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Cryogenic tolerance to freezing temperature and sperm motility characteristics of frozen-thawed spermatozoa of Philippine native pigs (*Sus scrofa*)

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Abstract Philippine native pigs (**PnPs**) in rural farming communities still thrive due to their adaptability and resilience to adverse environment challenges, perceived disease resistance traits and consumer demand for their tastier meat. These qualities make them likely candidates for gametes conservation for Animal Genetic Resources (**AnGRs**) Cryobanking. PnPs spermatozoa were cryopreserved using Lactose egg yolk-based **C**ooling **E**xtender (**CE**; 0.31M Lactose 80% v/v and 20% Egg yolk v/v) and **F**reezing **E**xtender **(FE)** solutions (**CE** 92.5% v/v, Glycerol 6% v/v and Equex paste 1.5% v/v). With the use of a Computer Assisted Sperm Analyzer (**CASA**) the average ± s.e.m sperm motility (CASA-MOT) and progressively motile sperm (CASA PMOT) were determined before and after cryopreservation. In study 1, epididymal spermatozoa (EpS) had a pre-freeze CASA MOT values of 71.75 ± 2.32 % and CASA PMOT of 44.0 ±1.96%, that decreased significantly to 30.1 ± 5.0% and 12.0 ± 3.0% after freezing, respectively (Student's T-test:$p<0.05$). In study 2, ejaculated semen of native Boar stud #1 and #2 registered a pre-freezing CASA MOT values of 53.1 ±3.6% and 52.3 ± 3.0% with sperm post thaw motility values of 29.1 ±4.1% and 31.0 ± 4.9%, respectively.The CASA PMOT values for Boar Stud #1 and #2 before cryopreservation was 45.9 ±3.6% and 46.4. ±3.3%, which manifested a significant decline to 25.0 ± 3.9% and 26.6 ± 4.6, respectively. Fluorescence microscopy using Live/Dead® Sperm Viability Kit revealed sperm head with green fluorescence confirming live sperm and viability indicative of intact plasma membrane integrity post thawing. These results demonstrated the cryogenic tolerance to freezing temperature and survivability of Philippine native pig spermatozoa for future research on *in vitro* fertilization and eventual AnGR cryo-conservation/cryobanking.

Keywords: Philippine native pig, Spermatozoa, Cryopreservation, CASA sperm motility

Introduction

Several breeds of farm animal species are selected intensively for a particular trait such as meat or milk to address the current demand for food

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protein that continues to rise with the growing human population. In order to avoid loss of genetic diversity caused by inbreeding depression, FAO member countries are encouraged to establish cryobank repositories as a reserve pool source of frozen gametes and embryos for breed conservation, reconstitution, repopulation of farm animal species and utilization thru basic and applied research to ensure food security.

The Philippines is currently home to 9.87 M head of the total pig population which manifested a significant reduction when compared with the 12.7M pig inventory in 2019 (Philippine Statistics Authority 2021 Swine Industry Report). Backyard hog raising comprise to about 72.1% of the total pig inventory while 27.9% are produced on a commercial scale in privately owned huge farms. The disease outbreak caused by the ASF virus largely affected the swine industry covering both the commercial and native pig population.

With the current government led re-population plan of action to increase swine production efficiencies there is a compelling need to complement such measures capitalizing on semen cryopreservation technology for frozen germplasm safekeeping thru cryobanking of Animal Genetic Resources. The National Livestock Cryobank facility at the Philippine Carabao Center premises currently comprise in its core collections frozen semen of water buffaloes, cattle, goat and aims to include among others frozen semen of pigs and poultry as well.

Native pigs are noticeable in most rural farming communities be in the upland or lowland areas. In spite of the introduction of imported foreign pig breed counterparts, they have managed to remain visible and existent in various localities with traditional and cultural ethnicities. Furthermore, the rising demand for the native pig commodity due to to its tastier meat has created a niche market for ‘wellness pork’ spelled organic as a protein source for the health conscious consumers after it was launched as an important commodity by the Philippine Native Animal Development (PNAD) of the Department of Agriculture-Bureau of Animal Industry.

