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Comparing the effectiveness of different extenders for preserving rooster semen

Comparação da eficácia de diferentes diluentes para preservação do sêmen de galos

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ABSTRACT: The present study aimed to evaluate the effectiveness of experimental extenders based on coconut oil (COC) and copaiba oil (COP) compared to extenders based on whole powdered milk (WPM) and powdered egg (PWE) in preserving rooster semen for up to 15 minutes at room temperature. The experimental design employed a factorial layout (4x4), encompassing four different extenders (extenders based on COC, COP, WPM or PWE), each applied across four distinct timeframes after semen collection (1, 5, 10, and 15 minutes). All evaluated variables displayed a significant impact ($p<0.05$). This investigation revealed that the extender containing COC led to a noteworthy reduction in the pH of the sperm samples, approximately 1.31 in semen pH, resulting in a more acidic environment, while COP caused a reduction of 0.25 in semen pH. This alteration created an unfavorable condition for the sperm, which inherently favors a more alkaline pH setting, subsequently compromising their quality. In contrast, extenders based on WPM and PWE yielded superior results in sperm quality and viability at room temperature, providing lower reductions (less than 0.25) in pH and, consequently, a better environment for the sperm. Conclusively, extenders based on WPM and PWE exhibited superior capability in preserving rooster sperm at room temperature for up to 15 minutes when compared to extenders formulated with COP or COC. These extenders demonstrated remarkable outcomes in terms of sperm quality and viability.

KEYWORDS: coconut oil; copaiba oil; poultry; semen extender; sperm quality.

RESUMO: O presente estudo objetivou avaliar a eficácia de diluentes experimentais à base de óleo de coco (COC) e óleo de copaíba (COP) em comparação com diluentes à base de leite em pó integral (WPM) e ovo em pó (PWE) na preservação do sêmen de galos por até 15 minutos à temperatura ambiente. O delineamento experimental utilizou um arranjo fatorial (4x4), abrangendo quatro diluentes diferentes (diluentes à base de COC, COP, WPM ou PWE), aplicados em quatro intervalos de tempo distintos após a coleta de sêmen (1, 5, 10 e 15 minutos). Todas as variáveis avaliadas apresentaram impacto significativo ($p<0,05$). Esta investigação revelou que o diluente contendo COC levou a uma redução notável no pH das amostras de esperma, aproximadamente 1,31 no pH do sêmen, resultando em um ambiente mais ácido, enquanto o COP causou uma redução de 0,25 no pH do sêmen. Essa alteração criou uma condição desfavorável para o esperma, que naturalmente favorece um ambiente com pH mais alcalino, comprometendo assim sua qualidade. Em contraste, os diluentes à base de WPM e PWE apresentaram melhores resultados na qualidade e viabilidade do esperma à temperatura ambiente, proporcionando reduções menores (menos de 0,25) no pH e, consequentemente, um ambiente melhor para o esperma. Conclusivamente, os diluentes à base de WPM e PWE exibiram superior capacidade de preservação do sêmen de galo à temperatura ambiente por até 15 minutos em comparação com os diluentes formulados com COP ou COC. Esses diluentes demonstraram resultados notáveis em termos de qualidade e viabilidade do esperma.

PALAVRAS-CHAVE: avicultura; diluente de sêmen; óleo de coco; óleo de copaíba; qualidade espermática.

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INTRODUCTION

Modern biotechnology is responsible for significant advances in poultry science, studying areas such as artificial insemination, embryo development, gene transfer, embryo sexing, and semen handling (Rutz *et al.*, 2007; Lavor; Câmara, 2012; Freitas *et al.*, 2018). For poultry farms, the dilution and preservation of semen for long intervals represent excellent support in breeder selection programs (Morais *et al.*, 2012), especially considering that rooster semen is highly concentrated, producing a low volume compared to other farm animal species. Additionally, rooster semen has a maximum service life at room temperature of up to 15 minutes (Rutz *et al.*, 2007; Bongalhardo, 2013; Rufino *et al.*, 2014; Feijo *et al.*, 2016; Freitas *et al.*, 2018; Partyka; Nizanski, 2021). Moreover, it can aid in developing more efficient procedures for preserving rooster semen for longer periods, using artificial insemination for breeder selection, and monitoring high-performance progeny results compared to the breeders (Rutz *et al.*, 2007; Bongalhardo *et al.*, 2009).

