PHILIPP AGRIC SCIENTIST
Vol. 103 No. 4, 322-336
December 2020

ISSN 0031-7454

Honey or Pineapple Juice as Extender Components for Quezon Native and Duroc Boar Semen at Different Storage Temperatures

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Computer-assisted semen analyzer (CASA) was used to assess the effectiveness of formulated boar semen extenders supplemented with honey and/or pineapple juice. Four boars (i.e., Duroc = 2 and Quezon = 2) served as semen donors, where four ejaculates from each animal were used in this study. Only samples that passed the preliminary quality evaluation were further analyzed and processed. Semen samples were divided and randomly assigned into one of the 11 treatment groups: (T1) a medium-term commercial extender with antibiotics as positive control, (T2) prepared base extender without sugar and antibiotics as negative control (NC), (T3) NC + 0.5% honey, (T4) NC + 1% honey, (T5) NC + 2% honey, (T6) NC + 0.5% pineapple juice, (T7) NC + 1% pineapple juice, (T8) NC + 2% pineapple juice, (T9) NC + 0.5% mixed (1:1) honey and pineapple juice, (T10) NC + 1% mixed honey and pineapple juice, and (T11) NC + 2% mixed honey and pineapple juice and stored at either low temperature (15–20°C) or room temperature (22–25°C). Results showed that Quezon native and Duroc boar semen diluted with T1 had a semen shelf life of 48 and 52 h, respectively, which is longer compared to those diluted using other treatments. For percentage (%) slow sperm, significantly lower values were seen from using T5, T6, T7, T9, T10 and T11 than T1 for Quezon boar semen; while T1, T6 and T7 showed significantly lower values than T2 in Duroc boar semen. In terms of sperm morphological parameters, results from all treatments were comparable in Duroc boar semen; while the use of T4 had significantly lower % coiled tail sperm compared to T7 in Quezon boar semen. Room temperature was observed to be more effective in storing diluted semen from Quezon native boars; however, both low and room temperatures were comparable in maintaining diluted Duroc boar semen. Collectively, 0.5% to 2.0% of honey and/or pineapple juice, or its mixture in a 1:1 proportion, are useful and economical substitute ingredients in boar semen extender.

Key Words: honey, pineapple juice, Quezon native boars, semen extension

Abbreviations: AI – artificial insemination, AMR – antimicrobial resistance, CASA – computer-assisted semen analyzer

Naturally occurring ingredients such as honey and pineapple can be used to replace some essential components of commercial semen extenders such as sugar and antibiotics to lessen their cost and boost the use of artificial insemination in swine production most especially among backyard raisers. The effectiveness of homemade extender recipe was evaluated using honey and pineapple juice to lengthen the shelf life of semen from Quezon native and Duroc boars. Results showed that semen extender formulations with 0.5–2.0% honey and/or pineapple juice are effective in prolonging the storage of Quezon native boar semen at room temperature and Duroc boar semen at either room or low temperature. Honey and pineapple juice are useful substitute ingredients for boar semen extender preparation.

INTRODUCTION

Artificial insemination (AI) is widely practiced in the swine industry due to its proven effectiveness in increasing swine production through the insemination of a considerably high number of sows using only one ejaculate from a superior boar (Pitcher 1997; Knox 2016). For widespread application of this process, dilution of semen in extender is performed to prolong the shelf life of the sperm cells. Semen extenders are composed mainly of sugars as energy source, buffers as pH regulators, salts as osmotic pressure regulator, and antibiotics as bacterial growth inhibitor (Bonet et al. 2013; Carrillo 2016; Holtgrew-Bohling 2016). These essential components are already supplied in commercially available boar semen extenders to minimize the preparation time. However,

there are some limitations with the use of commercial boar semen extenders. The availability and the high cost of these extenders can limit AI adoption most especially among small hold pig raisers. Moreover, the subtherapeutic use of antibiotics in extender preparation for AI could contribute to the emergence of antimicrobial resistance (AMR) and pose a threat to the animal industry (Mendez-Vilas 2010; Bresciani et al. 2014; Vickram et al. 2017).

For this reason, alternative ways to control bacterial growth in diluted semen were devised and reported. The addition of antimicrobial peptides was suggested to be a good alternative to antibiotics (Blondelle et al. 1995; Dathe and Wieprecht 1999; Sancho et al. 2017; Schulze et al. 2014); however, the toxicity of these peptides still needs to be assessed before using them as a semen extender component (Schulze et al. 2015). Another alternative is the physical elimination of bacteria by Single Layer Centrifugation (Nicholson et al. 2014; Morrell and Wallgren 2011). This may also be effective in controlling bacterial growth, but the tedious process could introduce stress to the sperm cells, thereby affecting their motility and viability (Banday et al. 2017). Preparation of extenders with the same ingredients as used in a commercial extender can also be costly. Therefore, it is necessary to find effective and economical substitute ingredients for semen extenders, possessing chemical compositions favorable for sperm viability and motility as well as with good antimicrobial properties.

Honey is known to contain sugars such as fructose (~38.5%), glucose (~31.0%), maltose (~7.1%), and a variety of other carbohydrates (Silva et al. 2016; Moustafa and Elkhawagah 2017). Other nutrient sources that are present in honey include minerals such as magnesium, potassium, calcium, sodium chloride, sulphur, iron, zinc, phosphates and vitamins B and C (Estevinho et al. 2008; Syazana et al. 2011; Igbokwe et al. 2013). Honey also has a good antimicrobial property due to the enzymatic production of hydrogen peroxide, which is harmful to a wide spectrum of bacteria (Doner and White 1980; Mandal and Mandal 2011). Moreover, the antibacterial activity of honey is reported to be effective against some microorganisms that already developed resistance against antibiotics commonly used in commercial semen extenders (Olayemi et al. 2011). Honey has also been reported to have antiseptic, antifungal, anti-inflammatory and antioxidant properties (Viuda-Martos et al. 2008; Tan et al. 2009; Igbokwe et al. 2013).