Native pigs thrive well when fed with just about anything from farm by products such as rice bran, the more commonly taro plant for some and vegetable trimmings of cabbages, broccoli and other cruciferious vegetable rejects in upland areas. Another unique feature about them is their ease of adaptation to any adverse environmental conditions with their inherent climate resiliency and disease resitant traits as perceived by local farmers. These qualities therefore make them likely good candidates for gamete cryopreservation and eventual germplasm cryobanking for future basic research

utilization in assisted reproductive biotechniques such as IVF and Artificial Insemination.

Semen cryopreservation technology in the Philippines is not new and is far more established locally in water buffaloes (Mamuad *et al*., 2004) and goats (Beltran *et al.,* 2013) which is in stark contrast for boar semen. Except for the two published research papers on boar semen cryopreservation using composite breeds of introduced/foreign breeds by Ancheta *et al.,* 2015 and Granadozin *et al.,* 2020 there is dearth of information on the cryogenic tolerance of native pig spermatozoa in the country. Furthermore the very limited number of pure native boar studs trained for semen ejaculate collection is one challenging feat to overcome in establishing a workable semen freezing procedure. This situation therefore has prompted us to use an alternative source of spermatozoa specifically the cauda epididymis from slaughterhouse-derived native pig post mortem testes.

Epididymal sperm (**EpS**) cryopreservation been demonstrated for commercial breeds of pigs (Kikuchi *et al*., 1998). Immediate recovery of EpS from the cauda epididymis of post mortem testes of slaughtered native pigs by Aquino *et al*., 2018 yielded spermatozoa of good quality in terms of sperm motility and high percentage of live sperm. In a related experiment by Dela Rosa *et al*., 2018, cauda epididymal sperm from post mortem testes of PnPs were analyzed using a computer assisted sperm analyzer (CASA) for sperm motility (MOT) and progressively motile (PMOT) spermatozoa registering 65.2%, (CASA MOT) with a subpopulation of 49.5% CASA-PMOT value.

The objective of the study was to cryopreserve native pig spermatozoa and determined the survivability after freeze preservation by evaluating post thaw sperm motility and plasma membrane integrity to indicate survivability and viability.

Materials and methods

The cryopreservation potential therefore of native pig EpS was performed preliminarily in Study 1. As a sequel in optimizing a workable cryopreservation procedure for native pig spermatozoa, ejaculated boar semen from two native boar was likewise subjected for experimentation in study 2. In order to achieve a more objective approach of semen evaluation, a Computer Assisted Sperm Analyzer (CASA IVOS II, Hamilton Thorne, USA) was used to measure the sperm motility (CASA MOT) and progressively motile sperm (CASA PMOT) characteristics and other sperm motion kinematics before and after cryopreservation. Fluorescence microscopy was performed. with the use of fluorescence probes in the Live/Dead Sperm^{TM} Viability Kit (Thermo Fisher

Cat L7011) for further viability confirmation and plasma membrane integrity of frozen-thawed native pig spermatozoa.

Setting up a workable 'Cold Room' set up

The fundamental cryopreservation steps during the slow phases of temperature cooling from 15℃ to 5℃ are usually undertaken in a 'thermostatically controlled **COLD ROOM'** with much precision in a temperature~ and time~ dependent manner that is commonly found in the cryobank facilities of the USDA-ARS NAGP, Fort Collins, Colorado, USA and the NARO Genebank in Ibaraki, Japan. Due to the unavailability of a cold room for conducting boar semen cryopreservation, practical approaches were carried out using a designated room with provisions of an air conditioning unit that can cool the area to 15-16℃ hereto referred as the **'Cold Room set up'**.

