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(Original Research)

Evaluating Sugarcane Water, Coconut Water, and Honey as Diluents for Philippine Native Chicken Semen at Two Storage Temperatures

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Abstract

Background: Natural extenders present a cost-effective, biodegradable and less toxic alternative to chemical extenders in semen extension and storage. The experiment was designed to find a suitable semen extension medium for Banaba native chicken by comparing the effects of four different extenders: egg yolk-citrate (EY-C), sugarcane water (SW), coconut water (CW), and honey solution (HS) under two storage temperatures: low temperature (3–5°C) and room temperature (25–27°C). **Methods:** The experiment employed a 4 x 2 factorial design in a randomized complete block design (RCBD) with four types of diluents and two storage conditions, using the collection period as a blocking factor. The quality of pooled semen samples from 10 collection periods was assessed using Computer-Assisted Semen Analyzer (CASA). Only samples with ≥ 70% total motility were further analyzed. **Results:** The type of extender had significant effect on sperm total motility, progressive motility, and morphology of the Banaba native chicken. Except sperm progressive motility, temperature had no significant influence on semen parameters. **Conclusions:** All extenders were effective in protecting the spermatozoa of the roosters at room temperature compared to that of low temperature. The sugarcane water diluent was numerically superior to the other diluents and can be used for semen extension.

Keywords

Banaba native chicken, Coconut water, Sugarcane water, Honey, Semen extension

1. Introduction

Poultry ejaculate has a unique characteristic, high concentration of spermatozoa in a limited volume, [1] and is one of the constraints in artificial insemination (AI) [2]. This limited volume necessitates an extension of the ejaculate for AI. AI is essential in poultry genetic improvement, presenting an incomparable control over breeding programs and allowing rapid propagation of desirable traits. Also, AI allows the use of cryopreservation and gene banking, preserving valuable genetic resources for future use, and therefore contributing to biodiversity preservation and the prevention of genetic loss [3].

Materials used for extension are collectively called extenders and are commercially available for poultry. These extenders provide requirements for sperm survival during fresh and storage at low temperature. Therefore, adding energy sources that support cellular metabolism, control pH and osmolality, prevent bacterial growth, and maintain a favorable microenvironment is essential for preserving good sperm motility and viability [4]. Although commercial chemical extenders are readily available, they are costly, require careful preparation, are environmentally

unfriendly, and are reported to be toxic to stored or extended semen. In contrast, extenders from naturally occurring ingredients are less expensive, can be easily adopted by local farmers and breeders, biodegradable, and less toxic.

Selecting the correct extender is a vital precondition of handling semen for AI. Natural extenders are alternatives to chemical extenders in semen extension and cryopreservation of various farm animals [5,6,7]. These may contain natural ingredients obtained from coconut, sugarcane, and honey. Coconut water (CW) is a clear liquid inside coconut fruits that contains various nutrients necessary for cell preservation [8]. In 2019, Rochmi and Sofyan [9] reported that CW is a good candidate for preservation of spermatozoa motility and viability in roosters for up to seven days. It is an alternative semen extender that is non-toxic, low-cost, practical, and effective for semen extension and cryopreservation [6,8]. Sugarcane water (SW), as a natural product, contains appreciable amounts of total sugars, minerals, vitamins, antimicrobial and cytoprotective effects, and antioxidants [10]. These components contribute to the maintenance of sperm motility and viability. Honey, on the other hand, is rich in sugars, proteins, lipids, vitamins, minerals, amino acids, enzymes, volatile chemicals, antioxidants, phenolic acids, organic acids, and flavonoids, all of which are helpful in the sustenance of sperm cells, leading to improved survivability and sperm motility quality [7,11].

Native chicken in rural areas contributes to protein supply, poverty alleviation, and additional income for poor rural farmers [12]. The Banaba is one of the Philippines' native chicken breeds, predominantly found in Batangas province. It is commonly used as a source of good-quality meat and eggs, and it is also favored for leisure activities such as cockfighting by locals. The Banaba chicken produces a relatively high volume of sperm cells. This high semen volume can be exploited in our quest to maximize poultry production efficiency to meet the increasing demands for sustainable human food resources through propagation using extended semen. Thus, the purpose of the study was to formulate an extension medium suitable for Banaba native chicken semen extension that could maintain and sustain sperm viability when processed for a short-term storage.

2. Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB) approved the experimental procedures with allotted procedure number CAFS-2018-006. Semen collections were done at the University Animal Farm (UAF) in Brgy. Putho-Tuntungin, Los Baños, College, Laguna, Philippines with location coordinates 14°09'24.4"N, 121°15'06.6"E. The semen samples were immediately sent to the Animal Physiology Laboratory, Villegas Hall, Institute of Animal Science (IAS), University of the Philippines Los Baños (UPLB) for pre-processing quality assessment, processing, and evaluation using a computer assisted sperm analyzer (CASA).

2.1 Experimental Design and Storage Temperature

The experiment employed a 4 x 2 factorial design in a randomized complete block design (RCBD) with four types of diluents and two storage conditions, using the collection period as a blocking factor. The types of extenders were: EY-C (egg-yolk citrate), SW (40% sugarcane water + 30% distilled water + 30% EY-C), CW (20% coconut water + 30% distilled water + 50% EY-C), and HS (15% honey solution + 20% distilled water + 65% EY-C). The storage temperatures used were low temperature (3-5°C) and room temperature (25-27°C).

2.2 Experimental Animals Management and Care

Twelve (12) 29-month-old Banaba native roosters with an average weight of 2.24 kg were obtained at five (5) months old from the National Swine and Poultry Research and Development Center, Bureau of Animal Industry, Tiaong, Quezon, Philippines. The roosters were housed in an open-sided housing system with temperature conditions at the farm between 21–26°C in individual cages with a floor space of 2.0 ft^2/bird. Birds were offered commercial chicken breeder feeds with clean drinking water provided *ad libitum*. The birds' cages were cleaned at regular intervals with the manual scraping of feces, washing with water, and disinfection of the floor to ensure good sanitary conditions on the farm. The roosters were trained for semen collection using the abdominal massage method.