It is described in the literature some extenders tested and available to improve the roosters' semen performance in the poultry industry, especially milk and egg products (Rutz *et al.*, 2007; Silva; Guerra, 2011; Lavor; Câmara, 2012). Despite these products being well-established in the literature regarding their effectiveness in preserving poultry semen, there are limitations to their use due to the fact that these products are also utilized for human consumption and other purposes, which reduces their availability in the market. Additionally, due to the limitations imposed by the fragility of the roosters' semen for handling at room temperature and its cell structural characteristics, new extenders continue to be tested searching maintain the fundamental properties of the seminal fluid for long time intervals, and providing stability and longevity for the sperm (Purdy *et al.*, 2009; Silva; Guerra, 2011; Bongalhardo, 2013).

Nissen and Kreysel (1983) pointed out that the use of lipid sources as semen extenders may represent an interesting alternative, especially due to the sperm membrane being constituted by more than 60% of polyunsaturated fatty acids of long-chain and presenting a natural predisposition to absorb lipids. Front this, the Brazilian biodiversity has several products presenting the potential to use as semen extender, especially essential oils rich in lipids that may increase the fluidity of the sperm membrane and its resistance to the damages caused by changes in the environment (Paulenz *et al.*, 2002; Iaffaldano *et al.*, 2007; Rutz *et al.*, 2007; Silva; Guerra, 2011; Lavor; Câmara, 2012; Adeyina *et al.*, 2017; Agostinho *et al.*, 2017).

It was hypothesized that the use of coconut oil and copaiba oil, due to their rich composition, could be used as semen extenders and have elevated the quality of roosters' sperm for long time intervals at room temperature compared to whole powdered milk and powdered egg, semen extenders commonly used (Rutz *et al.*, 2007; Lavor; Câmara, 2012). Therefore, the objective of the current study was to assess the effectiveness of different extenders (extenders based on coconut oil, copaiba oil, whole powdered milk or powdered egg) in preserving rooster semen over various time intervals at room temperature.

MATERIAL AND METHODS

The current study was conducted at the Research Poultry Farm of the Federal University of Amazonas, University campus located at Manaus city, Amazonas State, Brazil. All experimental procedures were performed according to the Local Experimental Animal Care Committee and were approved by the institutional ethics committee of the Federal University of Amazonas, Brazil (protocol number 011/2021).

Roosters and semen collection

The study was conducted on 16 breeder roosters Plymouth Rock Barred (40 weeks-of-age; average body weight of 2.32±0.21), of proven fertility, used for artificial insemination. All roosters were healthy and were ensured appropriate welfare conditions, being housed in an aviary with a density of 1 bird per m^2, feeding 115 g/bird per day of balanced diets (requirements according to Rostagno *et al.*, 2017) and water *ad libitum*.

Individual ejaculates were collected from each rooster by the method proposed by Burrows and Quinn (1937), involving an abdominal massage and movements along the sides of the cloaca. The tests took place in an aviary with an average air temperature of 27°C (80.6°F) and 65% relative humidity, recorded by a digital thermo-hygrometer. The ejaculates were collected in graduated tubes at room temperature, yielding an average semen volume of 0.95 ml per rooster.

Extenders preparation and experimental design

The collected ejaculates were immediately combined to create a homogeneous pool, ensuring equal distribution of sperm. This pool was then divided into 16 graduated Eppendorf tubes, each holding 3 mL. The tubes were assigned to four different treatments: coconut oil-based extender (4 tubes), copaiba oil-based extender (4 tubes), whole powdered milk-based extender (4 tubes), and powdered egg-based extender (4 tubes). The extenders were prepared at a ratio of 2 parts extender to 1 part semen, following the guidelines of the Brazilian College of Animal Reproduction (2013).

