

# Diluent and Storage Time Effect on Sperm Abnormality and MDA Level in Muscovy Duck Semen at 27°C

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## Abstract

This study aimed to determine sperm abnormality and MDA level in Muscovy duck semen in different dilution and storage at 27°C. This study was used in the level of dilution of glutinous semen with a long time store differently at 27°C. In this study, the parameters of the mass motility of spermatozoa were used individual motility, spermatozoa abnormalities, and activity test. A Muscovy duck, healthy and have a high libido were used then divided into treatment groups i.e. (A0) 0; (A1) 5 times; (A2) 10 times; and (A3) 15 times, the second factor as a subplot was the storage time (B0) 0; (B1) 60 minutes; (B2) 120 minutes; and (B3) 180 minutes stored at 27°C with 3 replications. In results, this study was reported abnormalities at a dilution rate in A1 group of 5 times with a shelf life in B1 group of 60 minutes and in B2 group of 120 minutes.

Keywords: abnormality, diluent level, Muscovy duck, semen, storage time

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## INTRODUCTION

Muscovy duck is one type of poultry that is widely reared by rural communities which is resistant to the environment and acts as a source of protein (Ismoyowati *et al.*, 2019). Crosses between male and female Muscovy duck can be done using artificial insemination (AI) technology (Chen *et al.*, 2016). This cross has the advantage that the duck has a large body while the duck has a lot of eggs (Quan and Benjakul, 2019). In the insemination process through AI the highest fertility that can be achieved is 80% and only 20–30% through natural breeding (Hanifah *et al.*, 2020). Through this technology, a male from which semen is collected can inseminate more females, where for fertilization of one egg only one sperm is needed, while muscovy ducks have a volume of 0.05–0.5 mL/ejaculate, a spermatozoa concentration of 1.6–7.4 billion/mL (Abadjieva *et al.*, 2023).

Cross breeding male and female Muscovy duck can be done by using IB technology. This cross has advantages, including muscovy having a large body while ducks have lots of eggs. The highest fertility that can be achieved in the

insemination process through AI is 80% and through natural breeding only 20–30%. Through this technology, a male whose semen is taken can mate with more females, where only one semen is needed for fertilization of one egg cell, whereas the volume of Muscovy duck is 0.05–0.5 mL/ejaculate, the spermatozoa concentration is 1.6–7, 4 billion/mL (Turnip *et al.*, 2018).

The success of AI is influenced by several factors including the level of diluent, storage time, and temperature of the semen to be inseminated into the female. Dilution aims to increase the volume of semen, non-toxic to semen, maintaining the viability, and the ability to fertilize (Chen *et al.*, 2016). This study aimed to determine sperm abnormality and MDA level in Muscovy duck semen in different dilution and storage at 27°C.

## MATERIALS AND METHODS

### Ethical Approval

The current study did not require animal ethics approval. However, this study was carried out according to standard operating procedures.

### Study Period and Location

This study was conducted in February 2020. The locations to evaluate semen in this study were at the Faculty of Health and Science, UN PGRI Kediri.

### Experimental Design

The Muscovy duck used in this study were aged about 1.5–2 years with a body weight of 3–3.5 kg. Semen collection was done with an artificial vagina. Using a female angler to stimulate the libido of male Muscovy duck, mating, and shelter was carried out when the male climbs the female and releases semen, and then holds it with a scale tube. The dilution of duck semen was carried out in the morning between 07.00–09.00 AM with a frequency of twice each week, diluted with Ringer's solution and egg yolk dilution with a dilution rate of 0, 5, 10, and 15 times can be seen in Table 1.

This study method was used an experimental method with a split-plot. The first factor as the main plot was the level of dilution, i.e. (A0) 0 times; (A1) 5 times; (A2) 10 times; and (A3) 15 times, the second factor as a subplot was the stored i.e. (B0) 0; (B1) 60 minutes; (B2) 120 minutes; and (B3) 180 minutes stored at 27°C. Four each groups consisted of 3 replication (Naes *et al.*, 2007).

### Spermatozoa Mass Motility

The number of spermatozoa in fresh semen from ejaculate that has been added with diluent and different storage times. Assessment of mass motility can be determined by dripping semen on a glass object and observed under a microscope with a magnification of 100x. If there were large waves, many, dark and thick like clouds, then the assessment of the spermatozoa was very good (+++). If there were small, thin, infrequent, indistinct, and slow-moving waves, the semen rating was good (++). If there were only progressive active individual movements then the assessment was moderate (+) and if there was no movement at all then the assessment was poor (0) guidance on instructions (Rochmi and Sofyan, 2019).

