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Assessment of Seminal Diluent Efficiency Using Different Extenders on Sperm Viability and Fertility of Muscovy Drake for *Anas-Cairina* Hybridization

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Abstract

BANGLADESH'S poultry industry offers rapid earnings and affordable employment opportunities, with a primary focus on chicken production. However, native duck productivity is low, leading to surplus drakes and spent ducks. Mule ducks, produced via artificial insemination (AI) between Muscovy and common ducks, are essential to global duck meat production due to their hybrid vigor and superior meat quality. However, optimizing AI techniques requires effective seminal diluents to preserve sperm viability and enhance fertility. This study evaluated the impact of different extenders and dilution ratios on the sperm quality and fertility of Muscovy drake semen in a tropical environment. Three extenders, Avian Universal (AU), Ringer's Acetate Semen Extender (RASE), and Commercial Poultry Semen Extender (CPSE), were tested at dilution ratio of 1:1, 1:3, and 1:5 on a productive F1 female (Pekin♂ × Rupali♀). Results indicated that AU extender achieved the highest fertility rate (67.8%), outperforming RASE and CPSE, while higher dilution ratios reduced sperm concentration and fertility. Temperature-humidity index (THI) analysis revealed a minimal impact on semen quality, although slight reductions in semen volume were observed. The findings underscore the significance of extender composition and dilution ratios in maintaining semen viability and optimizing AI success in hybrid duck production. Advancing artificial insemination (AI) in ducks requires refining semen collection methods, improving semen extenders, and enhancing storage techniques to boost fertility and efficiency.

Keywords: Hybrid duck, artificial insemination, semen extender, sperm quality, fertility

Introduction

The poultry industry in Bangladesh provides rapid earnings and local employment opportunities at affordable prices [1]. While the primary focus is on chicken production, the sector also raises guinea fowls, geese, quails, ducks, and pigeons, with ducks showing particular promise and significant potential within the poultry sector in Bangladesh [2]. However, the productivity of native ducks in the country is low in terms of meat production live weight (1385.34 ± 41.25 gm) [3]. In Bangladesh, the demand for duck meat has been partially met by the surplus drakes (the extra males that remain after selecting a few for breeding purposes) of egg-type ducks and often spent ducks, due to the lack of suitable meat-type duck breeds [4]. The mule duck is an interspecies hybrid duck created either through mating or artificial insemination (AI) of female domestic ducks (*Anas platyrhynchos*) with male Muscovy ducks (*Cairina moschata*), resulting in a combination of traits from both species. This hybrid is significant in global duck meat production due to its unique characteristics and high-quality meat [5,6].

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AI is essential for mule duck production due to natural mating challenges between Muscovy and domestic ducks, caused by behavioral and anatomical differences [7]. AI improves fertilization by bypassing natural barriers, increasing the number and duration of fertile eggs in intergeneric crosses, with selected duck lines showing fertility rates as high as 91% [8,9]. AI maximizes semen use, allowing one ejaculate to fertilize multiple females and improve hybrid production success [8]. Additionally, Quantitative trait locus (QTL) analysis has identified genetic markers associated with fertility duration, providing a foundation for targeted genetic improvements in mule ducks [10]. By boosting fertility and hatchability, AI in poultry enhances breeding efficiency, productivity, genetic selection, chick output, and economic viability, especially in commercial settings [8]. AI boosts commercial turkey production by optimizing semen use and timing, improving fertility, hatchability, and the yield of saleable chicks [11]. While AI offers numerous benefits, but requires proper training and regulation for optimal practices and timely insemination post-collection due to the poor cryopreservation response of poultry semen [8]. Muscovy and common ducks differ in mating behavior, are non-monogamous, and face high sperm competition and passive sperm loss, which impacts fertilization success.[12]. Crossbreeding Muscovy with common ducks often results in low fertility and shorter fertility duration due to intergeneric crossing [13,14]. Muscovy ducks show marked sexual dimorphism, with larger males influencing mating and reproduction, driven by neuroendocrine and genetic factors [15]. Hybrid ducks have a higher growth rate, but commercially, they have lower fertility compared to natural breeding, which yields 18-34% [16]. The main problem with hybridization is that the fertility rate is usually lower than that of pure breeds [17]. The genotype, age, and physiology of the Muscovy drake [18,19] and the environmental conditions [20,21] affect sperm quality. The quantity and quality of semen, particularly the semen quality factor encompassing three traits (volume, sperm concentration, and morphology), are critical indicators of male reproductive potential, especially regarding fertilization capacity [22].