Extenders based on whole powdered milk and powdered egg were utilized in a 3-to-1 ratio with soy lecithin (3 parts extender to 1 part soy lecithin). Soy lecithin served as a carrier and contributed synergistically to the structural and functional maintenance of the plasma membrane of roosters' sperm, enhancing its preservative effect. Copaiba oil and

coconut oil were exclusively used as components in the experimental extenders, sourced from commercial samples. Due to the naturally acidic pH of these oils, the extenders' pH levels were adjusted using Dibasic Sodium Phosphate to align with the recommended levels established by the Brazilian College of Animal Reproduction (2013).

The experimental method was implemented in a completely randomized factorial layout (4x4), where the factors comprised four extenders (extenders based on coconut oil, copaiba oil, whole powdered milk and powdered egg) and four-time intervals following semen collection at room temperature (1, 5, 10, and 15 minutes), resulting in a total of 16 treatments with four replicates each.

Studied parameters

Each ejaculate subjected to the extenders at their respective storage times had a droplet of semen solution placed between a slide and coverslip for evaluation under light microscopy at 400x magnification. Each sample was assessed for motility (percentage of motile sperm during analysis, ranging from 0 to 100%), vigor (straight and uniform movement of sperm on a scale from 0 to 5), wave motion (progressive mass movement of sperm on a scale from 0 to 5), pH (determined using a pH meter coupled with a fine-tipped probe directly in the semen samples), and sperm concentration (semen samples were diluted 1:800 in a methylene blue solution, and the sperm were counted using a Neubauer chamber). These semen characteristics were evaluated using a Nikon Eclipse E-50i microscope (Tokyo, Japan) connected to a camera and computer-assisted semen analysis (Microptic S.L., Barcelona, Spain). This evaluation followed the methods and reference values described by the Brazilian College of Animal Reproduction (2013).

Before the study was conducted (dilution of semen in extenders and evaluation at respective time), four semen samples were collected, stored for 1, 5, 10, and 15 minutes at room temperature, and assessed in terms of: motility, vigor, wave motion, pH, sperm concentration, and (Table 1). This analysis was performed to certify the quality of the semen to be used in the tests with the extenders.

Other slides were prepared using SpermBlue staining, according to the method described by van der Horst and Maree (2009). A thin semen smear was spread on a microscope slide heated to about 36 °C. After the smears had dried, they were stained for about 15 min with SpermBlue. Then the slide was washed with distilled water and dried at room temperature. In each slide the morphological structure of 300 sperm was evaluated. The number of sperm with normal structure and the number of morphologically abnormal sperm were determined, distinguishing forms with major and minor abnormalities according to the classification described by Blom (1981).

In addition, sperm with morphological abnormalities were divided into four subgroups: sperm with head defects (isolated head or deformed head) and sperm with tail defects (bent tail or curled tail). None of the sperm presented cytoplasmic droplet. Sperm morphology was also examined using the materials described above, but at 1000x magnification.

Statistical Analyses

Before performing statistical analysis, all datasets underwent normality tests, and if necessary, transformations were applied. Subsequently, one-way ANOVA was conducted using R software (version 4.1.3), following the methodology outlined by Logan (2010). Tukey's Honestly Significant Difference test was utilized to identify significant differences among mean values, allowing for a comparison of the effectiveness of coconut oil and copaiba oil as experimental extenders for roosters' semen against whole powdered milk and powdered egg. The results are presented as means, and the significance level for observed differences was set at $p<0.05$.

Table 1. Characteristics and morphology of *in natura* semen samples stored until 15 minutes[1].