### Individual Motility

Method of dripping sorghum semen which was diluted with Ringer's solution 10 times and different storage times at 27°C on a glass object and then covered with a cover glass. Individual motility assessment was carried out by calculating the percentage of spermatozoa whose movements were progressively moving forward compared to those that were stationary as much as  $\pm 100$  spermatozoa with units of percent (Tanga *et al.*, 2021) using a 400x magnification microscope.

### Spermatozoa Abnormalities

Observations were the same as observations of spermatozoa motility. Muscovy duck semen which was diluted with Ringer's solution 20 times with different shelf life at 27°C was dripped onto object glass and then stirred with eosin dye. The preparations were made as thin as possible with a cover glass. Abnormal spermatozoa were calculated as the percentage of normal spermatozoa between  $\pm 100$  times with an enlargement of 400 times in the observed spermatozoa (Fiqih *et al.*, 2021).

### Malondialdehyde (MDA) Level

The treated semen samples were tested. MDA activity was carried out using the spectrophotometer method. A total of 100 mL of treated semen sample was added with 100 mL of MDA solution (aquades 0.55 mL, TCA 10% 100 mL, HCl 1 N 0.25 mL, Na-triobarbiturate 1% 100 mL) centrifuged at 500 rpm for 10 minutes then heated for 30 minutes at 100°C, the supernatant was separated and its absorption was observed with a spectrophotometer with a wavelength of 580 nm and the absorbance was measured MDA (Purnama *et al.*, 2019).

### Data Analysis

Data were analyzed using ANOVA followed by the Duncan test at a 95% significant level.

## RESULTS AND DISCUSSION

### Evaluation of Muscovy Duck Semen

Macroscopic examination of semen includes volume, color, odor, consistency, and pH. These

observations is necessary to determine the quality of semen and male reproductive and the dilution rate of semen (Sharma *et al.*, 2015). While microscopic examination includes mass movement, concentration, motility, and the percentage of life or death (Wurlina *et al.*, 2020). The average results of fresh semen inspection can be seen in Table 2.

The average volume of sperm per ejaculate obtained during the study was  $2.17 \pm 0.29$  mL and the consistency of the semen obtained was more watery. It is thought to be produced when the stored semen mixes with the secretions of the folds of the spleen and vascular bodies in the cloaca. This is per the statement of Rodriguez-Martinez *et al.* (2021) that semen is divided into two solid parts called spermatozoa which are produced by the testes and the liquid part is called seminal plasma which is produced by the accessory glands of male 9 bulbourethralis, prostate, vesicular seminal, these secretions. serves as a buffer and medium for spermatozoa so that their vitality can be maintained normally after ejaculation.

The color of the semen obtained during the study was white to cloudy white which indicates a high concentration (Zimmerman and Mitchell, 2017). The smell of fresh Muscovy duck semen at the time of the study was found to have a characteristic smell of livestock. The smell indicates the semen is in normal condition and there is no contamination (Prayogo *et al.*, 2022). The acidity of the pH is thought to be the result of the activity of the phospholipase A enzyme because this enzyme is toxic to semen during the dilution process (Sobhnamayan *et al.*, 2015). The pH of semen obtained during the study was  $7.5 \pm 0.00$ . Zhou *et al.* (2015) stated that normal poultry semen has a pH in the range of 7.2–7.6. Dhumal *et al.* (2021) stated that fresh semen is slightly alkaline with an average pH ranging from 7.0–7.6. The high pH value during the study was caused by the influence of the transparent liquid or seminal plasma in the semen which caused the semen to become more alkaline.

The average concentration of fresh semen obtained during the treatment was  $1.12 \pm 0.25$  ( $10^7$ /mL). According to Tan *et al.* (2022), the

concentration of poultry semen ranged from  $3-7 \times 10^9$ /mL. Liu *et al.* (2016) explained that the concentration of poultry was influenced by its growth. Other factors that affect semen concentration include age, light, nutrition, genetics, and frequency of storage. The average concentration of fresh semen from the study showed that the above semen was in the moderate category because it had a semen amount of more than 1 billion/mL.