On the other hand, pineapple (*Ananas comosus*) also contains sugars such as sucrose and fructose, but at a lower concentration compared to honey (Sairi et al. 2004). It is a good source of natural antioxidant components such as carotenoids, vitamins, phenolic compounds, and flavonoids (Larson 1988; Daramola et al. 2016). Good antioxidant properties in semen extenders can be effective in avoiding lipid peroxidation in sperm cells due to oxidative stress (Donghue and Donoghue 1997; Daramola et al. 2016). The antimicrobial property in pineapple is attributed to the enzyme bromelain present in the core or stem of the fruit; bromelain is also commercially sold as an anti-inflammatory drug (Alli et al. 1987; Ahamed et al. 2016).

Philippine native pig production is gaining local popularity. The benefits of raising native pigs include their resilience to local environmental conditions, minimal maintenance, and provision of good income and high-quality protein food (Bondoc and Ramos 1998; Brion 2018). Moreover, the Philippine native pigs already have a local niche market. The Quezon native pig is one of the largest strains of Philippine native pigs found predominantly in Quezon province. Its local production for conservation, improvement, and wide utilization can be further realized with the adoption of AI. Thus, this study demonstrated the substitution of components such as sugar and antibiotics with natural ingredients such as honey and/or pineapple juice in the development of a cost-effective semen extender intended for use in Quezon native boars and commercial pig breeds such as Duroc, to determine what combination/s of extender and storage condition would work best per breed.

MATERIALS AND METHODS

All procedures related to animal use in the conduct of this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB) with assigned protocol number CAFS-2018-006. Semen collection was done in the University Animal Farm (UAF) of the University of the Philippines Los Baños, Tuntungin, Los Baños, Laguna, Region IVA, Philippines (14°09'24.4"N, 121°15'06.6"E). All semen processing and microscopic evaluation were done in the Animal Physiology Laboratory, Villegas Hall, Institute of Animal Science (IAS), College of Agriculture and Food Science (CAFS), UPLB. The experimental period lasted for 4 mo.

Experimental Design

The experiment was laid out using an unbalanced completely randomized design (CRD) with 11 types of extender and two storage temperatures. Data from Quezon native and Duroc boars were analyzed separately. Four sexually mature boars, two 2.5-yr-old senior Duroc boars with an average weight of 180 kg and two 1-yr-old junior Quezon native boars with an average weight of 58 kg, were used as semen donors. A total of 16 semen samples (i.e.,

four ejaculates from each boar) with at least 60% motile sperm were divided and randomly assigned into one of the 11 treatments. Components of these treatments are summarized in Table 1. Diluted semen samples were divided into equal volumes in 50 mL sterile conical tubes and stored at low temperature (15–20°C) inside a conventional refrigerator, and at room temperature (22–25°C) on a clean and properly disinfected table.

Management and Care of Experimental Boars

Two (2) 4-month-old Quezon native boars were acquired from the Bureau of Animal Industry-National Swine and Poultry Research and Development Center (BAI-NSPRDC) at Tiaong, Quezon, Philippines. The boars were raised to maturity at UAF. Meanwhile, the two senior Duroc boars which were regularly used as semen donors for the AI of commercial sows in UAF were previously purchased from a commercial breeder farm. All experimental boars were individually penned with concrete flooring and housed in an open-sided housing system under normal farm conditions (21–26°C). The Quezon native boars were fed daily with 700 g of breeder feeds and Trichantera leaves, while the Duroc boars were given only 2 kg of commercial breeder feeds daily. Water was provided *ad libitum*. All experimental boars were subjected to the same herd health program implemented in UAF.

Training of native boars for semen collection began when the boars reached the age of 6 mo. Every morning, the back or rump of the boars was gently tapped during feeding to tame them. During training, each boar was moved twice a week at a regular interval to the collection pen where a commercially available dummy sow was placed for the animal to be familiarized. Urine from a sow or gilt was applied around the surface of the dummy sow to increase the libido of the boar. Then, the preputial area of the boar was manually stimulated when the boar was sniffing and mounting the dummy sow. Training of boars took about 15–20 min each. When the boars had ejaculated successfully, semen collection was done more frequently in a span of 2 wk. This practice was done to help the boar associate with the dummy sow with successful ejaculation. Afterwards, semen collection was done once a week.

Table 1. Components of different treatments for semen samples of Quezon native and Duroc boars.

Treatment	Description
T1	Mulberry III® (positive control)
T2	Negative Control* (NC)
T3	Honey (0.50%) + NC
T4	Honey (1%) + NC
T5	Honey (2%) + NC
T6	Pineapple juice (0.5%) + NC
T7	Pineapple juice (1%) + NC
T8	Pineapple juice (2%) + NC
T9	(Honey + pineapple juice)** (0.5%) + NC
T10	(Honey + pineapple juice)** (1%) + NC
T11	(Honey + pineapple juice)** (2%) + NC

*Negative Control (i.e., composed of 9.88 g of sodium citrate, 2.50 g of sodium phosphate, 0.63 g of Ethylenediaminetetraacetic acid (EDTA), 0.38 g of potassium chloride and 500 mL of distilled water)
**At 1:1 volume preparation

Semen Collection

Semen collection was done once a week at 7:00 AM by one trained personnel inside a collection pen, using the gloved-hand technique as described by Youngquist and Threlfall (2007). The ejaculate was filtered using a sterile filter paper attached to a sterile beaker. Freshly collected ejaculates were placed in sterile bottles and immediately evaluated for volume, color, and consistency. The sterile bottles were placed inside a foam padded icebox for transport and immediately sent to the laboratory for further analysis.