Variables	Storage times (minutes)			
	1	5	10	15
Motility, %	95.00	79.25	63.15	35.50
Vigor	4.50	4.02	3.40	2.15
Wave motion	4.50	3.42	2.50	1.50
pH	7.00	6.50	6.30	6.00
Concentration, $x10^8$	1.80	1.60	1.50	1.30
Total sperm defects, %	4.50	6.75	7.50	11.00
Isolated head, %	2.00	3.00	3.25	5.00
Deformed head, %	0.35	0.50	0.50	0.50
Bent tail, %	1.80	2.75	3.25	4.75
Curled tail, %	0.35	0.50	0.50	0.75

[1]All data represent the mean of 4 replicates.

RESULTS

Motility (Table 2) decreased as the time of sperm exposure to all tested extenders increased. Following a 1-minute exposure, motility was higher ($p<0.05$) in sperm subjected to extenders containing coconut oil and powdered egg. However, after 5 minutes of exposure, sperm treated with experimental extenders based on copaiba oil and coconut oil demonstrated lower ($p<0.05$) motility compared to those treated with extenders containing whole powdered milk and powdered egg. Furthermore, among all the evaluated extenders, sperm treated with the coconut oil-based extender exhibited the poorest ($p<0.05$) motility results.

The vigor (Table 3) and wave motion (Table 4) results exhibited a similar pattern, wherein sperm treated with extenders based on whole powdered milk and powdered egg demonstrated improved ($p<0.05$) results across all evaluated exposure times. Additionally, sperm treated with the coconut oil-based extender once again exhibited the poorest ($p<0.05$) results among all the extenders assessed.

In the semen pH results (Table 5), samples treated with extenders based on whole powdered milk and powdered egg maintained a more alkaline pH ($p<0.05$) throughout all evaluated time intervals. Conversely, samples employing an extender based on coconut oil exhibited a more

Table 2. Sperm motility (%) of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Storage times (minutes)			
	1	5	10	15
Copaiba oil	93.75^{Aa}	91.25^{Aa}	89.75^{Bb}	82.25^{Bb}
Coconut oil	95.00^{Aa}	81.25^{Bb}	73.75^{Cc}	42.50^{Cc}
Whole powdered milk	94.25^{Aa}	94.00^{Aa}	93.75^{Aa}	90.00^{Ab}
Powdered egg	95.00^{Aa}	93.75^{Aa}	92.50^{Aa}	91.25^{Aa}
Effects	**p-value**			
Extender[2]	<0.001			
Time[3]	<0.001			
Extender x Time[4]	0.008			
CV[5], %	5.33			

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

Table 3. Sperm vigor of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Storage times (minutes)			
	1	5	10	15
Copaiba oil	4.50^{Ba}	4.20^{Bab}	4.12^{Bb}	3.87^{Bc}
Coconut oil	4.37^{Ba}	4.12^{Ba}	3.62^{Cb}	2.87^{Cc}
Whole powdered milk	5.00^{Aa}	4.50^{Ab}	4.50^{Ab}	4.37^{Ab}
Powdered egg	4.50^{Ba}	4.37^{Bab}	4.25^{Bab}	4.12^{Ab}
Effects	**p-value**			
Extender[2]	<0.001			
Time[3]	<0.001			
Extender x Time[4]	0.008			
CV[5], %	4.49			

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

rapid reduction in pH, resulting in an acidic pH across all time intervals.

Regarding sperm concentration results (Table 6), samples treated with extenders based on whole powdered milk and powdered egg demonstrated higher ($p<0.05$) sperm concentration across all evaluated exposure times. Furthermore, samples utilizing an extender based on whole powdered milk saw an increase in sperm concentration, while other samples experienced a reduction. Among all the extenders evaluated, sperm treated with the coconut oil-based extender once again exhibited the poorest ($p<0.05$) results.

Sperm defects, both overall (Table 7) and specifically in the head (Table 8) or tail (Table 9), increased with extended sperm exposure time to the extenders. Samples treated with extenders based on whole powdered milk and powdered egg presented fewer ($p<0.05$) sperm defects in nearly all evaluated time intervals. In contrast, sperm subjected to the extender based on coconut oil showed higher ($p<0.05$) percentages of sperm defects compared to all other evaluated extenders.

