# The Impact of Adding Egg Yolk in Various Concentrations into The Tyrode Extender on Muscovy Duck *(Cairina moschata)* Sperm Quality

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**ABSTRAK**

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Penelitian ini bertujuan untuk melihat pengaruh konsentrasi kuning telur yang berbeda dalam ekstender Tyrode terhadap kualitas semen Itik setelah pengawetan. Sampel semen dikoleksi dari Itik berusia 1,5 tahun. Sampel dievaluasi untuk motilitas sperma, integritas membran plasma, integritas akrosom, dan morfologi sperma sebelum dipisahkan menjadi tiga bagian yang sama dan diencerkan dalam ekstender Tyrode yang mengandung kadar kuning telur yang berbeda: $T_1$ (5%), $T_2$ (10%), dan $T_3$ (15%). Setelah 21 jam pengawetan pada suhu 4-5ºC, setiap sampel dievaluasi untuk parameter yang sama seperti semen segar. Hasil penelitian menunjukkan penurunan yang nyata pada motilitas sperma, mebran plasma dan akrosom pada $T_1$, $T_2$, dan $T_3$ dibandingkan semen segar yaitu $T_1$ (53.34%; 63.5%; 60.04% vs 84.16%), $T_2$ (48.7%; 57.8%; 55.3% vs. 85.6%), and $T_3$ (50.6%; 57.6%; 56.1% vs 85.4%), respectively ($P<0.05$). Sebaliknya, morfologi sperma abnormal meningkat secara signifikan pada $T_1$, $T_2$, dan $T_3$ dibandingkan dengan semen segar (6,69%, 6,89%, 7,89% vs 2,2%) ($P<0,05$). Lebih jauh, penambahan 10% kuning telur ke dalam ekstender Tyrode menghasilkan persentase terbaik dari motilitas sperma, integritas membran sperma, dan integritas akrosom setelah pembekuan. Akan tetapi persentase sperma dengan morfologi abnormal tidak berbeda secara signifikan ($P>0.05$) antara $T_1$, $T_2$, dan $T_3$ setelah periode pembekuan. Dapat disimpulkan bahwa menambahkan 10% kuning telur ke dalam ekstender secara substansial meningkatkan motilitas sperma, integritas membran, dan integritas akrosom pada sperma Itik setelah 21 jam pembekuan.

**Kata Kunci** : Kuning Telur, Pembekuan, Kualitas Semen, Tyrode

**ABSTRACT**

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This study investigated the impact of varying egg yolk concentrations in Tyrode extender on the quality of *Muscovy duck* semen after preservation. A sample of semen was collected from 1.5-year-old *Muscovy ducks*. The sample was evaluated for sperm motility, plasma membrane integrity, acrosome integrity, and sperm morphology before being separated into three equal parts and diluted in Tyrode extender containing increasing levels of egg yolk: $T_1$ (5%), $T_2$ (10%), and $T_3$ (15%). After 21 hours of preservation at 4-5ºC, each sample was evaluated for the same parameters as fresh semen. The study found a significant decrease in sperm motility, membrane integrity, and intact acrosome cap following preservation in $T_1$, $T_2$, and $T_3$ compared to fresh ejaculate (53.34%; 63.5%; 60.04% vs 84.16%), (48.7%; 57.8%; 55.3% vs. 85.6%), and (50.6%; 57.6%; 56.1% vs 85.4%), respectively ($P<0.05$). In contrast, abnormal sperm morphology increased considerably in $T_1$, $T_2$, and $T_3$ compared to fresh semen (6.69%, 6.89%, 7.89% vs 2.2%) ($P<0.05$). Furthermore, the addition of 10% egg yolk to the Tyrode extender resulted in the best percentages of sperm motility, sperm membrane integrity, and intact acrosome cap following preservation. Surprisingly, the percentage of sperm with abnormal morphology did not differ significantly ($P>0.05$) between $T_1$, $T_2$, and $T_3$ after the preservation period. It can be concluded that adding 10% egg yolk to the extender substantially improved sperm motility, membrane integrity, and intact acrosome cap in Muscovy duck sperm after 21 hours of preservation.