Semen Processing and Evaluation

Gross semen evaluation, sperm concentration calculation, and dilution rate determination were conducted as described by Capitan and Palad (1999). Semen volume was measured using the graduated sterile bottle itself, while color and consistency were observed by visual appraisal. Sperm concentration was determined using a conventional hemocytometer slide. After evaluation, semen samples were divided into equal volumes and randomly assigned to the different extender treatments. The different extenders were pre-warmed and made sure with the same temperature as in the fresh semen prior to extension. The diluted semen was then assessed using CASA (Ceros II, IMV Technologies, China) for initial percent (%) motile sperm and only samples with ≥ 60% were used in this study. The settings of the CASA were adjusted for the evaluation of boar semen which included a frame capture speed of 60 Hz and camera exposure of 4 ms. Diluted semen samples that passed the initial assessment were further divided into two volumes and stored at either low (15–20°C) or room (22–25°C) temperature. CASA-based examination of diluted semen on motility [percent (%) motile sperm, percent (%) progressive sperm, percent (%) slow sperm, and percent (%) static sperm] and morphology [percent (%) coiled tail, percent (%) bent tail, and percent (%) normal morphology] using 5 CASA-captured frames was done right after dilution and after 4 h of storage. Shelf life of each sample was

determined by evaluating sperm motility on a 4-h interval until percent (%) motile sperm drops below 50%.

Preparation of Honey

About 300 g of *Apis mellifera* honey was provided by the UPLB Bee Program in a tightly sealed sterile glass jar. It was secured inside the laboratory at room temperature (22–25°C). Its chemical composition, as analyzed by the National Center of Excellence for Bee Research and Development (2019) is listed in Table 2. Due to its viscosity, a sterile syringe without needle was used to aspirate and measure the needed amount for each extender formulation. Three milliliters of the base extender were used to initially dilute the honey prior to supplementation.

Preparation of Pineapple Juice

Pineapple fruits were purchased from a local fruit market in Grove, College, Los Baños, Laguna, Philippines. The pineapple was washed thoroughly with distilled water before cutting into small pieces using a sterile knife and then blended for approximately 5 min. The juice from the blended pineapple was extracted using sterile cheesecloth three times to separate residues and collected using a sterile 200 mL glass beaker. Lastly, parafilm was used to cover the glass beaker to avoid contamination. The general chemical composition of pineapple juice is listed in Table 3.

Preparation of Different Extenders

All extenders were prepared aseptically. A medium-term commercial extender called Mulberry III® was used as the positive control while a base diluent was formulated as the negative control (NC). The base diluent is composed of 9.88 g of sodium citrate, 2.5 g of sodium phosphate, 0.63 g of Ethylenediaminetetraacetic acid (EDTA), 0.38 g of potassium chloride and 500 mL of distilled water. Ingredients used in the NC formulation were based on common formulation for commercial boar semen extender (Carrillo 2016). Honey, prepared pineapple juice or a mixture of the two ingredients was used as supplement to NC. Eleven semen extenders, as described in Table 1, were prepared and used in this study. Except for T1, the pH of the prepared extender was adjusted to 7.0–7.2. This pH range was found to be the most conducive for boar sperm motility and viability (Capitan and Palad 1999). Moreover, the optimal osmolarity range of 240–380 mOsm (Weitze 1990; Johnson et al. 2000) was considered during the preparation of NC (base diluent, T2) alongside with pH adjustment using sodium citrate and sodium phosphate.

Table 2. Chemical composition and corresponding amount in a 300 g *Apis mellifera* honey (National Center of Excellence for Bee Research and Development 2019).

Chemical Composition	Amount
Glucose	32.63% (w/w)
Fructose	33.75% (w/w)
Total yeast	1.0 x 10^1 cfu/g
Total mould	3.0 x 101 cfu/g
Moisture	18.92% (w/w)
Hydroxymethylfurfural	11.85 mg/100 g

cfu – colony-forming unit(s)

Table 3. Chemical composition and its corresponding amount in a 100g of pineapple juice (Sairi et al. 2004).

Chemical Composition	Amount
Sugars (sucrose, glucose and fructose)	12-15%
Acid	0.60-1.20%
Citric acid	87%
Malic acid	13%
Calories	47-52 g
Water	85.30-87 g
Protein	0.40-0.70 g
Fat	0.20-0.30 g
Carbohydrates	11.60-13.70 g
Fiber	0.40-0.50 g
Ash	0.30-0.40 g
Calcium	17-18 mg
Phosphorus	8-12 mg
Iron	0.50 mg
Sodium	1-2 mg
Potassium	125-145 mg

Economic Analysis of the Different Extender Types

Cost in Philippine peso (Php) per liter of each extender type was calculated based on the price of the raw ingredients used such as the formulated base extender (Php 69.37/1L), Mulberry III® (Php 145.00/1L), distilled water (Php 25.00/1L), honey (Php 275.00/300 g), and pineapple juice (Php 60.00/1L). A processing fee of Php 2.00 was also added for the production cost of T2–T11. The cost in preparing T1 (commercial extender) was used to compare with the cost in preparing any of the other treatments.