The average mass motility obtained during the study ranged from ++ to +++ . Santoso *et al.* (2021) stated that the quality of semen is good when compared to the mass motility of semen which has (++) good value and (+) is not good. Muvhali *et al.* (2022) stated that mass movements are scored (+++) if large, dark, thick, and active waves are seen. A score (++) is given if there are small, thin, infrequent, slow-moving, and indistinct waves. A score (+) is given for semen that does not show clear mass movement of the lumps and only individual movements of the semen are visible. The average percentage of individual motility of fresh semen obtained during the study was  $81.67 \pm 2.89\%$ . According to Sayed *et al.* (2022), normal poultry semen has individual motility ranging from 60–80%.

The average percentage of live semen in fresh semen obtained during the study was  $87 \pm 1\%$ . According to Feyisa *et al.* (2018) in normal poultry semen, the percentage of fresh live is about 80%. The results obtained during the study showed that the accommodated Muscovy duck semen was of good quality and could be used for AI purposes.

### Abnormality of Muscovy Duck Semen

Abnormality is one of the indicators in determining the quality of spermatozoa because abnormal cell structures can cause disturbances and obstacles during fertilization, further causing low rates of implantation and pregnancy (Wajdi, 2021). Spermatozoa abnormalities are closely related to the ability to fertilize eggs and infertility in various species. Abnormal cell structure can cause disturbances and barriers at the time of fertilization that further cause low rates of implantation and pregnancy (Iskandari *et al.*,

2020). Wang *et al.* (2022) said that the forms of spermatozoa abnormalities were classified into two, namely primary and secondary abnormalities. Spermatozoa abnormalities that have not exceeded 20% of the semen sample, the semen can still be used for insemination (Suárez *et al.*, 2018). Average abnormalities of Muscovy duck semen in different dilution levels and different storage times at 27°C are in Table 3 below.

The result of this study showed that the highest mean of abnormality of glutinous semen occurred in no dilution or control at  $7.77 \pm 0.96\%$  with storage of 120 minutes at  $8.05 \pm 2.15\%$ . Control is the highest mean of abnormality, while the dilution level of 5 times is the lowest mean. This proves that egg yolk can suppress the abnormalities of egg yolk spermatozoa because egg yolk is an antioxidant that acts to bind unsaturated fatty acids and prevent chain reactions that cause high spermatozoa abnormalities. According to Forman *et al.*, (2014), antioxidants are nucleophilic compounds that can reduce, extinguish or suppress free radical reactions.

The highest average occurred in 120 minutes of storage, while the lowest average occurred in 60 minutes of storage. Long storage will cause spermatozoa to become non-isotonic which causes damage to the plasma membrane of surviving spermatozoa, thereby increasing spermatozoa abnormalities. This matter per the opinion of Silvestre *et al.* (2021) stated that longer storage causes the increase in dead spermatozoa. The number of damaged spermatozoa plasma membranes and dead spermatozoa makes abnormal spermatozoa increase. These results follow the study on squid conducted by Chankitisakul *et al.* (2022) that the dilution of gummy semen with physiological NaCl and storage time at 27°C affect the abnormality of spermatozoa, with the higher degree of dilution and the duration of storage, the higher the abnormality of the spermatozoa.

The lowest mean of semen abnormalities occurred at 60 minutes of storage  $6.8 \pm 0.96\%$  with a dilution rate of 5 times  $7.22 \pm 1.77\%$ . The level of abnormality of Muscovy duck

spermatozoa is strongly influenced by the composition of the cell structure and metabolism in the semen. Colagar *et al.* (2013) stated that lipid peroxidation will cause structural damage and disrupt the metabolism of spermatozoa which results in the death of spermatozoa.

The result of the highest mean abnormality of guinea pig semen occurred in no dilution or control at  $7.77 \pm 0.96\%$ , with storage of 120 minutes at  $8.05 \pm 2.15\%$ . This indicates that the addition of egg yolk can suppress semen abnormalities. This percentage is classified as normal, following the opinion of Kumar and Singh (2015) which states that in most ejaculation the percentage of abnormal spermatozoa ranges from 5–20%. Pokhrel *et al.* (2020) stated that if abnormal spermatozoa are more than 25% of the total spermatozoa in one ejaculation, it will reduce fertility. Meanwhile, according to Rengan *et al.* (2012), spermatozoa abnormalities that exceed 14% indicate the presence of symptoms of infertility or infertility in a male, whereas according to Kumar and Singh (2015), abnormal sperm counts in semen up to 20% will not cause a decrease in fertility rates. The level of abnormality of the gout semen is shown in the graph in Figure 1. Abnormality of spermatozoa at different storage times at 27°C and Figure 2. Abnormality of spermatozoa at different levels of diluent at 27°C.