2.3 Semen Collection

A trained person at the University Animal Farm was responsible for the semen collection. The semen collection was done twice a week at 7:00 AM. The roosters were fasted for 15 h (from 16:00 to 07:00) to reduce the chances of fecal contamination during semen sample collection. Additionally, feathers were occasionally plucked at the peri-cloacal region to avoid dirt contamination during collection. During collection, care was taken to ensure collection of clean semen. The duration of semen collection was between 10-15 min per collection. During collection, each collected semen sample was independently assessed for color and consistency through visual appraisal, while volume was determined using Indoplas® sterile disposable 1 mL syringe with 0.01 mL calibration. The 1 mL syringes containing the semen sample were put inside a clean, well-disinfected foam-padded ice chest and sent to the laboratory for processing and evaluation. The transportation time was usually 10–15 min. During the experiment, Electrogen® D+, an anti-stress supplement, was offered to the experimental birds for revitalization of energy. Semen samples were collected ten times, totalling 120 ejaculates.

2.4 Semen Processing and Evaluation

Semen samples were pooled to remove individual variability effects among the semen donor roosters. Subsequently, using a conventional hemocytometer slide, sperm concentration was determined according to the procedures of [13]. The pooled ejaculate was gently mixed and divided into equivalent volume and was arbitrarily allotted to each experimental treatment with a dilution rate of 1:25 (semen: extender) for CASA (Ceros II, IMV Technologies, China) evaluation. Semen with preliminary microscopic parameters of ≥ 70% were used in this study. The diluted semen of the various treatments (EY-C, SW, CW, and HS) was then divided into two parts (Part A and Part B). Part A was stored at 3–5^{o}C in the refrigerator, while part B was stored at room temperature of 25–27°C. About 2 µL sample from EY-C per batch was used for preliminary spermatozoa motility (%) evaluation. The Gallus setup/module of CASA was used in this study with a frame capture speed of 60 Hz and camera exposure of 4 ms. Normal microscope slides were used in analyzing the semen samples. The slides were put in a MiniTherm Stage Warmer, maintaining the sample temperature at 37 °C. Five (5) frames were taken for every single analysis, with an average time of 60 seconds to complete. The % total motility, % progressive motility, and % normal morphology were analyzed at 2 h intervals up to 10 h post extension. Sperm viability was determined using eosin-nigrosin procedure [13].

2.5. Semen Extender Preparation

2.5.1 Preparation of Sodium Citrate Solution (13.6 mL)

The sodium citrate solution (CS) was prepared by dissolving 0.37 g of sodium citrate and 0.20 g of D-fructose in 13.6 mL of distilled water. The mixture was then thoroughly mixed using a vortex mixer resulting in 13.6 mL of solution. This served as the base solution.

2.5.2 Preparation of Standard Extender (10 mL)

The standard extender (EY-C) was prepared using 8.5 mL of the base solution and 1.5 mL of egg yolk. The mixture in a 50-mL conical tube was then gently mixed, resulting in 10 mL of the standard extender (EY-C).

2.5.3 Preparation of Coconut Water Extender (10 mL)

The fresh young coconut fruits were bought from the open market throughout the experiment, but from the same source. The fruits were washed to remove any contaminants and wiped dry using paper towels. The fruit's pericarp (epicarp) was then cut open through the mesocarp to reach the endocarp, exposing the water-containing meat. The water of the fruit was then withdrawn using disposable 1-mL syringes piercing through the flesh of the fruit. The coconut water (CW) extender was made up of 2 mL of CW, 3 mL of distilled water, and 5 mL of EY-C (20% CW+30% dH_2O+50 % EY-C) and mixed carefully. This formed the coconut water extender (CW).

2.5.4 Preparation of Sugarcane Water Extender (10 mL)

The 2004-1011 sugarcane variety obtained from the Institute of Plant Breeding (IPB) was used for the experiment. The freshly cut sugarcane pieces with a maximum of four internodes were washed clean under running water. The skin of the sugarcane of one-piece internode was peeled off and rinsed under running water. Subsequently, the piece was divided into four quarters and placed in a juice extractor, pressed, and extracted the liquid. The extract was then sieved using the sperm filtering paper in conjunction with a cheesecloth. The SW extender was made up of 4 mL of sugarcane water, 3 mL of distilled water, and 3 mL of EY-C (40% SW+30% dH_2O+ 30% EY-C), which was gently mixed in a 15 mL conical tube. This was designated as SW extender.

2.5.5 Preparation of Honey Extender (10 mL)

The honey used in the experiment was purchased from Institute of Biological Science (IBS). The honey extender was prepared by dissolving 1 mL of honey in 9 mL of distilled water in a 15 mL conical tube, resulting in 10% honey solution. Then, 1.5 mL of the honey solution was added to 2 mL of distilled water and 6.5 mL EY-C (15% (10% HS) +20% dH_2O + 65% EY-C) and mixed carefully in a conical tube. This formed the honey extender (HS).

The optimized composition of the above natural extenders used in the experiment were obtained through the "trial and error method" of different concentrations before arriving at various inclusion rates.

2.6 Statistical Analyses

All data gathered were first tested for normality and homoscedasticity using Shapiro-Wilk's test and Levene's test, respectively. All data satisfying both assumptions were analyzed using analysis of variance (ANOVA) while Tukey's LSD was used as a post hoc analysis tool to determine the level of significance among the means at 5%. All statistical analyses were done using STATA V 15.

3. Results and Discussion

In this experiment, macroscopic and microscopic characteristics of Banaba native chicken semen were determined from 120 ejaculates during a three-month long experimentation. The macroscopic characteristics were semen color, semen consistency, semen volume and semen pH while the microscopic characteristics were sperm concentration, sperm motility (total motility and progressive motility), sperm morphology and semen viability. All observed values on macroscopic semen characteristics and sperm concentration are summarized in Table 1.