Statistical Analyses

Using the *rstatix* package (Kassambara 2020) in R 3.6.0 (R Core Team 2019), the normality and homoscedasticity assumption for ANOVA were tested using Shapiro-Wilk's Test and Levene's Test, respectively. With the same package, semen parameters that satisfied both assumptions (in Quezon native boars: % slow sperm, % bent tail, % normal morphology) were tested for significant differences across treatment (type of extender)

and storage temperature using Two-Way ANOVA and Scheffe's post-hoc test. When homoscedasticity is violated, semen parameters (in Quezon native boars: % motile sperm, % static sperm) were compared using White-adjusted Two-Way ANOVA with post-hoc Games-Howell Test. Results were presented as means ± SEM.

Non-parametric tests were used for semen parameters which violated both assumptions (in Quezon native boars: shelf life, % progressive sperm,% coiled tail; in Duroc boars: shelf life, % motile sperm,% progressive sperm, % slow sperm, % static sperm, % coiled tail, % bent tail, % normal morphology). A combination of Kruskal-Wallis and the post-hoc Dunn's Test of the *FSA* package (Ogle et al. 2020) were used for comparison across treatments and medians were presented as results while Mann-Whitney U Test was used to compare between temperatures. For all tests, the level of significance was set at 5%.

RESULTS

Quezon Native Boars

Fresh Semen Evaluation

Volume (mL), color, and consistency of semen ejaculates (n=8) from Quezon native boars were assessed immediately after collection. An average ejaculate volume of 101.88 ± 37.98 mL was recorded while gray to grayish white color was observed in all semen samples. Among the 8 ejaculates, 50% were creamy and 50% were watery. Lastly, a sperm concentration of 388.75 ± 186.35 × 10^6 sperm cells mL^{-1} was also recorded.

Semen Shelf Life

The shelf life of semen from the Quezon native boars diluted using different types of extenders and maintained at different storage temperatures was determined through CASA examination of % motile sperm on a 4-h interval until the observed reading falls below 50%. Results showed a significant (*P= 0.0000*) effect of extender type on shelf life of the diluted semen of Quezon native boars (Supplementary Table 1). Moreover, as shown in Supplementary Table 2, the diluted semen stored at room temperature was better (*P=0.0000*) in maintaining good sperm motility compared to diluted samples at low temperature. Meanwhile, the use of T1 in semen extension demonstrated a significantly longer shelf life than the other treatments (Table 4).

Motility

Percent (%) progressive sperm, % motile sperm, % static sperm, and % slow sperm of diluted semen from Quezon native boars were analyzed using CASA after 4 h of extension. Analysis of data on % progressive sperm showed no significant difference among extender types (Supplementary Table 1) but revealed a significant (*P=0.0000*) effect of storage temperature on diluted semen samples (Supplementary Table 2). Meanwhile, as shown in Supplementary Table 3, a significant effect of temperature storage on % motile sperm (*P=0.0077*), % slow sperm (*P=0.0035*), and % static sperm (*P=0.0077*) was observed. On the other hand, significant effect of treatment (*P=0.0061*) was only observed in % slow sperm data (Supplementary Table 3). Further analysis showed that highest average percent (%) slow sperm was observed in semen diluted with T1, which is comparable to T2, T3, T4 and T8 but significantly different from the rest of the other treatments (Table 5). Based on the significantly higher (*P=0.0077*) average for % motile sperm and significantly lower averages for (*P=0.0077*) % slow sperm and (*P=0.0035*) % static sperm (Table 6), storage of Quezon native boar semen at room temperature is better than storage at low temperature.

Morphology

A significant effect of extender type (*P=0.0283*) on % coiled tail was observed in diluted Quezon native boar

Supplementary Table 1. Kruskal-Wallis test of different parameters of Quezon native boar semen diluted using different types of extenders.

Parametrs	X^2	p-value
Shelf life (h) (n=166)	37.43	0.0000
% progressive sperm (n=166)	12.04	0.2825
% coiled tail (n=128)	20.10	0.0283

Table 4. Median of different parameters of semen in Quezon native boars diluted using different types of extenders.

Extender Type	Shelf Life (h)** (n=166)	% Progressive* (n=166)	% Coiled Tail* (n=128)
T1	48.00^a	15.30	0.45^{ab}
T2	18.00^b	11.40	0.60^{ab}
T3	18.00^b	16.90	0.40^{ab}
T4	16.00^b	17.55	0.20^b
T5	16.00^b	18.40	0.60^{ab}
T6	16.00^b	14.50	0.65^{ab}
T7	16.00^b	18.40	1.10^a
T8	16.00^b	14.50	1..05^{ab}
T9	16.00^b	19	0.60^{ab}
T10	16.00^b	24.30	0.40^{ab}
T11	16.00^b	18.20	0.50^{ab}
P-value	0.0000	0.2825	0.0283

Pairwise mean comparison was done using Dunn test; Values with the same superscript are not significantly different ($p > 0.05$).
*Observed after 4 h of dilution using CASA
**Shelf life was observed in CASA every 4 h until the total sperm motility (%) dropped by 50%.

Supplementary Table 2. Mann-Whitney U analyses of different parameters using the diluted semen samples collected from Quezon native boars and maintained at different storage temperatures.

Parametrs	Temperature	N	Mean Rank	Rank Sum	U	p-value
Shelf life (h)**	Low	81	64.48	5,223.50	1902.50	0.0000
(n=166)	Room	85	101.61	8,637.50		
% Progressive	Low	81	69.20	5,605.00	2284	0.0000
sperm* (n=166)	Room	85	97.13	8,256.00		
% Coiled tail*	Low	62	72.21	4,477.00	2524	0.0224
(n=128)	Room	66	57.26	3,779.00		

*Observed after 4 h of dilution using CASA
**Shelf life was observed in CASA every 4 h until the total sperm motility (%) dropped by 50%.

Supplementary Table 3. Two-way ANOVA of different parameters of semen in Quezon native boars diluted using different types of extenders (treatment) and maintained at different storage temperatures.