AI, using dilutors, overcomes mating challenges from genetic differences between Muscovy and common ducks, enhancing fertility and mule duck production [5], but it requires careful handling of semen to maximize fertility rates [23]. Dilutors are used to maintain semen quality and facilitate its distribution across multiple females, optimizing the use of each ejaculate [8]. The choice of dilutor is crucial for maintaining sperm motility, viability, and fertility outcomes in AI. Gerzilov *et al*. [24] mentioned, the commercial extender enhances sperm motility and viability, with dilutor choice, storage temperature, and duration affecting semen quality. In contrast, the AU extender maintains higher rates, while antioxidants mitigate oxidative stress.

Understanding these challenges is crucial for improving AI practices in poultry. Poultry semen shows a poor response to cryopreservation, necessitating immediate use after collection and this limits the flexibility of AI operations and requires precise timing to ensure semen viability [8]. Ewuola *et al*. [25] mentioned, vaginal insemination requires skill to avoid injury, while improper semen dosage or timing can reduce fertility and hatchability. Thus, this project aimed to assess the impact of dilutors on Muscovy drake semen fertility and AI adaptation in tropical environments for H1 meat-type hybrid duck production.

Material and Methods

Ethical statement

This research was approved by the Animal Experimentation Ethics Committee, Bangladesh Livestock Research Institute, Savar Dhaka-1341 (Reference no. 33.05.2672.303.05.007.19-1469).

Location and study design

The experiment was carried out at the ‘Waterfowl production research farm’ under the Poultry Production Research Division (PPRD) of Bangladesh Livestock Research Institute (BLRI) in Savar, Dhaka, Bangladesh, located at latitude 23°53'19" N and longitude 90°16'25"E and has an elevation of 10.62m (34.84ft). Initially, the parent Muscovy ducks (both sexes) and sound productive F1 were selected according to known methodology for semen collection, hatching egg collection, and artificial insemination activities. Semen was collected using the male stimulation method by a productive Muscovy female. Both macroscopic and microscopic semen evaluation was performed visually and with a computer-assisted semen analyzer (CASA).

Selection criteria with particulars of experimental ducks

The Muscovy duck (male and female) and F1 female, a crossbred between Pekin male and Rupali female (a native common duck, improved by BLRI through selective breeding) were chosen with no physical defects, healthy, free from external parasites, both Muscovy and F1 with production stage and Muscovy drake with sexually matured (Table 1).

Rearing management of Muscovy and F1 Duck

The experimental ducks (MD and F1) were kept in an open-sided semi-gable roof with a concrete floor poultry house, where both types of ducks were kept in separate houses. Throughout the experiment, all males (MD) were kept separately in cages with individual pens (4 cages, 4 pens/cage, each with 100

cm³/pen), specially designed for restricted breeding, where the females were kept on the floor. The female of F1 was kept at night in a nest box (6 separate boxes/nest with 50 cm3) to collect the egg individually according to treatment. Egg collection was conducted each morning within a 30-minute timeframe throughout the experimental period. Each cage was outfitted with its feeder and drinker. All of the ducks were raised in a poultry house with natural ventilation. A commercial feed with a specific feeding regime was supplied to the experimental ducks.

Temperature Humidity Index (THI) of the trial shed

The purpose is to collect temperature and humidity data to determine whether body metabolism activity has any impact on the features of sperm and semen. Data on the mean temperature (°C) and mean humidity (%) were collected between January 2024 and April 2024. The temperature-humidity index (THI) was computed utilizing a mathematical model [27,28].

Calculation: THI: (0.8×AT) + [(RH/100) ×(AT-14.4)] + 46.4; AT: Ambient Temperature (°C) RH: Relative Humidity (%) [66].

