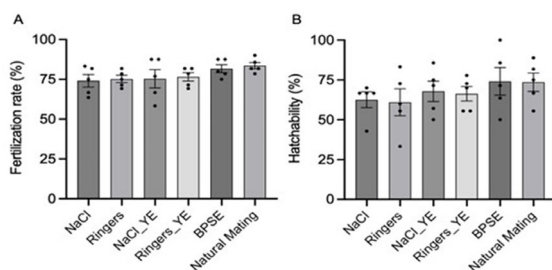


Fig. 2. Comparative Performance of Various Semen Extenders on Fertility rate (A) and Hatchability rate (B) in Nunukan Chickens. Data (Mean $\pm$ SEM) are from five independent experiments.

The results demonstrate that different extender compositions can effectively maintain sperm viability and fertilizing capacity under practical field conditions, with storage at 4°C for 60 minutes representing a realistic timeframe for artificial insemination implementation in poultry production systems. The superior performance of BPSE extender suggests that more complex formulations containing balanced electrolytes and nutritional components provide enhanced protection against storage-induced cellular damage (Laffaldano *et al.*, 2011; Getachew *et al.*, 2023). The maintenance of acceptable fertilization rates across all extender treatments indicates that the 60-minute storage duration at refrigeration temperature represents a practical window for semen utilization without significant deterioration of reproductive potential (Long & Kulkarni, 2004; Blesbois, 2007). Simple extenders like NaCl and Ringer's solution, despite lower performance metrics, achieved over 74% fertilisation rates which highlights their usefulness in budget-constrained environments lacking advanced extenders (Donoghue & Wishart, 2000; Santiago-Moreno *et al.*, 2009; Partyka & Nizański, 2022).

Incorporating yolk extract into basic saline solutions has shown differing impacts on reproductive performance. While Ringers_YE outperformed Ringers without yolk, NaCl_YE performed worse than NaCl alone. Such responses may arise from the interplay of yolk constituents with certain electrolytes, influencing the integrity of the sperm membrane as well as the metabolic activities of the spermatozoa during the storage period (Partyka *et al.*, 2017; Surai *et al.*, 2000). The uniformity in sperm quality parameters within all treatments over time indicates that the conditions monitored for these stored samples provide utility for practical spermatological application in artificial insemination programmes. Therefore, artificial insemination can be incorporated into Nunukan chicken breeding programmes, where the choice of extender can be made depending on the infrastructure, economy, and region while still maintaining good reproductive performance of the chickens.

Conclusion

This study assessed five semen extenders in relation to Nunukan chicken semen's reproductive performance, determining optimal combinations for practical use in artificial insemination (AI). With all extenders, sperm quality declined with increasing storage time; however, BPSE maintained superior storage capacities for sperm quality and reproductive performance relative to natural mating. In contrast, egg yolk-supplemented extenders had intermediate grades suitable for field use. Basic extenders, though their quality decreased rapidly, maintained acceptable reproductive success for short-term storage, making them useful in a variety of operational settings. These results suggest extender choice is influenced most by desired storage time, advancing Nunukan chicken artificial insemination strategies.

Conflict of interest

The authors have no conflict of interest to declare.

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