Table 1. Macroscopic semen characteristics and sperm concentration of Banaba Philippine native chicken.

Parameter	Results
Semen volume, mL	0.15±0.02
Semen color	Creamy
Semen consistency	Thin
Semen pH	7.27±0.06
Semen concentration, x10^9 spz/mL	4.99±1.32

spz- spermatozoa

3.1 Semen Characteristics of Banaba Native Chicken

3.1.1 Semen Volume

The semen volume values ranged from 0.07±0.01 to 0.34±0.03 mL with a mean value of 0.15±0.02 mL. The mean semen volume obtained in this current

study is higher than that in earlier study by [14] which was 0.13 ± 0.01 mL of Kampung broiler chicken. Similarly, Telnoni *et al*., in 2017 [15] reported a similar semen volume of 0.15±0.02 mL in Sentul Kampung Kedu (SK Kedu) chicken in Indonesia. On the contrary, the results in this report are lower than those obtained by [16] who reported an average semen volume of 0.24 ± 0.06 mL in Kampung chicken. The differences in semen volume could be attributed to breed variations, as meat-type chickens tend to produce more semen than egg-type chickens [6]. Other contributing factors include management practices, the condition of reproductive glands, and the extent of exploitation of the breed's genetic potential [17].

3.1.2 Semen Color

Majority of the ejaculates (98) representing 81.67% were creamy while 22 ejaculates (18.33%) were watery/clear. These findings collaborate with the findings of [18] who reported semen color of white milky/creamy from four different breeds of local chicken. They associated creamy color with high sperm concentration hence good quality while bright white color/clear semen was associated with lower concentrations of sperm and poor quality. Esguerra *et al*., in 2020 [6] reported 53.21% of the ejaculates being watery in Paraoakan native chicken which differs from the findings of this current study. This may be attributed to breed difference and the season in which the experiments were conducted [19].

3.1.3 Semen Consistency

The consistency of the various ejaculates observed were 60, 21.67 and 18.33% for thin, thick, and watery, respectively. This partly agrees with [20] who reported a consistency of fresh rooster sperm as viscous/thick using dorso-abdominal massage method in collecting the semen. Ideally, the normal semen consistency in chicken ranges from thin to thick creamy. The semen in this experiment can best describe as creamy thin.

3.1.4 Semen pH

The semen pH values ranged from 7.17 ± 0.06 to 7.35 ± 0.08 with a mean value of 7.27 ± 0.06. The semen pH recorded in this study agrees with the reported pH value of 7.31 ± 0.06 by [14]. In contrast, the pH value obtained in this study is lower compared to [21] who reported 7.70 ± 0.90 for the Ovambo breed but higher than the previous reports of [17]. Hambu *et al*., in 2016 [20] stated that the stimulation of the accessory sex glands and method of semen collection could be factors contributing to variations in semen pH. However, the findings in this report are within the normal pH of chicken sperm of 6.0 to 8.0 in most studies [22].

3.1.5 Sperm Concentration

Sperm concentrations vary within breeds and between species and range from 2.0-10.0 x 10^9 spermatozoa/mL. It is an essential indicator of the viability of the spermatozoa and provides information on the extent of dilution necessary to obtain required sperm numbers per insemination dose [23]. The sperm concentration of the pooled semen from the Banaba native chicken in this study ranged between 4.73 x 10^9 to 5.25 x 10^9 spermatozoa/mL with an average of 4.99 x 10^9 spermatozoa/mL. The values in this study are within the findings of [24], who reported range values of 3.0 x 10^9 to 8.0 x 10^9 spermatozoa/mL in Anak 2000 broiler breeder cocks. However, the results in this report are higher than that of [14] (2.62± 51.1x10^9 spermatozoa/mL), [19] (4.16-4.39 x 10^9 spermatozoa/mL), [16] (2.81 ± 0.40 x 10^9) in indigenous breeds of chicken. On the other hand, the results in the current study are lower than the results reported by [25](6.60 x 10^9 spermatozoa/mL) in Ross broiler breeder roosters. The variation in sperm concentration is attributable to strain genetic makeup, environmental adaptability, season, ejaculate volume and age [24].

3.2 Effects of Storage Duration and Storage Temperature on Sperm Motility of Banaba Native Chicken

Percent (%) total sperm motility and % progressive sperm of extended semen from Banaba native chicken were analyzed using CASA after extension at a 2 h intervals for 10 h. Irrespective of the extender, there was a decreasing trend observed in percentage total motility (TM) and progressive motility (PM) over time among the four experimental extender treatments and maintained at two storage temperatures (Fig. 1, 2, 3, & 4).

In Figure 1, CW extender showed a wave-like trend while the other three extenders showed linear trends. However, EY-C extender showed superior quality in maintaining the % TM of the sperm cells at room temperature closely followed by SW extender.

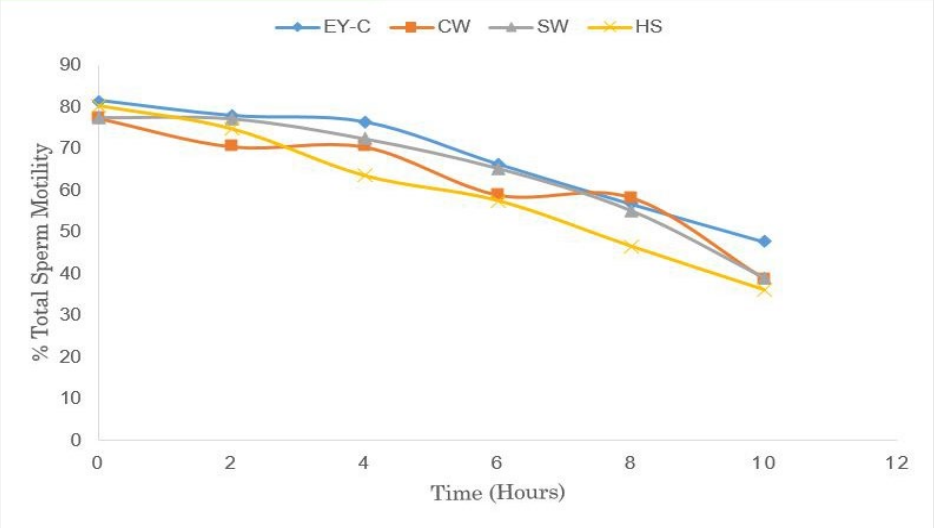

Figure 1. CASA-assessed percentage total sperm motility changes in Banaba native chicken semen diluted with four natural extenders and maintained at room temperature (n=10).

