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RELATIONSHIP OF SEMEN CHARACTERISTICS OF ITIK PINAS-KHAKI (*Anas platyrhyncos*) BEFORE AND AFTER CRYOPRESERVATION

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ABSTRACT

Itik Pinas (IP) is a superior breed of egg-type Philippine mallard duck developed for high egg production and consistent egg quality to meet the growing local demand for duck eggs. A clear and thorough understanding of IP's reproductive characteristics and performance can help boost its local production. Thus, this study assessed the semen characteristics of IP-Khaki through different techniques depending on the parameter. Here, pooled semen samples were collected from six drakes grouped in two batches for the comparison of semen parameters before and after cryopreservation. Pearson Correlation and Kendall's Tau Correlation were used to determine the relationship of the different semen parameters before and after cryopreservation. Overall, the only significant relationships are the positive linear relationships of initial motility with initial viability, change in motility and change in viability. This was consistent with the findings for the good batch and poor batch, individually. The poor batch generally showed weaker magnitudes and smaller significance of all the parameter relationships compared to those of the good batch.

Key words: cryopreservation, drake, semen parameters, *Anas platyrhyncos*, Itik Pinas

INTRODUCTION

Itik Pinas (IP), a new improved Philippine mallard duck (*Anas platyrhynchos* L.) is a product of continuous breeding and selection of the commonly raised traditional Pateros duck. IP has three developed strains: IP-Itim (pure maternal line), IP-Khaki (pure paternal line), and IP- Kayumanggi (commercial hybrid line). Itik Pinas has a uniform physical appearance and higher predictable egg production performance characterized by a consistent and higher egg quality compared to its traditional counterpart (PAB-IS, 2017). This improved performance is maintained well even when using low-cost housing and feeds (Parungao, 2016; Parungao, 2017; Aya, 2018; Pinca *et al*., 2019). Limited studies are available on the semen characteristics and response to cryopreservation of IP genetic lines which can help improve its commercialization (Lambio, 2000).

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Cryopreservation makes use of very low temperatures to preserve structurally intact living cells and tissues (Pegg, 2007; Abdelhafez, 2009; Benson *et al*., 2012). It aims to increase the diffusion and management of genetic progress, sustain conservation of genetic biodiversity and improve artificial insemination management (Blesbois, 2007; Zaniboni *et al*., 2014). Commercializing semen preservation has been fully established in some mammalian species industries where semen cryopreservation has been optimized, standardized, and automated (Gao *et al*., 1995; Long, 2006; Okazaki *et al*., 2009). Although in avian species, it is not as successful as these mammalian species. The fertility rates of cryopreserved avian sperm are substantially lower than any of the domestic mammalian species generally making it more sensitive to cryopreservation processes compared to mammalian spermatozoa (Long, 2006; Lemoine *et al*., 2009) and the reason for this might be related to its distinct morphology (Donoghue and Wishart, 2000; Pearson *et al*., 2001).

A better way to start assessing the characteristics, resiliency and suitability to freezing techniques of semen from a newly developed duck genetic group such as IP is by adopting protocols optimized and reported on the same species. A reported study on ducks by Han *et al*. (2005) has demonstrated that the use of permeating cryoprotective agent (CPA) like dimethyl sulfoxide (DMSO) during semen cryopreservation provided a higher motility rate than that of glycerol, N N-dimethylacetamide (DMA), and dimethylformamide (DMF). Adopting this same protocol, the optimum diluent, cryoprotectant, equilibration time, and thawing temperature were 10% DMSO, 15 minutes, and 37°C, respectively.

Thus, the overarching goal of this study is to contribute to the understanding of IP-Khaki's semen characteristics and their relationship before and after semen processing using a reported protocol on avian semen cryopreservation.

MATERIALS AND METHODS

All procedures 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 from IP-Khaki drakes was done at the University Animal Farm in Putho-Tuntungin, Los Baños, Laguna, Region IV-A, Philippines (14°09'24.4"N, 121°15'06.6"E). All semen processing and evaluation were conducted at the Animal Physiology Laboratory, Institute of Animal Science (IAS), College of Agriculture and Food Science (CAFS), UPLB. The study was conducted from September to November 2020.