**Key Words**: Egg Yolk, Preservation, Sperm Quality, Tyrode

## INTRODUCTION

The Muscovy duck (*Cairina moschata*) has the potential to improve its genetic quality by utilizing a genetic resource (*germplasm)* obtained from Central and South America and developed as indigenous livestock in Indonesia (Lan & Worowan 2021). Muscovy ducks have the potential to be developed as meat, egg, and feather-producing birds because of their excellent body and egg

weights and thicker feathers compared to chickens and ducks. However, the population is decreasing year after year. A reduced population of productive ducks has led to a decline in the number of ducks compared to the number slaughtered. Efforts must be made to accelerate the transmission of genetic potential and enhance the genetic quality of ducks by applying Artificial Insemination (AI) Technology with semen from superior males. The utilization of limited superior males can be achieved by preservation or cryo-preservation, which involves storing genetic material as chilled or frozen sperm.

A critical factor during sperm preservation or cryopreservation is the choice of the extender. The extender maintains sperm quality during cryopreservation or preservation by providing spermatozoa with an optimal environment and nutrition (Bustani & Baiee 2019). The extender could also preserve the integrity of the spermatozoa cell membrane during cryopreservation, thereby minimizing sperm injury caused by the ice crystal formation (Kumar et al. 2019). The extender combines a buffer, an energy source, and an anti-cold shock agent. Lipoprotein and lecithin act as anti-cold shock agents capable of maintaining and protecting the integrity of sperm cell membranes during preservation or freezing. Egg yolk is one of the sources of lipoproteins (Salmani et al. 2014). Moreover, egg yolk, as a cryoprotectant, functions as a medium for providing food, energy sources, and protection for extracellular spermatozoa from cold shock when freezing, a protective agent, provides the effect as a buffer against spermatozoa (Upadhyay et al. 2021)

Currently, there is a lack of information regarding the precise concentration of egg yolk that should be applied to the Tyrode extender for Muscovy *duck* sperm. This study aims to evaluate the quality of Muscovy duck sperm diluted with a Tyrode extender containing egg yolk at different concentrations.

Tyrode's diluent has several advantages in freezing poultry semen, primarily due to its ability to maintain sperm motility and viability. The addition of egg yolk to Tyrode′s diluent, for example, has been shown to increase sperm motility and reduce abnormality rates in frozen Muscovy duck semen. Tyrode diluents help maintain the ability of sperm to move actively. The correct use of Tyrode diluents can reduce in the abnormalities of sperm. It leads to a higher success rate of artificial insemination. Tyrode diluents can be combined with egg yolk for more optimal results. It is also relatively easy to use in the semen freezing process of poultry.

It is essential to note that selecting the correct diluent and freezing technique is crucial for maintaining the quality of poultry sperm. The use of Tyrode diluents combined with egg yolk, for example, has shown promising results in studies. The findings from this study can be utilized to enhance the quality of preserved and frozen duck sperm, hence improving the success rate of Artificial Insemination.

## MATERIAL AND METHODS

### Ethical approval

The Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran, No. 297/UN6, approved all the procedures in this study.KEP/EC/2023. Semen samples were collected from *Cairina moschata* at the Indigenous Ducks Breeding Station of Universitas Padjadjaran. A *Chairina moschata* was given 0.5 kg of mixed feed consisting of concentrate, bran, and ground corn.

### Semen collection and sperm dilution

Semen collection from one male duck was carried out twice a week, at 08:00–09:00 pm on Tuesdays and Thursdays, using an Artificial Vagina based on the procedure in a previous study (Watanabe & Sugomiri 1957). The collected semen was transferred into a 1.5 mL ampule using a pipette and immediately evaluated for sperm progressive motility, membrane integrity, intact acrosome caps, and abnormal morphology. Furthermore, each ejaculate is divided into three equal parts and diluted in a Tyrode extender with a different egg yolk concentration: T1 (5%), T2 (10 %), and T3 (15%). The sample semen was mixed with extender and various egg yolk concentrations and stored at 3-5°C for 21 hours, and then the sperm evaluation was performed (Hidayat et al. 2022).