### MDA Level in Muscovy Duck Semen

The MDA has been widely used as an indicator of the presence of free radicals and the occurrence of oxidative damage (Alvarez-Mon *et al.*, 2022). MDA is the final product of lipid oxidation which is toxic to cells, causing damage to the spermatozoa membrane. Damaged spermatozoa membranes will cause a decrease in the integrity of the spermatozoa membranes, which in turn causes a decrease in sperm quality (Agarwal *et al.*, 2014). According to Rao *et al.* (2021), MDA compounds cause damage to the semen membrane and a decrease in the integrity of the semen membrane, resulting in a decrease in semen quality. The average dilution rate and storage time of the average MDA results at 27°C after treatment are shown in Table 4.

**Table 1.** Dilution level and storage time for each treatment

Treatment	Dilution level	Storage time/minutes
A0B0		0
A0B1	0	60
A0B2		120
A0B3		180
A1B0		0
A1B1	5	60
A1B2		120
A1B3		180
A2B0		0
A2B1	10	60
A2B2		120
A2B3		180
A3B0		0
A3B1	15	60
A3B2		120
A3B3		180

**Table 2.** Muscovy duck cement evaluation results

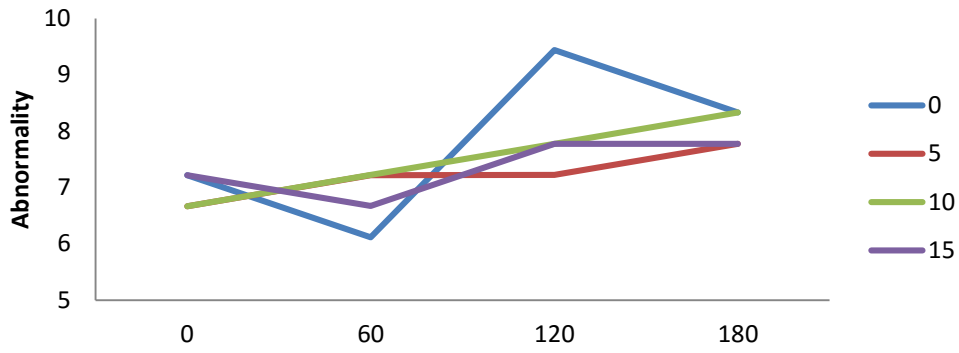
	Parameter	Average ± SD
Macroscopic	Volume	2.17–0.29
	Colour	White–Cloudy white
	Smell	Stench
	Consistency	Watery–Viscous
	pH	7.5–0.00
Microscopic	Concentration (10 <sup>7</sup> /mL)	1.12–0.25
	Motility of mass	++ s/d +++
	Individual motility (%)	81.67–1.00
	Abnormalities dilution rate (%)	7.46–1.52
	Abnormalities storage time (%)	7.46–1.48
	MDA activity dilution rate (umol/dL)	1313.01–48.35
	MDA activity storage time (umol/dL)	1313.01–48.35

**Table 3.** Average abnormalities in dilution levels and different storage times at 27°C

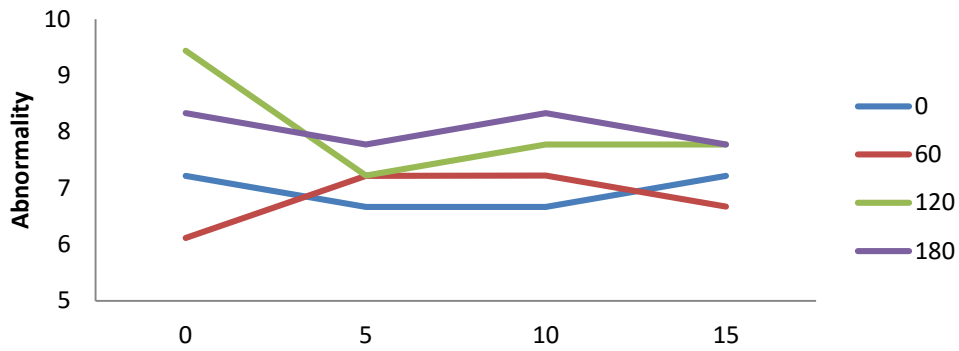
Dilution level	Abnormalities (%) ( $\bar{X} \pm SD$ )	Storage time (minutes)	Abnormalities (%) ( $\bar{X} \pm SD$ )
0	7.77 ± 0.96	0	6.94 ± 2.10
5	7.22 ± 1.77	60	6.80 ± 0.96
10	7.50 ± 2.01	120	8.05 ± 2.15
15	7.36 ± 1.36	180	8.05 ± 0.71
( $\bar{X} \pm SD$ )	7.46 ± 1.52	( $\bar{X} \pm SD$ )	7.46 ± 1.48