In Figure 2, EY-C, CW, and HS showed wave-like trends of declining progressive sperm motility while SW showed linear trend as in Fig. 1.

In Figures 3 and 4, SW showed superiority over the other extenders in the % TM and % PM in the extended ejaculate stored at low temperature. However, HS extender supported the survival of the sperm cells up to 8 h while the other extenders maintained the viability of sperms cells up to the 10 h designed experiment.

Similar results have been reported by

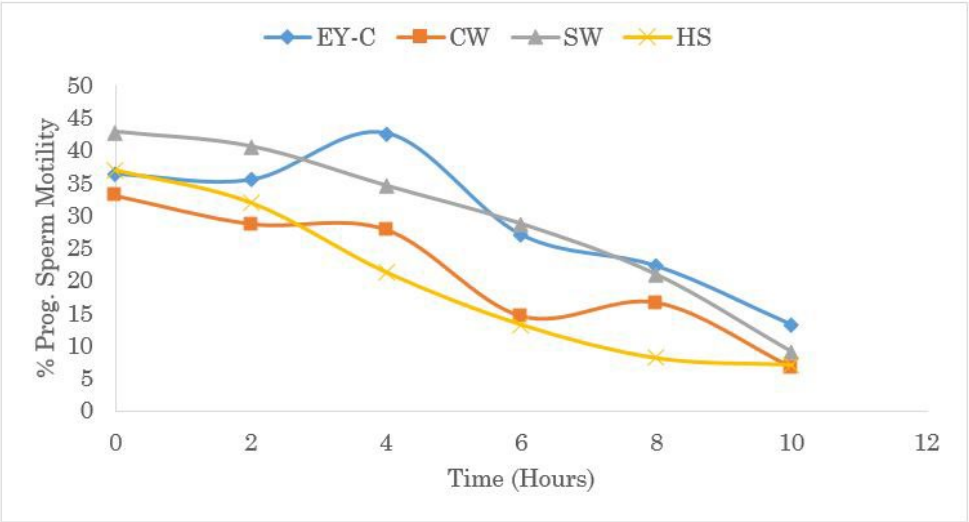

Figure 2. CASA-assessed percentage progressive sperm motility changes in Banaba native chicken semen diluted with four natural extenders and maintained at room temperature (n=10).

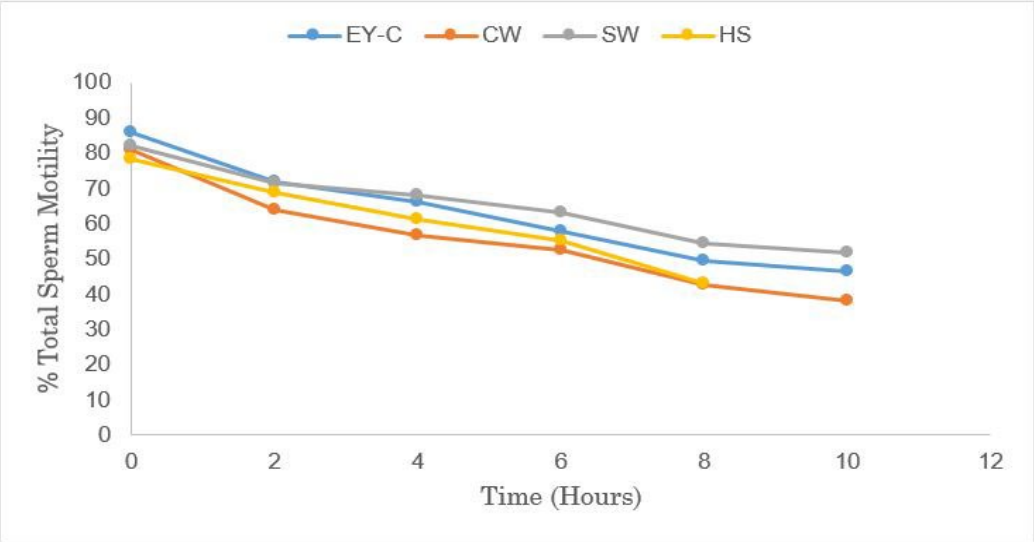

Figure 5. CASA-assessed percentage total sperm motility changes in Banaba native chicken semen diluted with four natural extenders and preserved at low temperature (n=10).