Twenty-five (25) 3-month-old IP- Khaki drakes labeled from IP-Khaki1 to IP-Khaki25, were acquired from the National Swine and Poultry Research and Development Center, Bureau of Animal Industry (NSPRDC), Tiaong, Quezon (13°56'37.9"N 121°22'23.6"E). They were fed with standard commercial duck feeds and water was provided *ad libitum* while being caged and allowed to mature individually. Upon sexual maturity, the IP-Khaki drakes were trained for semen collection daily using the dorso-abdominal massage method (Burrows and Quinn, 1935) which spanned for about 1-2 min for 3 consecutive weeks. After training, semen collection was done routinely every other day.

Semen collection was done every other day at 07:00 AM by one trained personnel at the farm. Measures to avoid dirt and fecal contamination during semen collection were regularly observed such as removal of feathers and 17-h feeding to collection interval. Semen samples were collected individually on a sealed and sterile glass funnel for semen

consistency evaluation. The volume of each sample was then measured using a sterile disposable 1 mL syringe with 0.01 mL calibration. After the initial evaluation, samples were immediately transported from the farm to the laboratory by placing them in a sterile beaker, lined with a sterile cloth and placed in a disinfected foam-padded icebox, at room temperature. Using pH strips, pH of each ejaculate was determined before further semen processing.

The semen samples collected were placed in a microcentrifuge tube per animal. Each semen sample was diluted 1:35 with AU extender, 1.5 μL from each was used to evaluate motility using the Computer-Assisted Sperm Analyzer (CASA). The animals were ranked from lowest to highest motility after several semen collections. Samples with close total motility values were grouped creating 4 batches: Poor, Mid1, Mid2, and Good. The samples were pooled according to their batch designation.

Upon pooling of poor and good batches, the samples were diluted 1:1 with AU extender for cryopreservation (Han *et al*., 2005) and sperm concentration determination. An aliquot (10% of total volume) of the pooled samples were diluted 1:35 with the AU extender optimized for Computer-Assisted Sperm Analysis (CASA) and viability testing. The AU extender is composed of: 0.40 g D-glucose, 0.80 g D-fructose, 0.80 g sucrose, 0.90 g sodium citrate, 0.84 g sodium glutamate (MSG), 0.40 g glycine, 0.04 g ethylenediaminetetraacetic acid (EDTA), and 100 mL distilled water (Gerzilov *et al*., 2011).

The pooled samples were characterized prior to processing, as previously mentioned. The color and consistency of individual sperm samples per batch were examined by eye, while the total volume was determined using a 1 mL syringe. The pH was determined using pH strips. Sperm count or sperm concentration was determined by manually counting the number of cells using Neubauer hemocytometer (Capitan and Palad, 1999). Here, the diluted semen sample was drawn up to the 0.5 mark of a dilution pipette and mixed with the staining solution (1.0% eosin in 3.0% NaCl solution) until it reaches the 101 calibrated mark on the dilution pipette. The pipette was gently swirled for thorough mixing, making sure that the sperm cells were evenly distributed. The mixture was placed on the counting chamber of a Neubauer slide with a cover slip for spreading. The slide was placed on the microscope stage and was observed under the high-powered objective lens and the number of cells was determined. Viability was determined by eosin-nigrosin staining. The diluted pooled samples were mixed with the stain (1.67g eosin and 10 g nigrosin in 100 mL sterile distilled water containing 2.9 g sodium citrate) at a 1:1 ratio on a glass slide. The stain and sample were mixed and spread by sliding another glass slide on top. Slides were immediately dried using a hotplate. The number of viable cells was counted under the oil immersion objective (OIO), and percent (%) viability was calculated by determining the number of live cells per 200 cells. The total motility was determined using Computer Assisted Sperm Analysis – Animal Breeder Software. A glass slide with 1.5μL of diluted semen sample (1:35 ratio) spread with a cover slip was placed on the stage of the microscope equipped with a high-performance camera connected to the Animal Breeder Software. Different settings were set (i.e., storage location, animal species, animal ID) and the data were presented after capturing. The average of 5 frames was recorded to ensure the accuracy of data on the motility of each sample.