### Sperm evaluation

#### *Sperm progressive motility*

The percentage of progressive sperm motility was observed under a binocular microscope (CX21 Olympus, Japan) at 400x magnification and calculated based on a previous study (Iskandar et al. 2022). Sperm motility was calculated by calculating the total sperm concentration and the concentration of dead sperm by placing one drop of semen in the *Neubauer* counting chamber. Sperm motility (Y) in percentage (%) was calculated by the formula below.

$$Y = \frac{\sum Total\ Sperm - \sum Dead\ sperm}{Total\ of\ Spermatozoa}\ x\ 100\%$$

#### *Sperm membrane intact*

The procedure was carried out to assess the integrity of the plasma membrane, as per previous research. Thirty grams of Sodium Chloride were dissolved in 100

ml of distilled water as a Hypo osmotic Swelling Test (HOS-test) solution. The HOS-test solution was added at a 1:6 or 10 µl: 60 µl ratio. The semen that had been mixed with the HOS-Test solution was then incubated at 38°C for 30 minutes. After incubation, the sample was prepared for review on an object glass, and 200 spermatozoa were counted. A circular or bent tail marked the intact Plasma Membrane, while a straight tail indicated that the plasma membrane was incomplete or damaged (Widyastuti et al. 2022).

***Intact acrosome cap of sperm***

A 100 µl NaCl physiological solution containing 1% formalin was mixed with 25 µl of cement. The mixture was gently shaken to ensure homogeneity and left to stand for 5 minutes. A light microscope was utilized to examine the thin stain preparation on a glass object at a magnification of 400x in the presence of at least 200 spermatozoa. Spermatozoa that have an intact acrosome cap have been identified by an enormous black point on their heads; conversely, spermatozoa that have been damaged lack this characteristic (Rizal & Herdis 2005).

***Sperm abnormal morphology***

Spermatozoa abnormalities were evaluated using an eosinnigrosine-stained smear under 1000x magnification. Furthermore, the evaluation of sperm morphology following the previous research with categories: a) Pear-shaped, b) Macrocephalus, c) Microcephalus, d) Detached Head, e) Head only, f) Circular Tail, g) Tail, and h) Stump Tail (Arifiantini 2012; Susilawati 2011).

**Statistical analysis**

Data was analyzed using GraphPad Prism (version 10)(La Jolla, USA). The normality of values distribution was first tested with the Shapiro-Wilk test. Sperm data were subjected to two-way factorial analysis of variance (ANOVA) followed by multiple pairwise comparisons using a post-doc (Tukey test). The threshold of significance was set at $P<0.05$.

## RESULTS AND DISCUSSION

The findings revealed that the presence of egg yolk in Tyrode extender significantly reduced the progressive motility of Muscovy duck spermatozoa in all treatment groups. Moreover, adding 5% egg yolk to Tyrode extender significantly inhibits the progressive motility of Muscovy duck spermatozoa after 21 hours of preservation by more than 20% compared to fresh semen. Interestingly, adding 10% egg yolk to Tyrode solution is capable of maintaining the progressive motility of Muscovy duck spermatozoa after 21 hours of preservation, as opposed to the other treatments (Figure 1a).

Enhancing spermatozoa quality can be achieved by incorporating egg yolk into the extender, which serves as an energy source for the spermatozoa (Allai et al. 2018). Egg yolk provides nourishment, energy, and protective substances for sperm during storage. The study demonstrated that supplementing egg yolk in different concentrations can help preserve progressive motility, membrane integrity, and intact acrosome cap and reduce abnormal morphology in Muscovy duck spermatozoa preserved for 21 hours at 4-5ºC.

The Tyrode extender has several functions, including increasing sperm motility and reducing abnormality rates in frozen Muscovy duck semen. Another function that we can contribute to a higher success rate of artificial insemination is relatively easy to prepare and use in the poultry semen freezing process.