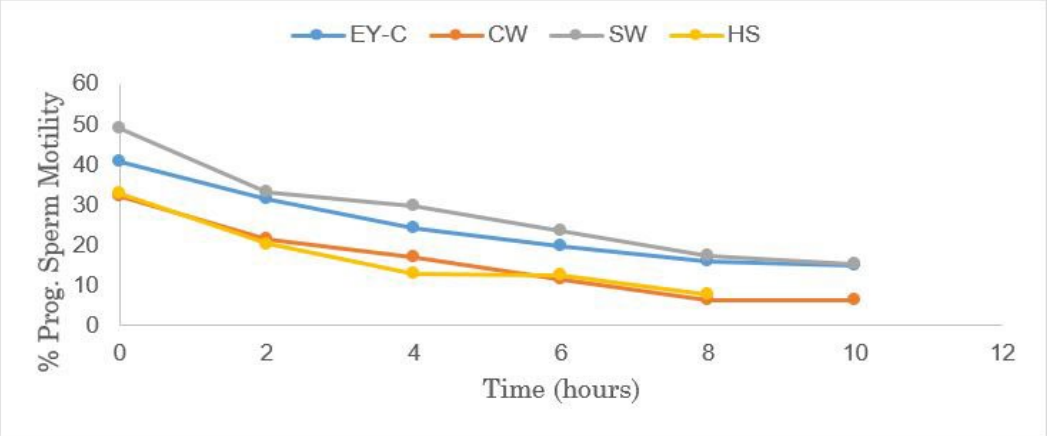

Figure 4. CASA-assessed percentage progressive sperm motility changes in Banaba native chicken semen diluted with four natural extenders and preserved at low temperature (n=10).

previous researchers. Masoudi *et al.*, in 2019 [26] observed significant reduction of all sperm parameters measured in Ross broiler semen stored at 5°C for 48 h. Rakha *et al.*, in 2016 [27] also recorded decreasing number of motile sperm cells in all extenders at any time measurements were taken during 48 h cold storage of semen from the Indian Red Jungle fowl. A decreasing sperm motility with increasing storage periods has been reported in all extenders for other birds [28,29,30]. Evaluation of progressive sperm motility of ejaculate indicates the reproductive efficiency of the cock.

3.3 Effects of Storage Duration on Extender pH Values

The initial pH values of the extenders were 7.39 for EY-C, 7.45 for HS, 6.79 for SW, and 6.99 for CW. After storing the semen samples in these extenders at 5°C for 10 h, the pH values in all extenders showed a slight increase. Specifically, the pH values increased by 0.54% for EY-C, 0.40% for HS, 1.91% for SW, and 1.72% for CW.

Low temperature storage of semen is a common technique used to decrease sperm metabolism and to maintain sperm viability over

an extended period [31]. Dilution and holding of semen in low temperatures reduce sperm metabolic activity as a result, conserving energy and prolonging the accumulation of sperm metabolites in storing media [6]. Also, the harmful effects of microbial contamination and competition are curtailed at low temperature, hence protecting sperm cells, and making nutrients available to them. The decrease in sperm metabolism and delayed accumulation of sperm metabolites may have contributed to the observed stable or slight increase in the pH.

Conversely, extenders with semen samples stored at 25-27°C exhibited a decrease in pH values. The percentage decreases were 2.30% for EY-C, 3.62% for HS, 5.30% for SW, and 2.15% for CW, with the most significant decrease observed in the SW extender. This decline in pH is often linked to increased sperm metabolic activity, which leads to the production of lactic acid and subsequently a reduction in sperm motility [7]. Additionally, factors such as changes in nutrient composition due to sperm metabolite release, alterations in medium osmolarity, increased chloride ion production in certain extenders, and potential physical damage to spermatozoa during the dilution process can also contribute significantly to reduced sperm motility [32]. Surprisingly, SW extender with the lowest pH value in both storage conditions performed comparable to that of EY-C at room temperature and better than all the other extenders at low temperature. The decrease in sperm motility during storage may be due to other factors rather than decreasing pH in this extender.

3.4 Effects of the Type of Extender and Storage Temperature on Sperm Characteristics of Banaba Native Chicken

3.4.1 Sperm Motility

The results on sperm motility (%) of the Banaba native chicken showed significant ($p=0.016$) difference among the type of extender (Table 2). However, the interaction effect between temperature and type of extender and the temperature main effect on sperm motility (%) were found to be insignificant.

Table 2. Mean (± SEM) motility (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two different storage temperatures (n=10).

	Temperature		Mean for type
Treatment	Low (3-5°C)	Room (25-27°C)	of extender
EY-C	63.21 ± 2.22[a]	68.24 ± 1.99[a]	65.70 ± 1.51
SW	65.18 ± 1.95[a]	64.95 ± 2.20[ab]	65.06 ± 1.46
CW	56.43 ± 2.54[b]	62.69 ± 2.29[b]	59.59 ± 1.73
HS	60.27 ± 2.34[ab]	64.471 ± 1.17[ab]	60.98 ± 1.85
Mean for Temperature	61.46 ± 1.14	64.47 ± 1.17	
		p-values	
Type of extender		0.0160	
Temperature		0.0569	
Type of extender x tem.		0.4497	

[a, b], Means in a column with different superscript letters differ significantly ($P<0.05$). Each value is the mean of 10 independent observations. Tem- Temperature.

Under the low temperature, the EY-C, SW and HS extenders were statistically similar in maintaining motility and viability of the spermatozoa. Though, there was similarity among the three extenders, SW was numerically superior compared to the EY-C and HS extenders. There was no significant difference between CW and HS while CW was significantly ($p<0.05$) lower compared to EY-C and SW extenders in maintaining percent sperm motility.

In the room temperature condition, EY-C was numerically superior to the three natural extenders but only statistically differs from CW extender. The SW, HS and CW were similar in maintaining sperm motility and viability under this temperature condition.

Low temperature storage of extended semen is commonly employed to reduce sperm metabolic activity and enhance sperm longevity, thereby preserving sperm quality during storage [33]. Higher temperatures, in contrast, can lead to rapid depletion of energy from the extension medium, reducing sperm motility and increasing the risk of bacterial growth [30]. Slanina *et al*., in 2015 [30] found that spermatozoa stored in chilled media (5°C) exhibited higher motility compared to those incubated at room temperature (22°C). However, this study found that extended semen from Banaba roosters stored at room temperature showed comparable results to those stored at low temperatures in terms of sperm motility and morphology, with significantly higher progressive sperm motility ($p<0.05$) at room temperature. This finding aligns with previous research by [34,35]. Castro *et al*., in 2020 [7] attributed this result to the adaptability of native animals to warmer environments or inherent characteristics that influence sperm longevity and motility.