Factors	Parameters				
	% Motile Sperm** (n=166)	% Slow* (n=166)	% Static** (n=166)	% Bent Tail* (n=166)	% Normal Morphology* (n=165)
Treatment x Temperature	0.9229	0.6323	0.9229	0.9306	0.9361
Treatment	0.9628	0.0061	0.9628	0.8371	0.7228
Temperature	0.0077	0.0035	0.0077	0.9469	0.9966

*Analyzed using ANOVA
**Analyzed using white adjusted ANOVA

semen evaluated after 4 h dilution (Supplementary Table 1). Dilution with T7 resulted in a % coiled tail significantly higher than that with T4 and comparable with those diluted with the remaining treatments (Table 4). A significantly (*P=0.0224*) higher % coiled tail was also observed when diluted semen was stored at low temperature (Supplementary Table 2). In contrast, no significant interaction between treatment and storage temperature, as well as treatment and temperature effect, was observed from the average % normal morphology and % bent tail of diluted Quezon native boar semen (Supplementary Table 3).

Duroc Boars

Fresh Semen Evaluation

Volume (mL), color, and consistency of semen ejaculates (n=8) from Duroc boars were also assessed immediately after collection. An average ejaculate volume of 103.75 ± 22.64 mL, and gray to grayish white semen color were observed. Meanwhile, recorded semen consistency

Table 5. Two-way ANOVA of different parameters of semen in Quezon native boars diluted using different types of extenders (treatment) and maintained at different storage temperatures.

Extender Type	% Motile Sperm (n=166)	% Slow (n=166)	% Static (n=166)	% Bent Tail (n=166)	% Normal Morphology (n=165)
T1	82.94 ± 2.05	16.92 ± 1.95^{a}	17.06 ± 2.05	11.42 ± 2.41	60.11 ± 5.98
T2	78.88 ± 3.04	12.11 ± 1.33^{ab}	21.12 ± 3.04	13.39 ± 2.00	51.70 ± 5.83
T3	83.95 ± 2.58	9.41 ± 1.21^{ab}	16.05 ± 2.58	13.71 ± 2.35	53.42 ± 5.52
T4	86.17 ± 3.07	9.31 ± 1.68^{ab}	13.83 ± 3.07	12.17 ± 1.87	54.64 ± 5.49
T5	80.32 ± 3.55	8.53 ± 1.52^{b}	19.68 ± 3.55	14.51 ± 2.18	50.99 ± 5.92
T6	77.94 ± 3.71	7.74 ± 0.99^{b}	22.06 ± 3.71	14.51 ± 1.77	49.73 ± 3.65
T7	77.92 ± 2.39	7.57 ± 0.78^{b}	22.08 ± 2.39	17.16 ± 2.38	46.82 ± 4.45
T8	75.85 ± 3.13	9.49 ± 1.22^{ab}	24.15 ± 3.13	15.48 ± 2.45	52.59 ± 6.77
T9	81.61 ± 2.94	7.17 ± 1.00^{b}	18.39 ± 2.94	14.43 ± 2.39	48.40 ± 5.01
T10	84.01 ± 1.96	7.18 ± 1.38^{b}	15.99 ± 1.96	13.99 ± 2.08	48.32 ± 5.42
T11	81.31 ± 2.16	6.08 ± 0.96^{b}	18.69 ± 2.16	12.65 ± 1.98	48.38 ± 5.42
p-value	0.9628	0.0061	0.9628	0.8371	0.7228

Pairwise mean comparison was done using Scheffe Test; Values with the same superscript are not significantly different (*p > 0.05*).

Table 6. Mean ± SEM of diluted semen parameters in Quezon native boars maintained at different storage temperatures and observed after 4 h of dilution using CASA.

Parameters	Temperature		p-value
	Low	Room	
% Motile Sperm** (n=166)	77.66 ± 1.32	84.42 ± 1.00	0.0077
% Slow* (n=166)	10.93 ± 0.68	7.75 ± 0.56	0.0035
% Static** (n=166)	22.34 ± 1.32	15.58 ± 1.00	0.0077
% Bent Tail (n=166)	13.31 ± 0.86	14.42 ± 0.96	0.9469
% Normal Morphology (n=165)	51.51 ± 2.08	51.47 ± 2.48	0.9966

*Mean comparison was done using Scheffe Test
**Mean comparison was done using Games-Howell Test

showed 80% were milky and 20% were watery. A sperm concentration of 397.5 ± 177.66 × 10^6 sperm cells mL^{-1} was also recorded.

Extended Semen Evaluation

Semen shelf life, motility, and morphology of diluted semen from Duroc boars were also observed using CASA after extension and using different types of extender and maintained at different temperatures. A one-way analysis was used to determine the significance of treatment effect on the different parameters of diluted semen from Duroc boars (Supplementary Table 4). A significant effect of extender type on diluted semen shelf life (*P=0.0066*), and % slow sperm after 4 h of storage (*P=0.0285*) was observed.

Table 7 shows the comparison of the observed values of semen parameters among treatments. T1 had the longest shelf life, with results comparable to T3, T5, T6, T8 and T9 and better shelf life than T2, T4, T7, T10 and T11. Moreover, T1, T6 and T7 had significantly lower % slow sperm compared with T2. Meanwhile, there was no significant storage temperature effect in the observed median values of different semen parameters among the diluted semen samples from the experimental Duroc boars (Supplementary Table 5).

Economic Analysis of the Different Extender Types

The price of each extender type is listed in Table 8. The cost to produce the honey-supplemented extenders T3, T4, and T5 was lower by 30%, 27% and 20%, respectively, compared with T1. Meanwhile, the production cost of pineapple juice-supplemented extenders such as T6, T7 and T8 were all lower by 33% compared with T1.