Our findings indicate that adding 10% egg yolk to the extender significantly improves the maintenance of spermatozoa's progressive motility compared to other concentrations. The results showed that adding 10% egg yolk to the extender adequately provides nutrients for preserving *Muscovy duck* spermatozoa during preservation. Egg yolk helps maintain *Muscovy duck* spermatozoa motility by providing proteins as an alternative energy source. Egg yolk contributes ATP and ADP, serving as an energy source for spermatozoa (Bilodeau et al. 2002). Amino acids found in egg yolks, like L-phenylalanine, L-tryptophan, and L-tyrosine, are involved in oxidative deamination to produce hydrogen peroxide, which helps sustain spermatozoa motility (Aitken et al. 2015).

Preservation at 4-5°C for 21 hours significantly decreased the percentage of intact membranes in *Muscovy duck* spermatozoa across all treatment groups ($P<0.05$, respectively). Comparing preserved semen to fresh semen, the percentage of intact spermatozoa membranes is reduced by 20-30%. In addition, 10% egg yolk added to the extender results in approximately 60% spermatozoa with an intact acrosome cap, whereas 5% egg yolk added results in only around 55% (Figure 1b).

The number of intact acrosome caps in *Muscovy duck* spermatozoa decreased by 25–30% after 21 hours of preservation at 4–5°C compared to fresh sperm. After 21 hours of preservation, the acrosomes remained intact when 10% and 15% egg yolk were added to the extender, compared to the 5% egg yolk addition. The proportion of intact acrosome caps in Muscovy duck spermatozoa preserved in extenders containing 10% and 15% egg yolk is approximately 60%. In contrast, the proportion is approximately 55% in spermatozoa preserved in extenders containing 5% egg yolk (Figure 2).

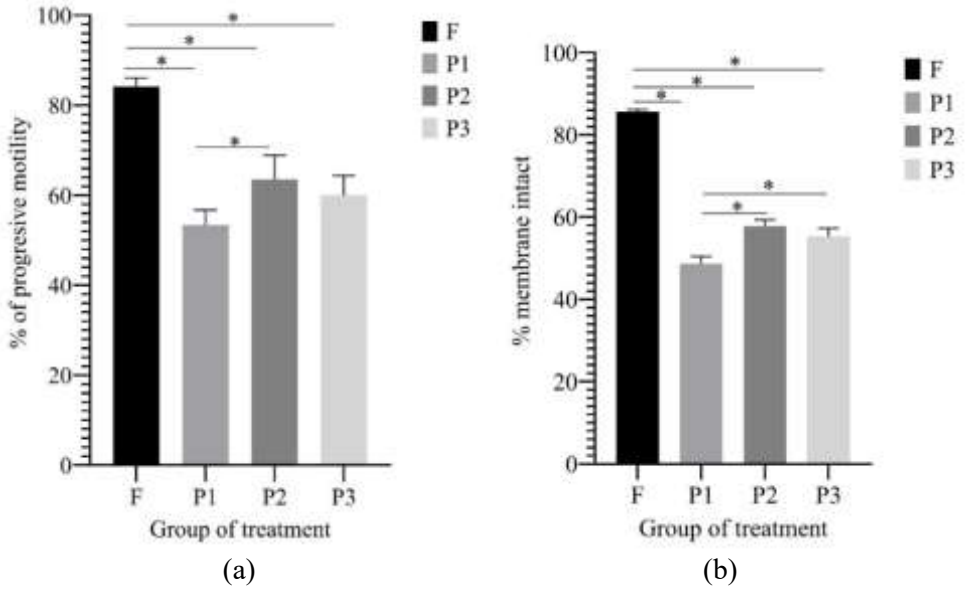



**Figure 1.** Spermatozoa progressive motility (a) and Spermatozoa membrane intact (b). F= fresh semen, P1= Addition of 5% Egg Yolk in Tyrode extender, P2= Addition of 10 % Egg Yolk in Tyrode extender, and P3= Addition of 15% Egg Yolk in Tyrode extender. Data presented as average bars with * means significant differences among the experimental groups (P<0.05).