Given that all extenders proved most effective for Banaba native chicken semen when stored at room temperature, refrigeration and its associated costs can be avoided. This finding offers a more practical and cost-effective solution for small rural farmers, facilitating the adoption of artificial insemination and enhancing the local rearing of Banaba native chickens.

In respect to percentage sperm progressive motility under low temperature condition in Table 3, the type of extender and temperature interaction did not significantly affect the percent progressive motility. However, the type of extender and temperature had significant effect on the percent progressive motility of the Banaba native chicken.

Table 4. Mean (± SEM) progressive sperm (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two different storage temperatures (n=10).

Treatment	Temperature		Mean for type of extender
	Low (3-5°C)	Room (25-27°C)	
EY-C	24.65 ± 1.74^{aB}	29.93 ± 1.95^{aA}	27.29 ± 1.32
SW	27.96 ± 1.85^{aB}	29.40 ± 1.88^{aA}	28.68 ± 1.32

Table 3. Mean (± SEM) progressive sperm (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two different storage temperatures (n=10).

Temperature	
	p-values
Type of extender	0.000
Temperature	0.002
Type of extender x temperature	0.629

^{a,b}, Means in a column with different superscript letters differ significantly ($P<0.05$).
^{A,B}, Means within a row with different uppercase superscript letters differ significantly ($P<0.05$).
Each value is the mean of 10 independent observations.

The SW and EY-C showed superiority in terms of maintaining sperm forward progressing and were significantly higher than HS and CW extenders, but HS and CW were similar statistically at 5% confidence level. The same trend was exhibited under the room temperature condition with SW and EY-C showing better performance over the HS and CW extenders. The observed significant effect of temperature on the % progressive motility is in line with the findings of [30,34].

Sugarcane water (SW) is a natural product rich in total sugars (glucose, fructose, and sucrose), minerals (potassium, phosphorus, calcium, magnesium, and iron), and vitamins (A, B1-6, C, and E). It also possesses antimicrobial, anti-inflammatory, cytoprotective, and antioxidant properties [36]. The sugar content in SW helps rehydrate spermatozoa and provides the necessary energy to maintain their motility and viability. Additionally, the high mineral content, particularly potassium, plays a crucial role in sperm viability [6]. SW also enhances natural immunity by protecting host cells from microbial damage, including bacterial infections, potentially contributing to its effectiveness as an extender [10]. This combination of beneficial properties may explain the superior performance of SW as an extender.

The continuous survival of the spermatozoa in SW extender may be attributed to the antioxidant properties of the sugarcane water. The phenolic and flavonoid compounds in SW are responsible for the high antioxidant activity and cyto-protection [37]. These properties can prevent the free radicals induced oxidative DNA and cell membrane damage. It is of no doubt that, the cytoprotective compounds in SW extender protected spermatozoa from oxidative stress resulting from variety of sources during the semen processing. In addition to the mentioned properties, SW has slightly low acidity in nature together with its antimicrobial effect is adequate to inhibit the growth of many types of bacteria and can therefore be used as an alternative to antibiotics in the preparation of extenders [7]. SW as an extender has shown to be successful in preserving the semen of pigs [38] and fishes [39]. Akandi *et al*., in 2015 [38] reported that the viability of boar semen stored in sugarcane juice was comparable to the honey-supplemented extender but significantly higher than that of tomato- and pineapple-supplemented extenders. They concluded that spermatozoa can be stored in extenders containing natural products especially sugarcane juice and honey.

Coconut water (CW) is sterile and slightly acidic natural solution composed of sugars, proteins, vitamins, salts, neutral fats, antimicrobial and antioxidant properties [5,6] and commonly consumed by people around the globe because of its health benefits. Esguerra *et al*., in 2020 [6] stated that CW is locally available and widely used in the Philippines and the natural buffering effect of CW led to the testing of its efficacy in semen extension and cryopreservation. It has been tested for preservation of semen in chicken [5], cattle [40], goats [41] African catfish [42], and dogs [43].

Daramola *et al*., in 2015 [42] reported that the improvement in sperm parameters in their study was attributed to sugar content in CW which provides energy and increases osmotic potential of spermatozoa thereby protecting their membranes against chilling-induced injury. It was further stated that improvement in sperm viability is due to the potassium levels in the holding medium and hence its positive effect on the viability of extended spermatozoa. Similarly, essential amino acids especially arginine and lysine in CW improve the shelf-life of sperm cells and sperm motility [6].

Honey is a natural product rich in nutrients like sugars, proteins, lipids, vitamins, minerals, amino acids, enzymes, volatile chemicals, antioxidants, phenolic acids, organic acids, and flavonoids [11,44]. All of these are beneficial to improving sperm motility quality and viability. Honey also contains defensin-1, an antimicrobial peptide and hydrogen peroxide and appreciable amounts of methylglyoxal upon water dilution. These inherent qualities of honey are responsible for the antimicrobial effects on bacteria contributing to the motility maintenance of spermatozoa stored in honey-supplemented diluents during extended period. The antimicrobial effect of honey-supplemented media reduces the competition for available nutrients between the sperm and bacteria, as a consequence improves viability of spermatozoa in the medium. It has also been reported that, honey has an antimicrobial effect on several bacteria species that are immune to antibiotics and can serve as an alternative for antibiotics in extender preparation.

Though HS extender did poorly in maintaining sperm motility and viability compared to EY-C and SW in this current study, it has been successfully used in preserving semen in many studies: chicken/bulls [11], rams [45] and stallions [46].

Castro *et al.*, in 2020 [7] reported nonsignificant differences between honey-supplemented extender and commercial extender in % sperm motility and % progressive motility in semen of Duroc and Quezon boars. Similarly, [11] recorded no significant differences ($p>0.05$) in sperm motility of native chicken between honey

sperm motility of boar semen stored under room temperature condition.