Collection of semen through male stimulation by productive females

Prior to semen collection, the sexually mature Muscovy drakes performed a two-week training regimen utilizing female stimulation by natural mating for semen retrieval [22]. Twelve of the eighteen drakes were chosen for semen collection purposes based on the male response (speed of climbing on a female and ejaculation, Table 2). Throughout the trial, the experimental ducks were grown in a natural setting with natural light and a dark photoperiod. Semen volume was measured by using a tuberculin glass syringe graduated to 1 ml [29]. During semen collection, the female Muscovy duck was initially caged with the target drake and then a skilled technician assisted the drake by helping it to climb the back of the female when the drake was excited. Semen was successfully collected by pressing the cloacal erection, showing that it was important to avoid contaminating the semen with cloaca/fluids. Semen was collected by stroking the belly and back above the testes to stimulate the copulatory apparatus to emerge. This was followed by pressing the tail forward with one hand while collecting semen from the organ's duct with the thumb and forefingers of the same hand. The sperm was collected in a clear, transparent glass vial, poured into an Eppendorf tube, and inserted into a hot water pot maintained at 37 ℃ for further analysis [29]. The semen was collected in two steps, after a two-week training regime, the semen was collected 3 times, as observation with 3 days intervals to evaluate the semen characteristics, and from the next 12 weeks onward, the semen was collected to assess the effect of dilutors in different ratio (1:1, 1:2 and 1:3) on reproductive performance (only fertility issue).

Characteristics of selected Muscovy female and F1 females

Table 3 shows the selection criteria of MD and F1 females. The MD females were around 40th weeks of age with egg production of around 50%. The female MD was only used to stimulate the male during semen collection. The F1 female was in peak production (60-65%) and both species were physically mature and sound.

Macroscopic evaluations of Muscovy semen

After semen collection, the initial task involved a macroscopic examination during ejaculation [30]. Semen was evaluated both macroscopically and microscopically, whereas visual inspection determined the color, consistency, and transparency macroscopically (Table 4). The properties of the semen were assessed immediately after collection [31,32,33,34].

Microscopic evaluations of Muscovy semen

Immediately after collecting the semen and placing it in a tube, 40µl of the semen was combined with 3.8 ml (3800 µl) of Dulbecco's Modified Eagle Medium (DMEM) solution. The semen mixture was then collected and immobilized on a slide using an automated micropipette. The solution mixture was prepared at a 1:100 ratio and transferred into a 20-micron standard count 4 chamber slide called a Leja slide. The slide was inserted into the analyzer, as described by Hoque *et al*. [34]. The CASA software (Hamilton Throne Ivos, Planer, United Kingdom) was used to assess various morphological characteristics of the semen, including motility, sperm concentration, and tail abnormalities such as bent tail, coiled tail, DMR (distal midpiece reflex), distal droplet, proximal droplet, and others (Table 4). The calculation of motility involved determining the percentage of sperm exhibiting moderate to rapid progressive movement, as defined by Otecko *et al*. [35].

Production of different extenders for screening the most suitable extender

The Muscovy drake semen was diluted using extenders for screening the most suitable extender. For this study, we used three different extenders having specific compositions like Avian Extender (AU) (Table 5), Ringer's Acetate Semen Extender (RASE) [36] (Table 6), and Commercial Poultry (Ovodyl TSS) Semen Extender (CPSE). The

composition of CPSE was unknown as their trade secret. The extenders were mixed with fresh semen and stored at 37ºC before insemination.

Dilution and identification of the insemination ratio at different doses of diluted semen

Immediately after collection, semen was kept in the water bath at 37ºC and diluted with all three dilutors in the ratio of 1:1 (50%), 1:3(25%), and 1:5(16.67%) according to Gerzilov *et al*. [37]. The pH of the dilutors was adjusted to 7.00. Four different F1 females were used for every dilutor treatment and ratio, kept in different pens and nest boxes. The individual F1 was tagged with wing band with a different identification number.

Insemination of common ducks (F1) with Muscovy semen

Fresh semen (as control) and three dilutors with three dilution ratios (1:1, 1:3, and 1:5) were set in eight different pens of F1 duck, where every two pens were fixed for each treatment and the insemination was followed according to Chen *et al*. [38]. The insemination was done within 20 minutes after dilution and 30 min. after collection. About 3 cm depth into the everted oviduct 0.3 ml diluted semen was introduced [16]. The F1 female was inseminated three times during the 12-day egg collection period. The eggs were collected daily from the 3rd day of post-insemination to the 12th day. An equal number of eggs artificially inseminated by diluted and raw semen groups were incubated to determine the percentage of fertility at the 10th day of incubation.