DISCUSSION

Fresh Semen Characteristics

The recorded semen volumes from both breeds fall within the normal range of ejaculate volume reported on commercial boars, which vary from 100 to 300 mL (Dagoon 2000). Variations in boar semen volume can be attributed to several factors which include age, selection, housing, nutrition, collection technique, breed, and season (Frunza et al. 2008; Knecht et al. 2014; Rodriguez et al. 2017). All semen samples showed normal semen color, which indicates very little to no contamination (Dagoon 2000; Frunza et al. 2008; Park 2013). Aside from semen color, the observed high frequency of milky to creamy

Table 7. Median of different parameters of Duroc boar semen samples diluted using different types of extenders.

Extender Type	Shelf Life (h)** (n=90)	% Motility* (n=90)	% Progressive* (n=90)	% Slow* (n=90)	% Static* (n=90)	% Coiled Tail* (n=88)	% Bent Tail* (n=90)	% Normal Morphology* (n=90)
T1	52^{a}	79.30	22.40	4.00^{b}	20.70	0.50	19.95	47.60
T2	14^{b}	71.40	12.65	7.90^{a}	28.60	0.95	15.25	45.95
T3	20^{ab}	74.40	20	7.30^{ab}	25.60	1	23	37.10
T4	16^{b}	77.10	12	7.10^{ab}	22.90	0.70	20.50	47.50
T5	18^{ab}	77.15	19.50	6.20^{ab}	22.85	1.10	16.85	44.80
T6	16^{ab}	74.10	15.15	5.40^{b}	25.90	0.85	14.65	49.10
T7	12^{b}	75.50	18	6.00^{b}	24.50	1.20	23.10	39.40
T8	16^{ab}	57.70	11.30	7.70^{ab}	42.30	3.80	27.60	30
T9	18^{ab}	75.70	17.30	4.90^{ab}	24.30	0.90	18.10	46.45
T10	14^{b}	78.05	13.95	7.60^{ab}	21.95	1.15	18.20	41.35
T11	10^{b}	76	17.10	7.45^{ab}	24	1.05	26.50	41.25
p-value	0.0066	0.0061	0.179	0.0285	0.6681	0.1924	0.4969	0.8863

Pairwise mean comparison was done using Dunn test; Values with the same superscript are not significantly different ($p > 0.05$).
*Observed after 4 h of dilution using CASA
**Shelf life was observed in CASA every 4 h until the total sperm motility (%) dropped by 50%.

Supplementary Table 4. Kruskal-Wallis analyses of different parameters of semen samples collected from Duroc boars and diluted using different types of extenders.

Parametrs	X^2	p-value
Shelf life (h) (n=90)	24.40	0.0066
% motile sperm (n-90)	7.60	0.6681
% progressive sperm (n=90)	13.87	0.1790
% slow sperm (n=90)	20.08	0.0285
% static sperm (n=90)	7.60	0.6681
% coiled tail (n=88)	13.59	0.1924
% bent tail (n=90)	9.38	0.4969
% normal morphology (n=90)	5.07	0.8863

consistency, which is correlated with sperm concentration (Sirois 2005), also suggested good semen quality.

The observed sperm concentrations from the experimental boars used in this study were found to be similar to the previous reports in Duroc boars: 147.0 ± 47 $\times 10^6$ sperm cells mL^{-1} (Kommisrud et al. 2002), 170.0 ± 15.1 $\times 10^6$ sperm cells mL^{-1} (Johnson et al. 2000), 574.1 ± 10.75 $\times 10^6$ sperm cells mL^{-1} (Kondracki 2003) and 297 ± 31 $\times 10^6$ sperm cells mL^{-1} (Conlon and Kennedy 1977). In this study, semen collection was done once a week since higher collection frequency results in lower sperm concentration (Frangez et al. 2005). Accurate sperm concentration determination is very important because it serves as the basis for the dilution ratio that is used in semen extension (Bonet et al. 2013).

Semen Shelf Life

Results showed that using T1 was the most efficient in prolonging the shelf life of the diluted semen samples from both Quezon native and Duroc boars. This was expected since Minitube MIII® (T1) is a commercially available semen extender that is reported to lengthen semen shelf life by 3–4 d (Gadea 2003). Nevertheless, other honey- and/or pineapple juice-supplemented extenders such as T3, T5, T6, T8 and T9 resulted in a comparable shelf life with T1 in experimental Duroc boar semen extension. This finding indicates that the minimal inclusion of 0.5% honey and/or pineapple juice (T3, T6 and T9) in semen extender preparation is enough to achieve the same shelf life as in the use of commercially available extender for Duroc boars.

A hyperosmotic extracellular environment, which is perceived harmful to sperm cells due to causing excessive intracellular dehydration via osmosis (Lemma 2011; Yimer et al. 2016), can happen with higher honey or mixed honey and pineapple juice supplementation in extenders. A range of 240–380 mOsm is considered good for boar sperm cells (Weitze 1990; Johnson et al. 2000). In other studies, honey has been proven effective as a non-permeable cryoprotectant at an inclusion rate of 1% to 4.5% (Yimer et al. 2016; Banday et al. 2017; Mohamed and Ahmed 2017; Zaghloul 2017) to provide a hyperosmotic extracellular environment prior to cryopreservation. The use of higher amounts of honey may be beneficial during cryopreservation, but it may be disadvantageous in semen extension since it may cause cell dehydration during prolonged storage. In addition, the use of lower amounts of natural ingredients will incur lower costs with economic advantage.