3.4.2 Sperm Morphology

As shown in Figures 5 and 6, EY-C and SW extenders were superior in maintaining

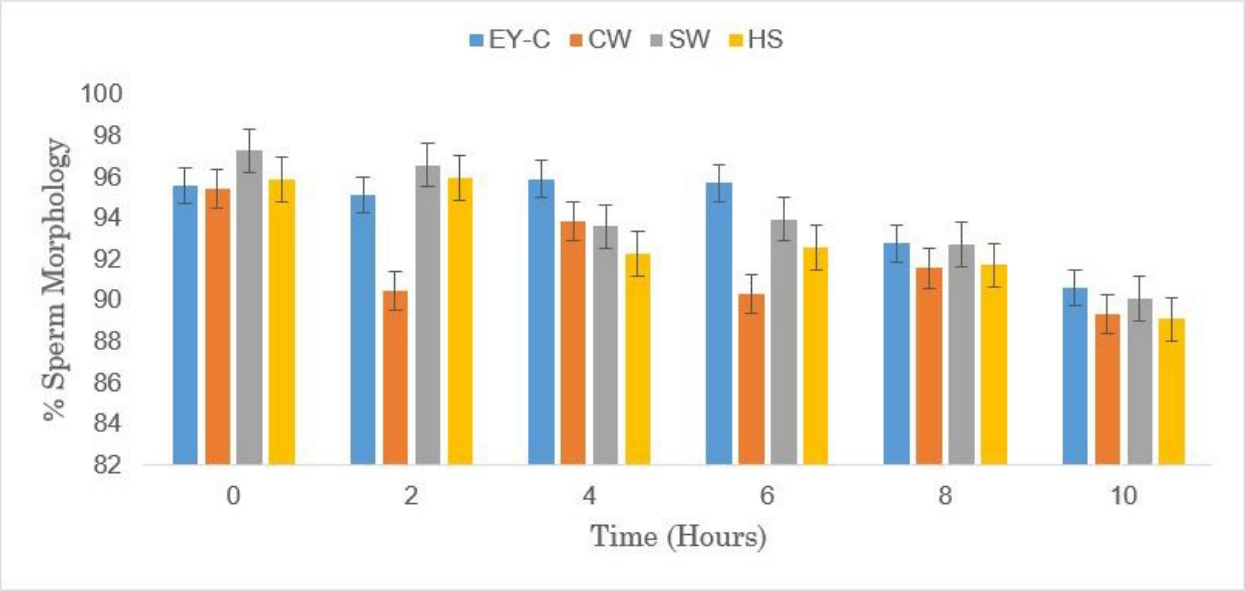

Figure 6. CASA-assessed percentage sperm morphology changes in Banaba native chicken semen diluted with four natural extenders and maintained at room temperature for 10 h (n=10).

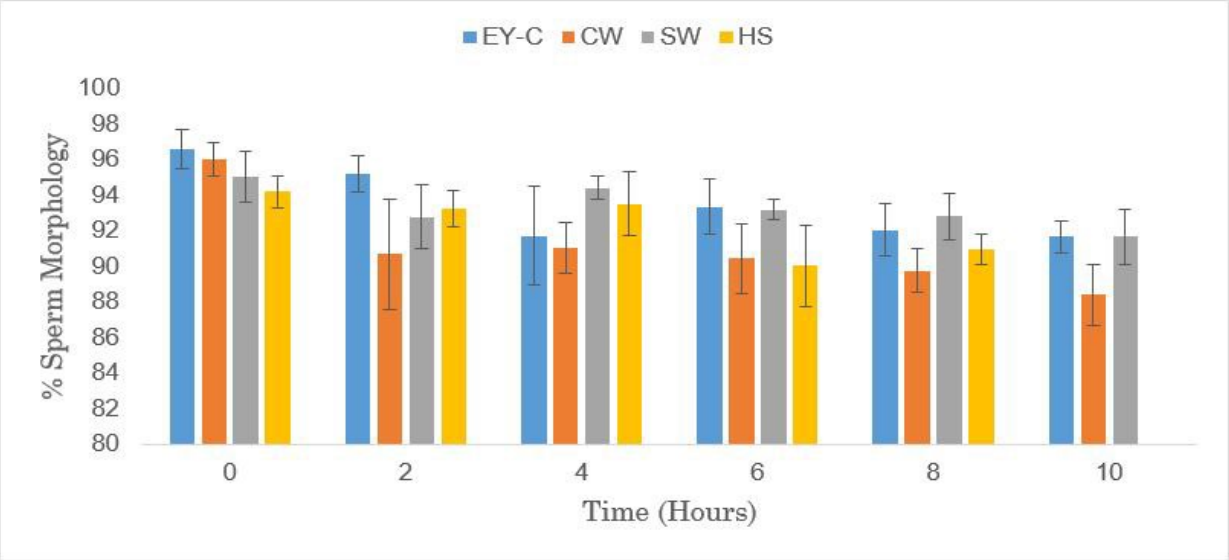

Figure 7. CASA-assessed percentage sperm morphology changes in Banaba native chicken semen diluted with four natural extenders and maintained at low temperature for 10 h (n=10).

extenders (4-6% inclusion rate) and the basic extender. Also, [38] reported honey extender superiority over other natural extenders in %

morphologically normal cells than CW and HS extenders. At hours 0 and 2 under the room temperature (Fig. 5), SW extender was superior in

maintaining morphologically normal sperm cells than the other three extenders. At hours 4-10, EY-C extender surpassed SW in keeping the sperm cell normal morphology while CW performed poorly throughout the 10 h period.

In Figure 6, EY-C extender out-performed SW, HS, and CW extenders in minimizing cell damage to sperm cells under the cold storage. However, SW performed better from the 4th h till the 10th h while HS could not maintain viability until the 10th h. It was generally observed that, morphologically abnormal spermatozoa increase with the passage of time.