**Table 4.** Average MDA level in dilution levels and different storage times at 27°C

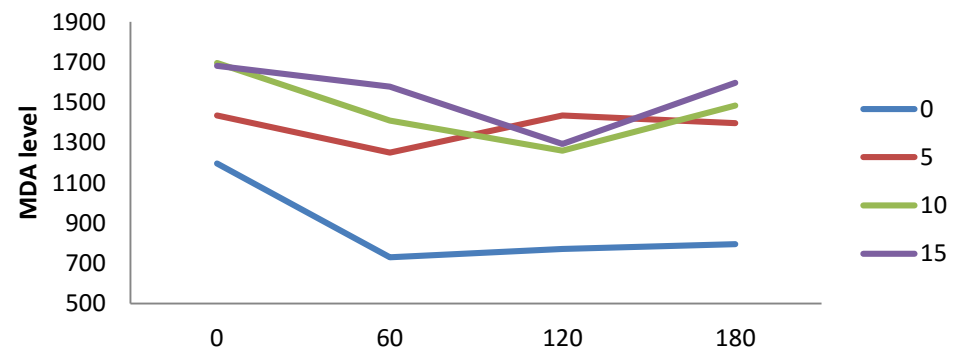
Dilution level	Abnormalities (%) ( $\bar{X} \pm SD$ )	Storage time (minutes)	Abnormalities (%) ( $\bar{X} \pm SD$ )
0	872.96 ± 2.72	0	1502.12 ± 36.77
5	1379.20 ± 94.08	60	1241.50 ± 60.02
10	1462.54 ± 34.57	120	1189.83 ± 39.35
15	1537.33 ± 62.00	180	1318.58 ± 57.06
( $\bar{X} \pm SD$ )	1313.01 ± 48.34	( $\bar{X} \pm SD$ )	1313.01 ± 48.34



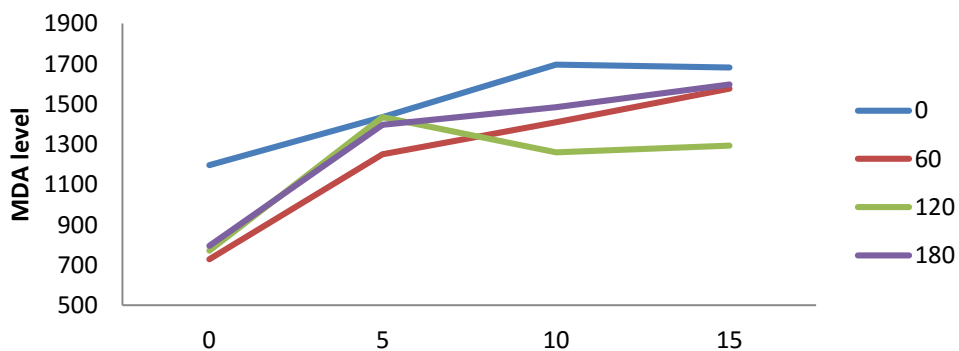
**Figure 1.** Spermatozoa abnormality at different storage times at 27°C.



**Figure 2.** Spermatozoa abnormality at different diluent levels at 27°C.



**Figure 3.** MDA level of different storage times at 27°C.



**Figure 4.** MDA level of different diluent levels at 27°C.

The results showed that MDA activity obtained the highest average in the no storage or control  $1502.12 \pm 36.77$  mol/dL, with a diluent level of 15 times  $1537.33 \pm 62$  mol/dL. The diluent is an antioxidant that can counteract the damage of lipid peroxidation that reacts with unsaturated fats in the dilution of fresh duck semen. From a dilution level of 15 times, it causes the formation of free radicals that react with cell membrane components in the dilution of egg yolk semen more quickly, this occurs in the control semen that has not experienced stress and after semen treatment, many are stressed due to treatment and the addition of egg yolk diluent so that the spermatozoa are damaged. Stress in semen is the main cause of semen dysfunction inhibiting the phosphorylation process (Sabeti *et al.*, 2016).