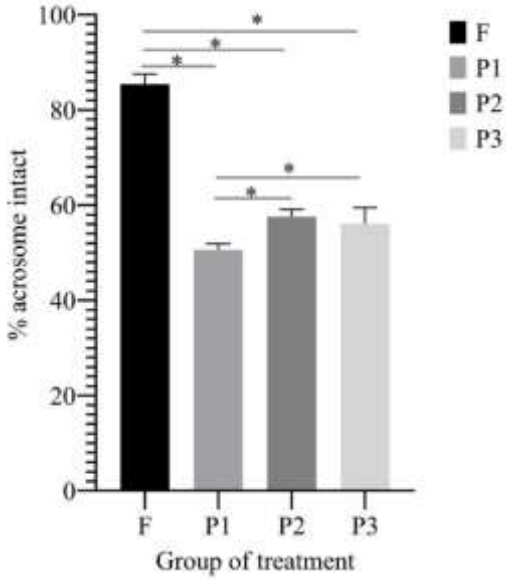



**Figure 3.** Intact acrosome cap of sperm. F= fresh semen, P1= Addition of 5% Egg Yolk in Tyrode extender, P2= Addition of 10 % Egg Yolk in Tyrode extender, and P3= Addition of 15% Egg Yolk in Tyrode extender. Data presented as average bars with * significant differences among the experimental groups (P<0.05)

Furthermore, there was a significant increase in the percentage of *Muscovy duck* spermatozoa showing abnormal morphology after being preserved at 4-5ºC for 21 hours compared to fresh semen. There was no significant difference in the increase of spermatozoa with abnormal morphology across all treatment groups (P>0.05, respectively). However, Muscovy duck spermatozoa preserved in the extender with the addition of 15% egg yolk showed the most significant percentage of spermatozoa with abnormal morphology (Figure 4).

A functioning membrane plays a role in the fertilizing ability of spermatozoa, as it is essential for spermatozoa capacitation, acrosome reaction, and adherence of the spermatozoa to the oocyte surface (Gadella & Luna 2014). Throughout this research, we utilize an HOS test to predict membrane integrity by assessing the spermatozoon's membrane ability to maintain equilibrium between the spermatozoon cell and its surrounding environment. Generally, preservation-induced stress on sperm occurs by altering the structure

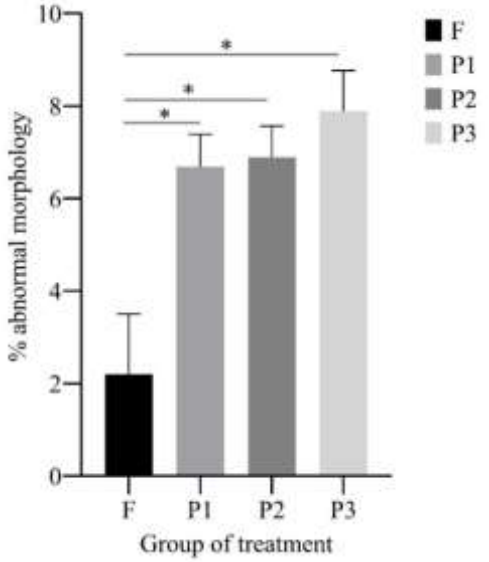



**Figure 4**. Spermatozoa with abnormal morphology. F= fresh semen, P1= Addition of 5% Egg Yolk in Tyrode extender, P2= Addition of 10 % Egg Yolk in Tyrode extender, and P3= Addition of 15% Egg Yolk in Tyrode extender. Data presented as average bars with * significant differences among the experimental groups (P<0.05)

of plasma membrane phospholipids, resulting in malfunction and increased membrane permeability (Cotter et al. 2005; Wongtawan et al. 2006). Tyrode diluent, which is adding egg yolk, will undoubtedly contain cholesterol, which comes from the egg yolk, provides a denser plasma membrane structure to shield spermatozoa from cold shock and help retain fluidity (Bathgate et al. 2006). The research found that adding 10% egg yolk to the extender provided better protection for the membranes of Muscovy duck spermatozoa during preservation. Moreover, adding 10% egg yolk to the skim milk extender resulted in better sperm motility than adding 15% egg yolk (Yuniar et al. 2021). Adding 10% egg yolk to Ringer Lactate diluent in Pelung's chicken sperm (Hidayat et al. 2022) and spermatozoa in Boer goats improved motility more than adding 15% or 20% egg yolk (Ihsan 2011).