There was no significant interaction observed between the type of extender and storage temperature on morphologically normal sperm (%) in Banaba native chicken semen. Also, storage temperature condition has no significant effect on normal sperm morphology. However, the type of extender significantly influenced percent sperm morphology (*p*-value = 0.001) in Banaba native chicken semen in both storage conditions (Table 4), showing that some extenders are better than others in maintaining sperm morphology during semen dilution. The EY-C and SW extenders were significantly higher than CW but was similar to the HS extender.

Sperm morphology gives a useful information about the quality of the semen collected, efficiency of collection and handling and the effectiveness of storage conditions of fresh and frozen semen [22]. Generally, it is accepted that, higher percentage of morphologically normal spermatozoa in semen corresponds with higher fertility rates [47].

Spermatozoa of chicken are filiform in shape [48]. A normal chicken sperm is made of the head (long and narrow) approximately 10.48 µm long and 1.39 µm wide, midpiece approximately 9.51 µm and tail 82 µm long [49]. The dilution processes, preservation, osmotic variance and storage time are known to cause structural damages to sperm morphology [6]. During storage, a decrease in live and an increase in morphologically abnormal dead spermatozoa with bent heads have been reported in earlier studies on avian [28]. Time-dependent decrease in viability and an increase in abnormal sperm morphology of chilled rooster semen are due to osmotic pressure, reactive oxygen species (ROS) and lactic acid produced during storage which cause damage to sperm plasma membrane hence reduces quality in chilled-stored semen.

Table 5. Mean (± SEM) morphologically normal sperm (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two storage temperatures (n=10).

Treatment	Temperature		Mean for type of extender
	Low (3-5°C)	Room(25-27°C)	
EY-C	93.46 ± 0.68[a]	94.38 ± 0.54[a]	93.92 ± 0.43
SW	93.32 ± 0.53[a]	94.13 ± 0.64[a]	93.72 ± 0.41
CW	91.23 ± 0.76[b]	91.93 ± 0.65[b]	91.58 ± 0.49
HS	92.27 ± 0.68[ab]	93.19 ± 0.69[ab]	92.74 ± 0.48
Mean for Temperature	92.62 ± 0.33	93.44 ± 0.32	
		p-values	
Type of extender		0.001	
Temperature		0.067	
Type of extender x temperature		0.998	

[a, b], Means in a column with different superscript letters differ significantly (P<0.05). Each value is the mean of 10 independent observations.

3.4.3 Sperm Viability

As indicated in Figure 7, HS and SW showed superiority over EY-C and CW in maintaining the % viability in semen of the Banaba native chicken.

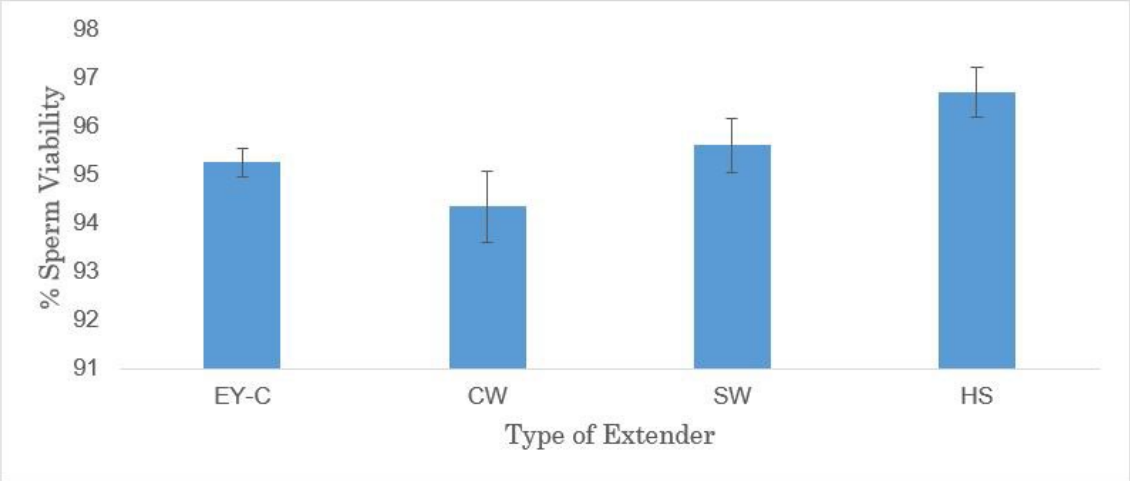

Figure 8. Percentage viability through staining of Banaba native chicken semen extended with four natural extenders and incubated for an hour (n=10).

The results showed sperm viability of 95.25, 94.34, 95.60, and 96.70 % for the EY-C, CW, SW, and HS extenders, respectively. This report agrees with [15,16]. Sperm viability is one of the most vital semen quality parameters used to determine the sperm quality and fertilizing potential of the semen [50]. From the practical viewpoint, [51] stated that, the most critical indicators of male fertilizing potency is the number of live, morphologically intact spermatozoa.

4. Conclusion

The study results indicate that the choice of extender significantly affects sperm total motility, progressive motility, and morphology. However, aside from progressive motility, the holding temperature did not significantly impact semen parameters. Storing semen at room temperature proved more effective in preserving Banaba native chicken spermatozoa. Natural extenders were successful in maintaining sperm motility, morphology, and viability. Among these, the sugarcane water (SW) extender demonstrated the highest suitability for diluting Banaba native chicken semen. Consequently, it can be concluded that SW extender is ideal for processing semen intended for short-term storage.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, A.R.S.S., and P.P.S.; Methodology, A.R.S.S., P.P.S., and M.M.L.; Investigation, A.R.S.S., G.A.D., and M.M.L.; Writing – Original Draft, A.R.S.S.; Writing – Review & Editing, A.R.S.S., and P.P.S.

Ethics Approval and Consent to Participate

The current research has followed the accepted principles of ethical conduct by The Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB), Philippines, protocol number CAFS-2018-006.

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Conflict of Interest

The authors declare no conflict of interest.

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