Statistical analysis

All data were recorded and analyzed using a Completely Randomized Design (CRD) with the Generalized Linear Model (GLM) procedure in SPSS 26.0, while mean comparisons were conducted using Duncan's Multiple Range Test (DMRT).

Results

Temperature Humidity Index (THI) of the experimental shed

During the experiment, the temperature and relative humidity index (THI) were recorded to determine the heat stress condition of the experimental shed throughout the experimental tenure (3rd November ’23 to 3rd March’ 24). THI is a commonly used index to measure heat stress in poultry and it combines temperature and humidity to provide a single value indicating the level of heat stress. Table 7 shows the average temperature (°C), RH% (relative humidity), and THI of the shed throughout the period.

Characteristics of Muscovy semen (macroscopic)

Macroscopic examination of semen includes volume, pH, color, consistency, and transparency. These observations are necessary to determine the quality of semen and male reproductive efficiency and the dilution rate of semen [40]. The macroscopic characteristics of Muscovy semen are presented in Table 8.

Characteristics of Muscovy semen (Microscopic)

The microscopic examination includes average concentration, mass movement, motility, and the percentage of live or dead [41]. The effects of seasonal variation in THI on semen quality parameters were assessed from February to April. A statistically significant ($p<0.05$) increase in THI was observed across the months, rising from 18.57 in February to 26.21 in April. Semen volume, sperm concentration, pH, and total motility did not show statistically significant differences. Table 9 denotes the microscopic characteristics of different semen parameters of Muscovy duck, and Figure 1 shows the sperm movements for different ratio and dilutors.

Effects of different dilutors' ratio on the morphology of Muscovy semen

Table 10 presents the effects of three ratios of AU extender (1:1, 1:3, and 1:5) on sperm quality parameters, including sperm concentration (M/ml) and different morphological parameters. Only sperm concentration showed significant differences ($P<0.05$) and non-significant on all other parameters. No statistical significance ($P>0.05$) was found in all morphological parameters.

Effects of different dilutors on the fertility of Muscovy semen

Fresh semen, serving as the control group, exhibited consistently high fertility across all ratios. Among the extenders, AU consistently resulted in higher fertility rates across all ratios, followed by RASE (moderate fertility rates), and CPSE exhibited the lowest fertility rates (Table 11). The statistical analysis revealed significant differences ($p<0.05$) in fertility outcomes among the extenders and dilution ratios. The fertility rate showed significant differences ($p<0.05$) among the treatments, where the fresh semen being the control group was recorded as the highest (73.69%).

Discussion

Between February to April 2024, the thermal environment had a gradual increase in temperature and heat stress conditions, with maintaining a comfortable temperature within the comfort zone. This trend is consistent with findings of Bouraoui *et al.* [42], who indicated that higher ambient temperatures and relative humidity levels significantly affect the thermal comfort index. The THI is a widely used indicator that combines air temperature and humidity to estimate heat stress, especially in occupational health, animal welfare, and environmental studies [43]. According to the

National Research Council [44], a THI above 24 often corresponds to mild heat stress, while values exceeding 26 indicate moderate to severe heat stress levels depending on the duration and exposure conditions. In this study, the rise in THI values from February to April suggests a seasonal shift toward hotter and more humid weather, contributing to increased thermal discomfort and potential health risks. Prolonged exposure to such conditions, particularly during peak daytime hours, may impair physical performance, concentration, and well-being [45]. While THI is a crucial measure for assessing thermal comfort in poultry sheds, it is important to consider other environmental factors such as air velocity and shed design. These factors can influence the effectiveness of THI as a sole indicator of heat stress. Additionally, regional variations in climate and the specific needs of different poultry breeds may require tailored approaches to managing thermal environments in poultry production [46]. These findings underscore the importance of heat mitigation strategies, such as shaded work areas, hydration protocols, and adjusted working hours during high-heat periods, especially as climate variability increases the frequency of extreme weather events.