Results of the study demonstrated the importance of adding appropriate amounts of antimicrobial agents and sugar to semen extenders to prolong semen shelf life. In this study, longer shelf life (16–18 h in Quezon native and 10–20 h in Duroc boar) with above 50% total sperm

Supplementary Table 5. Mann-Whitney U analyses of different parameters of diluted semen samples collected from Duroc boars and maintained at different storage temperatures.

Parametrs	Temperature	N	Mean Rank	Rank Sum	U	p-value
Shelf life (h) (n=90)	Low	48	45.75	2,196	1020	0.9249
	Room	42	45.21	1,899		
% motile sperm (n-90)	Low	48	46.27	2,221	1045	0.7678
	Room	42	44.62	1,874		
% progressive sperm (n=90)	Low	48	44.77	2,149	973	0.7802
	Room	42	46.33	1,946		
% slow sperm (n=90)	Low	48	46.18	2,216.50	1040.50	0.7957
	Room	42	44.73	1,878.50		
% static sperm (n=90)	Low	48	44.73	2,147	971	0.7678
	Room	42	46.38	1,948		
% coiled tail (n=88)	Low	48	44.88	2,064.50	983.5	0.8869
	Room	42	44.08	1,851.50		
% bent tail (n=90)	Low	48	45.04	2,162	986	0.8619
	Room	42	46.02	1,933		
% normal morphology (n=90)	Low	48	45.86	2,201.50	1025.50	0.8506
	Room	42	45.08	1,893.50		

All semen parameters except for shelf life were observed after 4 h of dilution using CASA.
*Shelf life was observed in CASA every 4 h until the total sperm motility (%) dropped by 50%.

Table 8. Cost-benefits analysis of each treatment (extender type).

	Amount (PhP)/ Quantity	Price (PhP) per liter										
		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Prepared base extender	69.37/1 L	-	69.37	69.37	69.37	69.37	69.37	69.37	69.37	69.37	69.37	69.37
MIII (positive control)	120/300 g	120	-	-	-	-	-	-	-	-	-	-
Distilled water	25/1 L	25	25	25	25	25	25	25	25	25	25	25
Honey	275/300 g	-	-	4.79	9.58	19.17	-	-	-	2.40	4.79	9.58
Pineapple juice	60/1 L	-	-	-	-	-	0.30	0.60	1.20	0.15	0.30	0.60
Processing fee	2/L	-	2	2	2	2	2	2	2	2	2	2
Total Amount (PhP)		145	96.37	101.16	105.95	115.54	96.67	96.97	97.57	98.92	101.46	106.55

motility was observed, compared to a similar study by Akandi et al. (2015) using honey and pineapple juice at 1.0%, 1.5% and 2% inclusion rates in the semen of crossbred boars maintained at room temperature, where the observed shelf life lasted only for less than 12 h. Honey, which is commonly used as a natural sweetener, contains concentrated amount of sugars such as fructose and glucose (De-Melo et al. 2017) that can be utilized as energy source for the sperm cell's flagellum movement and longer shelf life (Gadea 2003). It also has antimicrobial properties that can inhibit the growth of around 60 strains of bacteria (Hannan et al. 2004) and can be used as an alternative for antibiotics in extender preparation. More so, honey has been found effective on bacterial species that have developed resistance to antibiotics (Molan and Russell 1988). The use of honey has also been found beneficial in the semen extension of other animals such as bulls (Malik 2018), rams (Zaghloul 2017) and stallions (El-Sheshtawy et al. 2016).

Pineapple has also been used in semen extension of other animals, e. g., bucks (Daramola et al. 2016). It is less common than honey due to its acidic pH that can be harmful to sperm cells if used in large amount. Nonetheless, it has been known to contain antioxidants that can improve the motility and survivability of diluted semen (Cao et al. 1996; Wang et al. 1996; Velioglu et al. 1998). Pineapple juice also contains glucose and fructose, although at lower amount compared to honey, which can play an important role for the metabolism of sperm cells (Alli et al. 1987).

Surprisingly, the use of T2 in this study, which has neither antibiotics nor sugar supplementation, resulted in a significantly shorter shelf life than T1 but comparable shelf life with the rest of the other treatments during semen extension of samples from both breeds. This finding may seemingly be attributed to two possible factors, a balanced osmotic extracellular environment, and a good level of asepsis during semen extension. However, this may not be the case for T2 in an environment where good sanitation is not completely guaranteed as in a backyard setting where unwanted microbial growth from environmental contamination may happen. Microbes are known to compete for sugars in media during their exponential growth, thereby depleting sperm cells of their energy source which is vital for their metabolic needs and motility. Thus, the inclusion of honey and/or pineapple juice with previously mentioned sugar components and reported antimicrobial properties (Kwakman et al. 2010; Praveen et al. 2014; Akandi et al. 2015) during semen extender preparation may have advantages in such situation.

Motility

Despite the superiority of T1 in terms of shelf life, results have shown that some treatments with natural ingredients had comparable or better results in terms of motility. From the results of the study, it can be observed that honey- and/or pineapple juice-supplemented extenders performed well in terms of % slow sperm of diluted semen from both breeds. In fact, T5, T6, T7, T9, T10 and T11 had averages in % slow sperm with significantly lower values compared to T1 in the extension of Quezon native boar semen. Meanwhile, significantly lower % slow sperm was observed in T1 and some pineapple juice supplemented extenders (T6 and T7) with comparable results from the other treatments except T2 during the extension of Duroc boar semen.

Sperm motility is expected to decrease over time during extension (Estienne et al. 2007; Gogol et al. 2009). This decrease can be attributed to several factors such as mechanical damage in sperm cells during the extension process, changes in the osmolarity of the solution, and decrease in pH (Capitan and Palad 1999; Baguio and Capitan 2008). While the study did not monitor the pH of the diluted semen samples over time, the decrease in sperm motility can be attributed to the decrease in pH, since long storage of semen leads to lower pH when

lactic acid accumulates due to sperm anaerobic metabolic activity (Abutu 2015).