The cryopreservation protocol used in this experiment was entirely based on the study of Han *et al*. (2005). The series of experiments determined the best diluent, cryoprotectant (10% DMSO), equilibration time (15minutes) and thawing time and temperature (40°C, 1 minute). Uniform cell concentration of sperm cells was placed in the

microcentrifuge tubes, the sperm concentration set at 400M cells for each microcentrifuge tube was achieved by referencing the sperm concentration via hemocytometer technique as previously described, adding the appropriate volume of the diluted sperm cell to be diluted by the cryoprotectant and AU extender. Briefly, samples were diluted to 1:1 ratio placed in 1.5 mL microcentrifuge tubes and incubated in the refrigerator set at 4°C to 9°C for 2 hours for the first equilibration. After the first equilibration, 10% dimethyl sulfoxide (DMSO) in AU extender was added up to 0.5mL total volume. The mixture was mixed and placed in the refrigerator for another 15 minutes for the second equilibration. After the second equilibration, the microcentrifuge tubes were subjected to blast freezing, immediately submerged in the liquid nitrogen until thawing.

The cryopreserved samples were thawed at 37°C for 1 minute instead of 40°C indicated in the study of Han *et al*. (2005) due to the observed drop in the motility of the thawed semen samples. The post-thawing motility was determined using the same procedure used for the determination of motility before cryopreservation. The 1:35 dilution optimized for CASA is achieved by diluting the thawed samples with AU extender. The post-thawing viability was determined using the same procedure used for the determination of viability before cryopreservation.

The statistical analyses used for the correlation of different semen parameters were Pearson Correlation and Kendall Tau. Pearson Correlation assumes no outliers, bi-variate normality and homoscedasticity. The ceiling value for each variable was computed as the third quartile plus 1.5 of the interquartile range while the floor value was computed as the first quartile minus 1.5 of the interquartile range. Wilk-Shapiro Test was used to test Normality while Levene's Test was used to test for Homoscedasticity.

Kendall Tau Correlation was used for the parameters not having a normal distribution of data and are heteroscedastic. After assumption testing for normality and homoscedasticity, it was found out that Post Viability of the poor batch was not normally distributed and the Post Motility and Post Viability for all observations were heteroscedastic. Although the data for Spearman correlation is available, Kendall Tau was used for it is more appropriate for small data sets.

RESULTS

From a total of twenty-five (25), samples were collected from sixteen (16) Itik Pinas-Khaki drakes due to inconsistent semen ejaculation by the other animal semen donors. However, four (4) of those collected animals were still considered extraneous or have no batch designation because the contamination (excrement) was homogeneous with the semen in the samples obtained (i.e., IPK2, IPK8, IPK13, and IPK23). For the twelve (12) remaining IPK ducks, semen samples were collected thrice a week for 4 weeks, which were then immediately analyzed in the Animal Physiology Laboratory. The quantitative and qualitative semen characteristics evaluated were color, consistency, pH, sperm count, initial sperm motility and initial sperm viability. The initial motility evaluated using CASA was the sole criterion in grouping the IPK ducks. For the sperm count, the highest obtained value was 1480, from the good batch, while the lowest was 358 from the poor batch. The initial motility values were found to be fluctuating, with mean values of roughly 62% for the good batch and 32% for the poor batch. Lastly, the initial viability values were rather more consistent, with mean values of 88% and 64% for the good and poor batches, respectively, and thus an

overall mean of roughly 76%. Those with similar initial percent total motility were grouped to create batches, particularly Good, Poor, Mid1, and Mid2, summarized in Table 1.

Samples from only good and poor batches were pooled and obtained for further observation. Particularly, 10 collections were made from the good batch (pooled IPK6, IPK10, and IPK11) and 10 collections were made from the poor batch (pooled IPK1, IPK24, and IPK25). The initial motility of those pooled batches was again obtained. The values were observed to be fluctuating; the poor batch has 16.20% as the lowest initial motility and 49.00% as the highest while the good batch has 50.30% as the lowest and 79.30% as the highest. These are included in Table 2 below.

The color of the samples from both good batches and poor batches was white. Of a total of 60 samples collected, only three were yellowish — two from the good batch and one

Table 1. Batch designation of the twelve (12) IPK ducks.

Good	Mid1	Mid2	Poor
IPK6	IPK14	IPK9	IPK1
IPK10	IPK16	IPK18	IPK24
IPK11	IPK17	IPK19	IPK25

Table 2. Range values and means of semen characteristics.