Table 4. Sperm wave motion of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Storage times (minutes)			
	1	5	10	15
Copaiba oil	4.12^{Aa}	4.00^{Aa}	3.87^{Bb}	3.50^{Bb}
Coconut oil	4.25^{Aa}	3.87^{Bb}	3.62^{Bb}	1.87^{Cc}
Whole powdered milk	4.87^{Aa}	4.75^{Aa}	4.25^{Aa}	4.25^{Aa}
Powdered egg	4.37^{Aa}	4.37^{Aa}	4.12^{Aa}	3.87^{Bb}
Effects	**p-value**			
Extender[2]	<0.001			
Time[3]	<0.001			
Extender x Time[4]	0.008			
CV[5], %	4.35			

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

Table 5. pH of roosters' semen stored until 15 minutes using copaiba oil and coc9onut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Storage times (minutes)			
	1	5	10	15
Copaiba oil	6.99^{Ba}	6.84^{Bb}	6.78^{Bb}	6.74^{Bb}
Coconut oil	7.15^{Aa}	6.80^{Bb}	5.84^{Cc}	5.84^{Cc}
Whole powdered milk	7.21^{Aa}	7.08^{Aa}	7.04^{Aa}	6.98^{Bb}
Powdered egg	7.07^{Aa}	7.06^{Aa}	7.05^{Aa}	7.03^{Aa}
Effects	**p-value**			
Extender[2]	<0.001			
Time[3]	<0.001			
Extender x Time[4]	0.008			
CV[5], %	5.12			

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

DISCUSSION

Initially, it was observed that extenders based on whole powdered milk and powdered egg presented better results for preserving the roosters' sperm. These results were similar to those described in the literature using milk or egg derivatives to preserve animal semen (Bustamante Filho *et al.*, 2009; Pugliesi *et al.*, 2012; Badr *et al.*, 2021; Bustani; Baiee, 2021; Silva *et al.*, 2021), where the authors reported average motility percentages between 80 and 90% after 15 minutes of storage at room temperature, vigor results close to maximum values, and high pH values maintaining the sperm medium in an alkaline state.

It is important to mention that over the last 60 years, extenders based on milk and derivatives have been used in preserving sperm from both domestic and wild animals, especially those using egg derivatives (Bustani; Baiee, 2021). However, the biochemical mechanisms responsible for explaining how milk and egg derivatives preserve sperm integrity are still unknown, even though some studies attribute their action to the low density of their lipoproteins (LDL), which favor strengthening the sperm cell membrane, providing protection against damage caused by the environment, and consequently maintaining sperm integrity for longer (Manjunath, 2012; Rahman *et al.*, 2018; Bustani; Baiee, 2021).

Table 6. Sperm concentration of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Storage times (minutes)			
	1	5	10	15
Copaiba oil	1.78^{Ba}	1.76^{Ba}	1.76^{Ba}	1.52^{Bb}
Coconut oil	1.54^{Bb}	1.80^{Ba}	1.60^{Bb}	0.88^{Cc}
Whole powdered milk	1.99^{ABab}	2.05^{Aa}	2.32^{Aa}	2.56^{Aa}
Powdered egg	2.11^{Aa}	2.11^{Aa}	2.10^{Aa}	2.10^{Aa}
Effects	**p-value**			
Extender[2]	<0.001			
Time[3]	<0.001			
Extender x Time[4]	0.008			
CV[5], %	4.62			

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

Table 7. Total sperm defects (%) of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Storage times (minutes)			
	1	5	10	15
Copaiba oil	2.62^{Bc}	4.62^{Bb}	5.37^{Bb}	8.50^{Ba}
Coconut oil	4.50^{Ac}	6.00^{Ab}	7.00^{Ab}	10.62^{Aa}
Whole powdered milk	2.13^{Bb}	2.38^{Cb}	3.75^{Cb}	7.13^{Ca}
Powdered egg	1.88^{Cc}	4.25^{Bb}	6.25^{Bb}	7.00^{Ca}
Effects	**p-value**			
Extender[2]	<0.001			
Time[3]	<0.001			
Extender x Time[4]	0.008			
CV[5], %	4.65			