The present study examined the influence of rising THI on semen quality parameters from February to April. Most parameters, including concentration, pH, and motility, did not differ significantly across months. Despite a significant increase in THI from 18.57 in February to 26.21 in April, there was a minimal impact on semen quality, with the exception of a slight variation in semen volume, as the previous studies have reported that elevated THI values correlate with deteriorating semen quality in various species. As the semen volume is particularly sensitive to environmental stressors, especially heat and heat stress can reduce activity of the accessory reproductive glands, which contribute to seminal plasma, resulting in lower ejaculate volume without necessarily impacting sperm concentration or motility in the short term [47]. In birds, including ducks, seminal fluid is largely derived from the epididymal region and vas deferens; thermal stress can transiently impair their secretory function without directly damaging spermatogenesis [48]. Previous studies on poultry and waterfowl have also reported that mild to moderate heat stress primarily affects semen volume before other quality traits are altered. For example, in Muscovy and Pekin drakes, environmental heat increased the time required for ejaculation and slightly reduced semen volume, while sperm viability and motility remained stable over short periods [49,50].

Additionally, the thermal threshold for changes in semen parameters may not have been crossed. While the THI increased during the study period, it likely remained below the critical level required to disrupt testicular thermoregulation or spermatogenic function. Birds have some thermoregulatory adaptations, such as panting and reduced physical activity, that mitigate short-term heat effects [51]. Thus, the unchanged semen quality in this study, despite a modest decrease in volume, is consistent with published literature and can be explained by early, reversible physiological responses to mild heat stress rather than damage to sperm production or maturation.

In the current study, while total and progressive motility did not differ significantly across months, slow motility decreased markedly in March, which may be an early indicator of heat-induced stress on sperm metabolic function, similar findings were reported in Murrah buffaloes, were heat stress reduced sperm membrane integrity and motility, despite no significant changes in semen volume [52]. These subtle motility changes may arise from thermal stress affecting testicular thermoregulation or epididymal function [53]. Interestingly, semen volume and sperm concentration remained stable throughout the study period. These parameters are often less sensitive to short-term or mild heat stress but may decline with prolonged exposure [54]. This could suggest that the duration or intensity of heat stress during the study was not sufficient to impair spermatogenesis significantly.

The findings indicate that the AU extender ratio significantly impacts certain sperm parameters, particularly sperm concentration. The significant decline in concentration at higher extender dilutions (1:5) may suggest that an excessive dilution of seminal plasma reduces the viability or integrity of spermatozoa. Previous studies, such as those by Chen *et al*. [55], emphasized the importance of optimal extender concentration in preserving spermatozoa characteristics. While sperm and progressive motility were highest in the 1:3 ratio, these improvements were not statistically significant. This suggests that the 1:3 ratio provides an optimal balance between dilution and preservation of motility, corroborating earlier research findings [56]. However, the 1:5 ratio, with higher static sperm percentages, indicates possible adverse effects on sperm metabolism or structural integrity. The morphological parameters, including bent tails, coiled tails, and distal droplets, exhibited no significant variations across extender ratios, aligning with findings by Foote *et al.* [57]. This stability suggests that morphology is less sensitive to dilution ratios, as long as extenders maintain adequate osmotic pressure and nutrient availability. Interestingly, the DMR (disrupted membrane rate) was highest in the 1:3 ratio, indicating better preservation of membrane integrity. This ratio might optimize the biochemical environment

necessary for maintaining membrane functionality, as observed in similar extender studies [58].

The study revealed that the fertility of Muscovy semen is significantly influenced by the choice of extender and the dilution ratio. Fresh semen exhibited the highest fertility rate (73.69%), which aligns with previous findings that undiluted semen generally maintains optimal sperm viability and motility due to the absence of processing-related stresses [59,60]. Among the extenders tested, AU showed the best performance (67.8%), followed by RASE (59.97%) and CPSE (54.99%). The superior performance of AU may be attributed to its composition, which likely provides better osmotic balance, pH stabilization, and energy substrates essential for maintaining sperm integrity [61]. In contrast, the lower fertility rates with RASE and CPSE suggest a potential incompatibility with Muscovy semen or suboptimal nutrient support during storage. This finding is consistent with studies indicating that extender composition significantly impacts sperm motility and fertility [62,63]. The dilution ratio significantly influences fertility outcomes, as variations in extender concentration can affect sperm viability by altering the availability of protective agents and nutrients. For example, higher dilution ratios may reduce the protective effects of extenders, leaving sperm more vulnerable to oxidative damage or membrane destabilization [64]. The interaction effect between extender type and ratio was not significant ($p>0.05$), indicating that the effects of these two factors (extender type and ratio) are independent. Therefore, each factor can be optimized separately for artificial insemination applications. These results are consistent with broader findings in poultry reproduction research, where extenders are known to differ in their ability to preserve sperm function and fertility. For example, Akhtar *et al.* [65] reported that extenders with antioxidants and membrane stabilizers are more effective in maintaining sperm motility and viability during storage.