Parameters		PARAMETER RANGE/ VALUE	MEAN	SD
Initial Motility* (%)	Good	50.30 – 79.30	61.98	± 8.364
	Poor	16.20 – 49.00	31.96	± 11.03
Color	All	White**	N/A	N/A
Consistency	All	Creamy***	N/A	N/A
pH	All	6.2 – 7.4	6.61	± 0.3990
	Good	6.2 – 7.2	6.54	± 0.3682
	Poor	6.2 – 7.4	6.68	± 0.4221
Sperm Count ($x10^9$)	All	3.58 – 14.80	8.598	± 3.086
	Good	5.97 – 14.80	10.351	± 2.796
	Poor	3.58 – 12.30	6.845	± 2.337
Ejaculate Volume (ml)	All	0.08 – 0.46	0.235	± 0.1030
	Good	0.08 – 0.46	0.23	± 0.1037
	Poor	0.12 – 0.41	0.24	± 0.1076
Initial Viability (%)	All	49.00 – 99.00	75.925	± 15.90
	Good	72.00 – 99.00	88.00	± 8.907
	Poor	49.00 – 88.50	63.85	± 11.42

*samples (after batch designation)
**95% white, 5% yellowish
***98.3% creamy, 1.7% milky

from the poor batch. The consistency was also the same for all samples, which are described to be 'creamy', except for one sample from the poor batch, which is described to be 'milky'. The pH range of the samples from the good batch was 6.2 to 7.2, while that of the poor batch was 6.2 to 7.4. The mean pH for the good and poor batches were 6.54 and 6.68 respectively, with an overall mean pH of 6.61. For the sperm count of the samples diluted to 1:1 ratio, the highest and lowest in the good batch were 1480 and 597, respectively, with a mean of 1035, while that in the poor batch were 1230 and 358, respectively, with a mean of 684.5.

Also shown in Table 2 are the initial viability of the semen samples. Viability data were obtained in terms of per 200 cells but are reported as a percentage. The data showed higher consistency in values for both batches. This is apparent in the relatively close means of the good and poor batches, with values 88.00% and 63.85%, respectively. The poor batch has 49.00% as the lowest initial viability and 88.50% as the highest while the good batch has 72.00% as the lowest and 99.00% as the highest. All the mentioned and tabulated characteristics were identified prior to cryopreservation and thawing.

The sperm motility of each animal was evaluated using Computer-Assisted Sperm Analysis (CASA), while the viability was assessed using eosin-nigrosin staining or live-dead staining. These tests are commonly employed in assessing boar and avian sperm evaluation (Gadea, 2005; Peterson *et al*., 2007). This was done prior to cryopreservation and after thawing. In determining the relationship of these two parameters, the variables are set to be the following: initial motility (IM), post motility (PM), initial viability (IV), post-viability (PV), change in percentage motility (CIM), and change in percentage viability (CIV).

The post-thawing motility values for the good batch and poor batch were observed to be highly consistent apparent in the very close means, with values 11.12% and 11.11%, respectively. The good batch has 2.50% as the lowest post-thawing motility and 22.20% as the highest while the poor batch has 6.50% as the lowest post-thawing motility and 25.10% as the highest.

The post-thawing viability values were observed to be less consistent for the good batch and poor batch. This is apparent in the difference of mean values 19.56% and 10.9% respectively. The good batch has 4.0% as the lowest post-thawing viability and 34.5% as the highest while the poor batch has 7.0% as the lowest post-thawing viability and 19.0% as the highest. Post-thawing observations suggest that despite close mean motility values between the batches, there are still more live sperm in the good batch than in the poor batch. In comparison to previous studies of duck semen cryopreservation, using 7 muscovy drakes, viability values ranged from 40.5% to 71.1% (Chen *et al*., 2016). Range values and means of post-thawing semen characteristics are listed in Table 3.

Table 3. Range values and means of post-thawing semen characteristics.

Parameters		PARAMETER RANGE/ VALUE	MEAN	SD
Post-thawing Motility (%)	Good	2.50 – 22.20	11.12	± 0.063
	Poor	6.50 – 25.10	11.11	± 0.051
Post-thawing Viability (%)	All	4.00 – 34.50	14.75	± 8.200
	Good	4.00 – 34.50	19.56	± 9.320
	Poor	7.00 – 19.00	10.90	± 4.250

To determine the relationship of the sperm motility and viability before and after cryopreservation, all possible pairs of the six parameters were taken and the corresponding relationships are accounted for. This was done using Pearson correlation and Kendall Tau correlation. However, the data for post motility (PM) and post-viability (PV) did not follow the homoscedasticity assumption of the Pearson correlation, which is why Kendall Tau correlation for small data sets was instead used for these variables. Generally, correlation coefficient values obtained are much lower in Kendall Tau correlation than those obtained in Pearson correlation.