Honey contains an antimicrobial agent known as peptide bee defensin-1 and produces high levels of methylglyoxal and hydrogen peroxide when diluted in water (Kwakman et al. 2010; Akandi et al. 2015). Improvement in the quality of motility and spermatozoa survivability can be credited to the sugar components in honey such as monosaccharides, disaccharides, oligosaccharides and polysaccharides (Bogdanov et al. 2008; Yimer et al. 2016) which act as energy source to maintain sperm motility and viability. In addition, bromelain that can be found in pineapples is widely used as an anti-inflammatory and has also been proven to be antimicrobial (Praveen et al. 2014). Pineapples are also known to contain vitamins and phenolic compounds that can improve progressive motility of spermatozoa by reducing free radical damage to sperm cell membranes (Zuo et al. 2002; Gebhardt and Thomas 2002; Lee et al. 2003; Cutler et al. 2008; Daramola et al. 2016). These beneficial characteristics of honey and pineapple may have contributed to the control of bacterial growth and improved motility in some honey- and/or pineapple juice -supplemented extenders.

Despite the availability of reports on antimicrobial properties of honey and pineapple, it is still recommended to assess their antimicrobial effects when added to semen extenders. A dose-response analysis in a challenged experimental setup can further elucidate the minimal inclusion at which antimicrobial effect in media is demonstrated by these natural ingredients. The amount of antimicrobial compounds present in these ingredients can also be quantified using high-performance liquid chromatography (HPLC) (Pappalardo et al. 2016; Karasawa et al. 2017; Yantih et al. 2019) to assess their antimicrobial potential.

Morphology

It is generally accepted that the higher the percentage of morphologically normal sperm cells in semen, the higher the likelihood of fertility (Capitan and Palad 1999). A normal boar sperm is usually characterized by a head part that is approximately 9 μm long and 5 μm wide and a tail, together with a midpiece, that is approximately 30–35 μm long (Skinner 2018). Structural damages in the morphology of spermatozoa may be affected by factors such as processes in dilution, preservation, and duration of storage (Manee-In et al. 2014; Wysokińska and Kondracki 2014; Wysokińska et al. 2015).

In this study, no significant differences can be found among treatments in terms of morphological parameters, except in % coiled tail in extended Quezon boar semen. Dilution using 1% honey-supplemented extender (T4) led to a significantly lower % coiled tail compared to T7, which exhibited the highest % coiled tail, and the rest of the other treatments which are all comparable. This result implies that sperm cells (live and dead) were able to maintain structural integrity over time with the use of any of the formulated extender preparations, except T7, resulting in a better or similar sperm morphological profile with that found in semen diluted using a commercial extender (T1). The antioxidant properties in honey (Erejuwa et al. 2012) and pineapple (Lee et al. 2003; Spanos and Wrolstad 2004; Reza et al. 2011) might have prevented oxidative stress that can cause sperm abnormality (Daramola et al. 2016; Yimer et al. 2016). Comparable results may also be reflective of the good level of asepsis implemented during the semen extension process.

Storage Temperature

Storage temperature during semen extension also affects the quality of sperm cells with respect to parameters such as shelf life, motility, and morphology (Acton 2012). Normally, the diluted semen samples are stored under low temperature (15–20°C) to slow down the sperm metabolic activity (Gadea 2003) since higher temperature causes sperm cells to rapidly consume the energy component in the extender, resulting in early reduction in sperm motility; also, higher temperature increases the risk of bacterial growth (Youngquist and Threlfall 2007; Dariusz et al. 2015). However, it may not be the case in this study, since storage at room temperature of the diluted semen from Quezon native boars demonstrated a significantly longer shelf life and better sperm motility and morphology. A possible reason could be the adaptability of native animals to warmer environment (Baguio 2017) or inherent boar characteristics influencing the longevity and motility of the diluted semen (Zimmerman 2012). More so, no significant differences in storage temperature on different parameters can be observed in extension of semen from Duroc boars.

Economic Analysis of the Different Extender Types

In this study, the supplementation of at least 0.5% inclusion rates of honey and/or pineapple juice in semen extenders for both Quezon native and Duroc boars have been effective for substitution of sugar and antibiotic components in commercial extenders with respect to shelf life, motility and morphological parameters of semen. However, it should be noted that higher inclusion rates are not recommended to avoid disadvantageous risks on the viability of boar semen. Affordability would also be an advantage in lower

inclusion rates due to lower costs. However, despite being 33.53% lower than T1, the effect of T2 on shelf life in Quezon native boar semen and on both shelf life and % slow sperm in Duroc boar semen were inferior to T1 compared to honey- and/or pineapple juice-supplemented extender formulations. Considering that all extenders were most effective on Quezon boars when stored in room temperature, expensive cooling facilities are not anymore necessary for its semen extension; this situation is more convenient for small hold farmers for AI adoption to further boost the local production of the Quezon native pigs. In addition, antibiotics supplementation increases the cost of semen extenders and raises risks of AMR occurrence (Morell and Wallgren 2014). Treatments formulated in this study implemented the use of naturally occurring ingredients such as honey and pineapple in order also to address these issues.

ACKNOWLEDGMENT

This study was supported by the Department of Agriculture - Bureau of Agricultural Research (DA-BAR) through the DA-BIOTECH program funded project entitled, "Development of cryopreservation prototypes as biotechnological interventions for the conservation of genetic diversity of Philippine native pigs, chickens, and ducks" implemented by the IAS, CAFS, UPLB.

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