Conclusion

This study highlighted the critical role of dilutor ratios and types in influencing the morphological performance and reproductive outcomes of Muscovy duck semen. The AU extender ratio significantly impacted sperm concentration, with a 1:3 ratio offering an optimal balance between dilution and preservation of motility. Higher dilution ratios (1:5) negatively affected sperm concentration and viability, whereas morphological parameters remained stable across different extender ratios. The fertility of Muscovy semen was highest with fresh semen, followed by the AU extender, which provided superior osmotic balance and nutrient support. Higher dilution ratios reduced fertility due to lower protection against oxidative damage and membrane destabilization. Future studies should focus on the long-term storage and post-thaw fertility of Muscovy semen, as well as the optimization of extender formulations that can better support sperm quality over extended periods. The role of antioxidants and cryoprotectants should be explored further to enhance the stability and fertility of stored semen, to improve the efficiency and success of artificial insemination in Muscovy ducks.

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Conflict of interest

No conflict of interest

TABLE 1. Different selection criteria for experimental ducks

Parameters	Genotype	Features	
Age	MD	40th, 60th and 90th week	
	F1	46th week (production stage)	
Plumage	MD	White and mixed	
	F1	White	
population size (no.)	MD	18 (Male), 12 (Female)	
	F1	48 in 08 (06 in each pen)	
Weight (kg)	MD	Male (3.5±), Female (1.6±)	
	F1	Female (1.7±)	
Physical characteristics	MD (male & female)	Sound	No physical defects, healthy, free from external parasites, drake's squatting behaviour with matured morphometric measurements, production stage (Muscovy female)
		Defective	Bumblefoot or any problem in leg, unhealthy, absence of drake's squatting behaviour, with immature morphometric measurements, moulting stage/declined egg production (F1)
	F1 (only female)	Productive	The ducks are in the peak/off-peak egg production stage
		Moult	Interim period of the laying cycle
		Non-productive	No production/irregular egg production
Male's reaction to stimulation (The way and speed of the male's reaction to semen collection) [26]		The best	As soon as the female is put in the male's box, the drake jumps and tries to mate; it takes about one to two minutes from introducing the female to ejaculation of sperm
		Good	The reaction is the same as the first type, but it lasts up to three minutes longer
		Poor	The male shows interest and doesn't mate or ejaculate
		No reaction	Shows no interest in females

Note: MD - Muscovy Duck (male and female); F1 - female, first filial generation (2-way crossing)

TABLE 2. Characteristics of Selected Muscovy Drake for semen collection

No.	Tag No.	Age (wk)	Plumage color	Body wt (kg)	Body width (cm)	Body length (cm)	Shank length (cm)	Health status	Male response	Selection
01	MD-07		white	4.26	34.49	36.03	6.83	Sound*2	Good	Selected
02	MD-19		white	4.08	34.12	35.89	6.58	Sound	Best	Selected
03	MD-32	90th	white	4.35	34.49	36.03	7.02	Sound	Poor	Rejected
04	MD-27		Mixed*1	3.92	35.02	35.88	6.84	Sound	NR	Rejected
05	MD-16		white	3.78	34.98	35.98	6.75	Sound	Best	Selected
06	MD-22		white	4.12	36.67	35.82	6.86	Sound	NR	Rejected
07	MD-39		Mixed	4.12	35.56	35.23	6.95	Sound	Good	Selected
08	MD-56		white	3.52	33.04	35.4	6.48	Sound	Best	Selected
09	MD-43		white	3.96	35.23	35.76	6.76	Sound	Best	Selected
10	MD-47		Mixed	3.52	34.18	35.08	6.62	Sound	NR	Rejected
11	MD-48	60th	white	3.86	35.23	34.72	6.77	Sound	Best	Selected
12	MD-37		white	3.92	34.92	35.35	6.71	Sound	Good	Selected
13	MD-52		Mixed	3.84	33.87	34.82	6.46	Sound	Best	Selected
14	MD-53		white	4.02	35.23	36.04	7.08	Sound	NR	Rejected
15	MD-54		white	3.74	33.76	34.94	6.32	Sound	NR	Rejected
16	MD-57		white	3.02	34.09	34.03	6.56	Sound	Good	Selected
17	MD-58	40th	Mixed	3.23	33.12	33.8	6.26	Sound	Best	Selected
18	MD-61		Mixed	2.98	32.98	33.21	6.12	Sound	Good	Selected

Note: *1Muscovy ducks with mixed black and white plumage distributed across various parts of the body; *2Clinically healthy ducks, free from any visible diseases or physical abnormalities.