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

Additionally, some studies also attribute the great ability of milk and egg derivatives to preserve sperm to the fact that they contain the five major nutritional constituents of sperm cells: water (87.5%), proteins (3.2%), sugars (4.6%, mainly lactose), lipids (3.7%), and minerals (0.8%) (Manjunath, 2012; Emamverdi *et al.*, 2013; Rahman *et al.*, 2018). Their ability to buffer semen pH and potentially chelate any heavy metal ions naturally improves the sperm environment during storage (Manjunath, 2012; Emamverdi *et al.*, 2013; Bustani; Baiee, 2021), which may explain the favorable results obtained in this study using extenders based on whole powdered milk and powdered egg.

On the other hand, it was also observed that the use of extenders based on copaiba oil and coconut oil, both vegetable oils, resulted in lower motility, vigor, wave motion, and concentration, in addition to higher sperm defects (head and tail) compared to extenders based on whole powdered milk and powdered egg. Individual analysis indicated that

Table 8. Defects in the sperm head (%) of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	Isolated head				Deformed head			
	Storage times (minutes)				Storage times (minutes)			
	1	5	10	15	1	5	10	15
Copaiba oil	1.38^{Bc}	2.50^{Ab}	2.38^{Bb}	3.75^{Ba}	0.00^{Bc}	0.00^{Cc}	0.25^{Ab}	0.75^{Aa}
Coconut oil	2.00^{Ac}	2.50^{Ac}	3.50^{Ab}	4.87^{Aa}	0.50^{Ab}	1.00^{Aa}	0.25^{Ac}	0.50^{Bb}
Whole powdered milk	1.00^{Bb}	1.13^{Bb}	1.75^{Cb}	3.38^{Ba}	0.00^{Bb}	0.00^{Cb}	0.00^{Bb}	0.25^{Ca}
Powdered egg	0.75^{Cc}	1.88^{Bb}	2.75^{Ba}	2.88^{Ca}	0.13^{Ac}	0.25^{Bb}	0.38^{Ab}	0.50^{Ba}
Effects	**p-value**							
Extender[2]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Time[3]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Extender x Time[4]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
CV[5], %	2.82	4.13	4.75	4.15	4.63	4.54	2.14	2.32

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders. ns = non-significant ($p>0.05$).
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

Table 9. Defects in the sperm tail (%) of roosters' semen stored until 15 minutes using copaiba oil and coconut oil as experimental extenders compared to whole powdered milk and powdered egg.[1]

Extender	**Bent tail**				**Curled tail**			
	Storage times (minutes)				**Storage times (minutes)**			
	1	**5**	**10**	**15**	**1**	**5**	**10**	**15**
Copaiba oil	1.12^{Ac}	1.87^{Bc}	2.12^{Bb}	3.25^{Ba}	0.12^{Cb}	0.25^{Bb}	0.62^{Ba}	0.75^{Ba}
Coconut oil	1.63^{Ac}	2.25^{Ab}	3.00^{Ab}	4.50^{Aa}	0.37^{Ab}	0.25^{Bb}	0.25^{Cb}	0.75^{Ba}
Whole powdered milk	0.88^{Bc}	0.88^{Cc}	1.38^{Cb}	2.38^{Ca}	0.25^{Bc}	0.38^{Bc}	0.63^{Bb}	1.13^{Aa}
Powdered egg	0.63^{Bc}	1.25^{Bb}	2.25^{Ba}	2.38^{Ca}	0.38^{Ac}	0.88^{Ab}	0.88^{Ab}	1.25^{Aa}
Effects	**p-value**							
Extender[2]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Time[3]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Extender x Time[4]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
CV[5], %	4.85	5.21	4.79	5.56	5.42	4.53	5.49	4.28