The acrosome reaction is essential for mammalian spermatozoa to penetrate the zona pellucida (Gerton, 2002). Spermatozoa undergo the acrosome reaction before binding to the zona pellucida in particular species, whereas in others, binding to the zona can only be initiated by spermatozoa that possess an intact acrosome cap (Fazeli et al. 1997). Based on our findings, the percentage of intact acrosome caps in Muscovy duck spermatozoa was substantially higher when 10% and 15% egg yolk were added to the extender during preservation at 4-5ºC, as compared to the addition of 5% egg yolk. The findings suggested that a 10% egg yolk addition was adequate to prevent the capitulation of Muscovy duck spermatozoa after the spontaneous acrosome reaction while preserving the spermatozoa, in contrast to the addition of 5% or 15% egg yolk in the extender.

Moreover, current research results indicate that adding egg yolk at 5%, 10%, and 15% of the extender significantly decreases the number of spermatozoa with abnormal morphology in Muscovy duck spermatozoa following 4-5ºC preservation. Moreover, adding 15% egg yolk in the extender resulted in a higher percentage of spermatozoa with abnormal morphology. The results indicated that the concentration of egg yolk is too high, causing an imbalance in osmotic pressure, which can damage the shape of spermatozoa. The addition of 5% and 10% egg yolk to Tyrode diluent can prevent abnormalities by protecting and maintaining the protein coat of spermatozoa from incoming fluids, which have low osmotic pressure. Furthermore, recent research findings have shown that integrating egg yolk at 5%, 10%, and 15% in the extender significantly reduces the percentage of Muscovy duck spermatozoa with abnormal morphology after preservation at 4-5ºC. The results indicated that the concentration of egg yolk is too high, causing an imbalance in osmotic pressure, which can damage the shape of spermatozoa.

The current research found that preserving Muscovy duck spermatozoa for 21 hours decreased the progressive motility, membrane integrity, acrosome intactness, and normal morphology of the spermatozoa. However, adding egg yolk to the extender helped slow down this decrease. The presence of lecithin and lipoprotein in egg yolk acts as an antioxidant capable of protecting spermatozoa and fighting free radicals so that damage to spermatozoa due to oxidative stress can be reduced by the Reactive Oxygen Species (ROS) process (Alvarez-Rodriguez et al. 2013; Mehdipour et al. 2018; Yustiti et al. 2021). The decrease in spermatozoa motility, intact membrane, intact acrosome cap, and aberrant

morphology in the high concentration of egg yolk extender is due to excess fat granules that hinder sperm movement by increasing energy consumption; this leads to a depletion of energy reserves in spermatozoa cells, causing a buildup of lactic acid due to metabolism, resulting in damage to the sperm cell membrane and a rise in abnormalities (Zamira & Aitken. 2016).

Moreover, spermatozoa undergo anaerobic metabolism due to an insufficient energy supply, resulting in the production of lactic acid and a reduction in pH and motility. Disruption of the plasma membrane causes damage to metabolic reactions within the cell membrane (Santoso et al. 2020). Plasma membrane damage results in the disappearance of the aspartate aminotransferase enzyme, leading to a lack of energy reshuffling and subsequent loss of motility in spermatozoa (Septiyani et al. 2013). Lastly, adding egg yolk into the extender can be beneficial and may be suggested as a standard practice for preserving *Muscovy duck* spermatozoa.

## CONCLUSSION

It can be concluded that adding 10% egg yolk to the extender substantially improved sperm motility, membrane integrity, and acrosome integrity in *Muscovy duck* sperm, because it is cheaper, especially when applied to small farmers.

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