Based on Table 4, sperm quality parameters before cryopreservation (IM and IV) for all observations have a strong or very strong positive linear relationship with Change in Motility (CIM) and Change in Viability (CIV) given by Pearson correlation. Change in Viability vs. IM and CIM have relatively less Pearson coefficient values compared to IV vs. IM and CIM because the change in viability is less related to sperm motility than the initial viability. These six relationships are shown to be significant at 5% level of significance, meaning the strong or very strong positive relationship is expected to be observed in at least 95% of any given set of samples. On the other hand, sperm quality parameters after thawing (PM and PV) generally have a very weak positive linear relationship with IM, IV, CIM, and CIV given by Kendall Tau correlation. Exceptions include PM vs. CIM and CIV, and PV vs. CIV wherein a very weak negative relationship was found. However, these nine relationships are not significant at 5% level of significance.

Table 5 and Table 6 show the correlation coefficients for good batch and poor batch observations, respectively. The poor batch generally showed weaker magnitudes and smaller significance of the parameter relationships compared to those of the good batch.

For good batch observations in Table 4, IM is shown to have a strong positive relationship with IV and CIM, significant at 5% level of significance, but a very weak negative relationship with CIV and otherwise not significant. Also using Pearson correlation, IV vs. CIM and CIV, and CIM vs. CIV have moderate or weak positive relationships but are not significant. Given by Kendall Tau correlation, IM and IV vs. PM and PV, and PM vs. PV are shown to have a positive relationship, while PM and PV vs. CIM and CIV have a negative relationship, of fluctuating magnitudes (very weak to strong). However, these are generally not true 95% of the time.

Table 4. Pearson and Kendall Tau correlation coefficient for all observations before and after cryopreservation.

All	IM^P	IV^P	CIM^P	CIV^P	PM^K	PV^K
IM^P	1.0000					
IV^P	0.8782*	1.0000				
CIM^P	0.9464*	0.8453*	1.0000			
CIV^P	0.6732*	0.8232*	0.7564*	1.0000		
PM^K	0.0865	0.0547	-0.1030	-0.2066	1.0000	
PV^K	0.1867	0.1671	0.0963	-0.1769	0.4329*	1.0000

^{P}Pearson correlation

^{K}Kendall Tau correlation

*significant at 5% level of significance

For poor batch observations in Table 5, IM and IV have a moderate or very strong positive relationship with CIM and CIV but are not significant given by Pearson correlation. All other relationships analyzed using Kendall Tau correlation, which involves PM and PV, have generally very weak relationships and are also not significant.

Individual drakes have different fertilizing capacities which makes it important to evaluate initial sperm parameters before proceeding to cryopreservation and/or artificial insemination (Tai Liu and Tai, 1984). Overall, the present study generally shows that higher IM results in higher IV, CIM, and CIV for all observations. This was consistent even for the individual good and poor batches. The study also demonstrated a need for further IPK semen cryopreservation optimization since relatively lower post-thawing motility was observed in using a reported protocol in avian.

Table 5. Pearson and Kendall Tau correlation coefficient for good batch observations before and after cryopreservation.

Good (>50%)	**IM^P**	**IV^P**	**CIM^P**	**CIV^P**	**PM^K**	**PV^K**
IM^P	1.0000					
IV^P	0.7120*	1.0000				
CIM^P	0.6594*	0.4514	1.0000			
CIV^P	-0.1293	0.2592	0.4627	1.0000		
PM^K	0.1798	0.2299	-0.2697	-0.5682*	1.0000	
PV^K	0.4045	0.3678	-0.0449	-0.5227*	0.7500*	1.0000

^{P}Pearson correlation
^{K}Kendall Tau correlation
*Significant at 5% level of significance

Table 6. Pearson and Kendall Tau correlation coefficient for poor batch observations before and after cryopreservation.

Poor (<50%)	**IM^P**	**IV^P**	**CIM^P**	**CIV^P**	**PM^K**	**PV^K**
IM^P	1.0000					
IV^P	0.5946	1.0000				
CIM^P	0.8888*	0.5966	1.0000			
CIV^P	0.5132	0.9335*	0.4738	1.0000		
PM^K	0.1380	0.0000	-0.1163	0.0233	1.0000	
PV^K	-0.1380	-0.2353	0.0233	-0.3955	-0.2619	1.0000

^{P}Pearson correlation
^{K}Kendall Tau correlation
*significant at 5% level of significance

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