Disrupted phosphorylation oxidation causes an increase in reactive oxygen species (ROS) in semen, high levels of ROS in cells can oxidize lipids, proteins, and DNA. Semen plasma membrane lipids have high levels of phospholipids, making semen very susceptible to ROS (Shan *et al.*, 2021). Storage 0 minutes or control is the highest average MDA, this is due to the influence of the treatment during semen storage, which results in stress accompanied by an increase in free radicals. The absence of time resulted in the antioxidants in semen not being able to play an active role so the increase in MDA levels could not be stopped. Antioxidants are nucleophilic compounds that can reduce, extinguish, or suppress free radical reactions (Wijayanti *et al.*, 2023).

The results showed that too much and unbalanced addition of egg yolk as an antioxidant would increase MDA levels in semen. The addition of an excess level of diluent results in a lot of lipid damage, it is suspected that the damage between antioxidants in spermatozoa that cannot receive antioxidants from the outside eventually results in pro-oxidants. This is following the opinion of Ayala *et al.* (2014) that MDA is the end product of lipid oxidation, high levels of MDA are influenced by lipid peroxidation levels which also indicate the number of free radicals.

The addition of egg yolk diluent as an antioxidant in the experiment had slightly different levels. The MDA test can be seen in the graph of Figure 3. MDA of gout semen at different storage times at a temperature of 27°C and Figure 4. MDA of gout semen at different diluent levels at a temperature of 27°C.

### **Correlation of Sperm Abnormality and MDA Level in Muscovy Duck Semen**

Abnormality is one of the indicators in determining the quality of spermatozoa because abnormal cell structures can cause disturbances and obstacles during fertilization, further causing low rates of implantation and pregnancy (Saputro *et al.*, 2022). MDA is the process of forming fat peroxidation which has bonds in unsaturated fatty acids triggered by free radicals. Damage to the plasma membrane of spermatozoa causes abnormalities in the head and tail that affect locomotion and decrease the viability of semen so that it affects spermatozoa abnormalities. Spermatozoa head abnormalities are caused by the presence of free radicals that enter at the time of ejaculation (Benko *et al.*, 2022). MDA has been widely used as an indicator of the presence of free radicals and the occurrence of oxidative damage, Cui *et al.* (2018). Semen that is subjected to oxidative stress due to free radicals in its plasma membrane contains unsaturated fatty acids that cause cell damage. Damaged spermatozoa membranes will cause a decrease in the integrity of the spermatozoa membranes, which in turn causes a decrease in sperm quality (Wysokińska and Szablicka, 2021).

The highest MDA at 27°C was in the treatment of adding 15 times the diluent level without storage or control. The decrease in motility followed by abnormalities is thought to be due to metabolic processes so the amount of energy used to move causes a decrease during storage and the possibility of changes in the physiological properties of semen in the medium. The high level of MDA in the 15 times diluent is due to the formation of free radicals that react with cell membrane components in the faster dilution of the egg yolk semen, this occurs in the control semen that has not experienced stress and

after semen treatment many are stressed due to treatment and the addition of egg yolk diluent so that spermatozoa undergo damage accompanied by increased abnormalities of spermatozoa. Alahmar (2014) stated that lipid peroxidation will cause structural damage and disrupt the metabolism of spermatozoa which results in the death of spermatozoa.

The addition of different levels of diluent and shelf life at 27°C, the addition of diluent levels as antioxidants can counteract the damage of lipid peroxidation that reacts with unsaturated fats in the dilution of fresh duck semen, so that with the MDA test there is a decrease in damage to the presence of pink discoloration deposits. Muscovy duck semen provides an average of the percentage of individual semen abnormalities. Based on table 2 average abnormality of Muscovy duck semen in dilution levels and different storage times at 27°C showed that the average diluent level of  $7.46 \pm 1.52$  and storage time of  $7.46 \pm 1.48$  were included in the category of semen suitable for the AI process. The average abnormality of spermatozoa has not reached 20% of the semen, so the semen can still be used for insemination (Perry, 2021).

## CONCLUSION

It can be concluded that abnormalities at a dilution rate in the A1 group of 5 times with a shelf life in the B1 group of 60 minutes and B2 group of 120 minutes showed a significant effect for suitable Muscovy duck semen regarding insemination requirement.

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## AUTHORS' CONTRIBUTIONS

SA: Conceptualization and drafted the manuscript. AT, EY, and ARK: Performed sample evaluation. ARK: Validation, supervision, and formal analysis. AT and SA: Performed the

statistical analysis and the preparation of tables and figures. All authors have read, reviewed, and approved the final manuscript.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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