TABLE 3. Characteristics of selected Muscovy female and F1 female

No.	Duck	Age (wk)	No. of duck	Plumage color	Body wt (kg)	Egg production	Body width (cm)	Body length (cm)	Health status
01	MD-Female	36^{th}-40^{th}	12	White/mixed[*1]	1.62	40-55%	31.46	33.17	Sound[*2]
02	F1-Female	46^{th}-50^{th}	48	white	1.68	60-65%	31.65	33.29	

Note: *[1]Muscovy ducks with mixed black and white plumage distributed across various parts of the body; *[2]Clinically healthy ducks, free from any visible diseases or physical abnormalities.

TABLE 4. The different semen criteria for macroscopic and microscopic evaluation

Criteria	Features
Color[*1]	Assessed visually through transparent collection plastic vial, looking likely white/milky white/creamy white/cloudy white
Consistency[*1]	Thicky: Movement of semen in a cup is slow; Watery: Movement of semen in a cup is quick
Transparency[*1]	Opaque: Good quality (semen is not transparent); Cloudy: Semen mixed with fecal and blood; Clear: Semen with low quality (not mixed with fecal and blood
Volume[*1]	ml, collected in a measuring tube (cryo vial/ eppendorf)
Concentration[*2]	M (million)/ml, measured in CASA machine
Motility[*2]	Percent of sperm showing any movement (Static: percent live sperm with no movement; Progressive: percent of sperm with rapid movement in a straight path; Slow: percent of sperm with slow movement
Bent tail (curved)[*2]	A tail that deviates from the straight path, can affect the sperm's ability to swim properly
Coiled tail[*2]	A tail that forms a spiral or coil, can also hinder the sperm's motility
DMR[*2]	A distal mitochondrial droplet is a droplet containing mitochondria located near the end of the sperm tail.
Distal droplet[*2]	A small droplet of cytoplasm situated near the end of the sperm tail
Proximate droplet[*2]	A small droplet of cytoplasm located close to the sperm head

*[1](Visual measurement): [31,33,34]
*[2](Measured by CASA machine/microscopic): [32]

TABLE 5. Extender 1- Composition of AU (Avian Universal semen Extender)

Reagent Name	Unit	Proportion /Amount	Comment
D-Glucose	g w/v	0.40	EDTA (ethylene diamine tetraacetic acid disodium salt dihydrate) Osmolarity: 320 mOsmol/kg pH: 7 Store: 4°C for 6 hrs DDW (Double Distilled Water)
Sugar	w/v	0.80	
D-Fructose	w/v	0.80	
Sodium Citrate	v/v	0.90	
Sodium Glutamate	v/v	0.84	
Glycocol	v/v	0.40	
EDTA	µl	0.04	
DDW	ml	100	

TABLE 6. Extender 2- Composition of Ringer's Acetate Solution (BLRI improved)

Reagent Name	Unit	Proportion/Amount	Comment
Sodium acetate	g	1.9	Osmolarity: 320 mOsmol/kg
Sodium Chloride (NaCl)	g	3.0	pH: 7
Potassium Chloride (KCl)	g	0.15	Store: 4°C for 6 hrs
Calcium Chloride (CaCl2. 2H2O))	g	0.1	DDW (Double Distilled Water)
Water for injection/ DDW	ml	500	

TABLE 7. The thermal environment of the shed throughout the experiment

From 10 AM-4 PM	Thermal environment							
	Min. temp(°C)	Max. temp(°C)	Avg. temp(°C)	Avg. RH (%)	Avg. dry bulb (°C)	Avg. wet bulb (°C)	THI[*2]	HS condition[*1]
February'24	18.83	31.98	26.29	57.0	19.1	21.8	18.57[a]	Absence of Heat stress
March'24	23.08	36.57	31.04	57.0	23.3	25.3	22.04[b]	Absence of Heat stress
April'24	27.35	38.98	34.25	66.0	27.6	29.7	26.21[c]	Moderate heat stress
Average	23.08	35.84	30.53	60	23.33	25.6	22.22	Moderate heat stress