[1] All data represent the mean of 4 replicates per treatment.
[2] Means followed by capital letters (columns) show a significant difference ($p<0.05$) between extenders.
[3] Means followed by lowercase letters (lines) show a significant difference ($p<0.05$) between the different times evaluated.
[4] p-value above 0.05 demonstrates a direct influence of one factor on the result of the other and vice versa.
[5] CV = Coefficient of variation.

the copaiba oil-based extender exhibited results close to those observed with whole powdered milk and powdered egg across most evaluated parameters. In contrast, the coconut oil-based extender led to significantly diminished sperm quality. While vegetable oils are expected to offer reproductive metabolism benefits to roosters when ingested through their diets, utilizing them directly in a diluent did not yield similar advantages, as suggested by the obtained results (Freitas *et al.*, 2007; Rufino *et al.*, 2018).

Both copaiba oil and coconut oil were considered as potential bases for extenders for rooster semen due to their rich fatty acid composition, associated with a positive impact on sperm maintenance against environmental damage by adsorbing these fatty acids into the plasma membrane. This process enhances the natural protective barrier of the cells, extending their viability outside the roosters. However, contrary to expectations based on previous literature (Biavatti *et al.*, 2006; Orsavova *et al.*, 2015; Paulenz *et al.*, 2002; Rutz *et al.*, 2007; Lavor; Câmara, 2012), the results of this study indicate that extenders based on these oils did not protect sperm as effectively as those based on whole powdered milk or powdered egg, particularly over the long term. Thus, the direct exposure of sperm to the content of these oils in their environment may be considered detrimental (Freitas *et al.*, 2007; Rufino *et al.*, 2018).

The semen pH results obtained can help explain how these environmental changes affect sperm viability. It was observed a significant reduction in pH values in samples using extenders based on copaiba oil or coconut oil, meaning that the use of these oils provided a more acidic pH to the sperm environment. The literature indicates that acidic pH environments are adverse for sperm, as they tend to prefer an environment with a more basic pH (Oliveira *et al.*, 2006; Rutz *et al.*, 2007; Lavor; Câmara, 2012; Agostinho *et al.*, 2017).

A more acidic pH gradually oxidizes and destroys the lipid cell membrane of the cells, making them more sensitive to the environment and more likely to be destroyed. Consequently, this reduces their motility, vigor, and wave motion, as well as increases the percentage of dead or defective cells (Rutz *et al.*, 2007; Lavor; Câmara, 2012), just as observed in the results obtained in this study. Furthermore, another important point observed was that the greater the drop in pH as the time of sperm exposure to extenders increased, the greater the reduction in sperm quality and the percentage of defective sperm cells. This confirms the information described in the literature and cited above.

The fact that the rooster semen samples used in this study were exposed to the extenders at room temperature is also an important point to consider, as many factors related to the environment can limit rooster sperm quality, whether fresh or unfrozen. Some studies have reported that these characteristics generally decline in quality within an hour after collection, even when stored in a cold environment (Dumpala *et al.*, 2006; Das *et al.*, 2016). However, at room temperature, these characteristics tend to deteriorate more quickly (Rutz *et al.*, 2007). The results of this study align with the literature, as sperm quality significantly reduced with increased exposure time of rooster semen to the extender at room temperature, regardless of the extender used.

CONCLUSIONS

The study concludes that extenders based on whole powdered milk and powdered egg outperformed those based on copaiba oil or coconut oil in preserving rooster semen at room temperature for up to 15 minutes. The former demonstrated superior outcomes in terms of sperm quality and viability. Copaiba oil-based extender showed efficiency comparable to extenders based on whole powdered milk or powdered egg, while the use of coconut oil-based extender resulted in inferior results. The rapid acidification of the seminal medium induced by extenders based on these oils, especially coconut oil, is identified as a crucial factor contributing to the observed inferior outcomes compared to extenders using whole powdered milk or powdered egg.

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