Note: *[1]Oguntunji *et al.* [66]; *[2]found significant difference

TABLE 8. The macroscopic characterization of Muscovy semen

Observation (Frequency %)	Color (%)			Consistency (%)		Transparency (%)		
	White	Milky white	Creamy white	Thicky	Watery	Opaque	Cloudy	Clear
30 (100)	56.67	10	33.33	0	100	70	0	30

Note: These are the average data, the raw data of the observation are given in the appendix chapter

TABLE 9. Effect of month of the experimental period on semen quality of Muscovy duck

Months	Volume (ml)	Conc (M/ml)	pH	Motility (%)	Progressive (%)	Slow (%)	Static (%)
February	1.22	162.735	7.275	69.61	46.35	28.25	30.39
March	1.21	150.938	7.05	67.03	46.3	18.23	32.97
April	1.12	161.72	7.35	69.12	43.125	23.50	30.88
SEM	0.05	10.51	0.06	1.81	2.81	1.86	0.05
LS	0.73	0.90	0.10	0.85	0.89	0.07	0.85

Note: 'SEM' Standard error of mean; LS, Level of significance

TABLE 10. The sperm concentration, morphology and motility using the AU extender

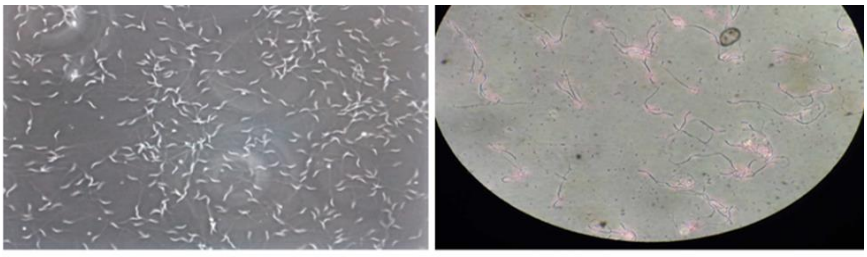
Ratio (AU extender)	1:1	1:3	1:5	SEM	LS
Sperm concentration (M/ml)	76.75^{b}	69.39^{ab}	45.63^{a}	5.64	0.04
Motility (%)	61.24	68.49	59.15	3.41	0.06
Progressive (%)	38.44	42.85	40.18	3.09	0.27
Slow (%)	30.40	29.49	26.16	1.11	0.80
Static (%)	38.80	31.50	32.00	2.47	0.45
Bent Tail (%)	23.40	27.38	27.17	2.60	0.68
Coiled Tail (%)	1.82	1.68	1.77	1.98	0.55
Distal Droplet (%)	12.90	12.25	14.30	0.28	0.40

Note: SEM, standard error of the mean; AU, Avian universal; LS, Level of significance;
[a, b] Means the difference among ratio is significant ($p<0.05$) shown with different letters;

TABLE 11. Effect of extender, ratio and interaction on fertility of MD semen

Parameter	Ratio (R)	Extender (E)				SEM	LS		
		Fresh	AU	RASE	CPSE		E	R	E×R
Fertility %	1:1	74.31^{c}	67.09^{b}	60.77^{a}	57.64^{a}	2.04	0.000		
	1:3	72.76^{d}	67.92^{c}	60.14^{b}	51.44^{a}	2.87	0.03		
	1:5	74.00^{c}	68.41^{b}	59.03^{a}	55.91^{a}	2.67	0.04		
	Overall	73.69^{c}	67.8^{b}	59.97^{a}	54.99^{a}	1.44	0.000	0.781	0.978

Note: SEM, standard error of the mean; AU, Avian universal; RASE, Ringer's Acetate semen extender; CPSE, Commercial poultry semen extender; E, Extender; R, ratio; ExR; interaction between extender and ratio
[a, b, c, d, e] means the difference among different dilutors is significant ($p<0.05$) shown with different letters



A B

Fig. 1. Sperm movement of Muscovy drake: A (CASA); B (phase-contrast microscopy, magnification about 400x)

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