A horizontal type refrigerator with a pre-calibrated temperature setting at 5-6℃ was placed inside this room that will function as the 'cold handling cabinet' and/or working area for the LN_2 vapor cooling procedure performed in a simple polystyrene box containing liquid nitrogen at the Reproduction and Physiology Section, Philippine Carabao Center (PCC), Science City of Muñoz, Nueva Ecija Philippines.

Preparation of Cryoprotectant Solution(s)

The cryoprotectant solutions that were used for boar semen cryopreservation were prepared in advance following the USDA boar semen cooling and freezing extender solution composition. The base medium Lactose:Egg Yolk consisted of 0.31M Lactose solution (80%, v/v) and Egg Yolk (20%,v/v) as the Cooling Extender solution (**CE**) supplemented with antibiotics (Gentamycin sulfate at 25ug/ml). The Freezing Extender (**FE**) solution was composed of the CE at 92.5%, v/v, Glycerol at 6%, v/v and Equex paste at 1.48% v/v as the final solution to be added to complete the whole process of semen dilution in preparation for the cryopreservation procedure.

Computer Assisted Sperm Analyzer CASA

Sperm motility using the Computer Assisted Sperm Analyzer (CASA) taking into account the Sperm Motility (CASA MOT) and the Progressively Motile sperm (CASA-PMOT) was employed for the processed boar semen samples for a more objective approach of evaluation. CASA MOT pertains to the proportion of motile sperm or sperm motility expressed in percent (%)

values while CASA PMOT refers to the subpopulation of progressively motile spermatozoa that can travel from point A to point B. These represented the sperm that can move in response to the target site where the fertilizable egg cell resides waiting to be fertilized. The CASA software technical settings specific for the porcine species for semen analysis was used to include stage temperature setting at 39℃; Kinematic parameters; Progressive STR (45%); Progressive VAP (45μm/s); Slow VAP (20μm/s); Slow VSL (30μm/s); Static VAP (4μm/s), static VSL (4μm/s). Video was captured at a frame capture speed of 60Hz, with frame count of 30 frames per second.

Cryopreservation of Native pig spermatozoa

Epididymal spermatozoa (EpS) were recovered from the cauda epididymis using the retrograde flushing technique. The aim was to determine the cryopreservation potential of epididymal sperm and to compare two thawing temperature conditions to be suitable for frozen thawed epididymal sperm. The EpS extract was diluted at a 1:4 ratio with Beltsville Thawing Solution (BTS) as the semen extender solution. The EpS: BTS mixture was allowed to cool to about 15-16℃ overnight in the "Cold Room Set up".

After an overnight cooling at 15-16℃ in the 'Cold Room" the samples (n=8) were centrifuged at 550g for 3 minutes to harvest the sperm precipitate /pellet and remove the semen extender solution/supernatant. The sperm concentration was determined using a Neubauer hemocytometer while the sperm motility was determined thru the use of the CASA equipment in order to compute for the total volume of extender (TVE) to be used following the method of Granadozin *et al*., 2020 with some modifications in the desired final sperm concentration per ml (Ravagnani *et al.,* 2018). The Cooling Extender solution (**CE**) was then carefully added into the sperm pellet to dilute the sperm initially to obtain a sperm concentration of $500x10^6$ sperm per mL. The CE: sperm mixture was allowed to gradually cool to for 2.5-3h inside the horizontal type refrigerator to attain the 5-6℃ temperature. As soon as the temperature of the sample reached 5℃, the pre-freeze CASA MOT and CASA PMOT values were determined.

The second dilution was done in a stepwise manner by adding the equal proportion of the freezing extender (**FE**) solution to achieve the desired final sperm concentration of $250x10^6$ sperm per mL. Pre sterilized 0.5ml semen straws (IMV, France) were loaded with the sperm mixture and sealed at one end with colored sealing powder and were arranged horizontally on a metal tray. Cryopreservation was performed using simple polystyrene box with liquid nitrogen that was placed inside the horizontal type refrigerator. The

horizontally arranged semen straws on the metal tray were subjected to Vapor Cooling 3cm above liquid nitrogen level for 10 minutes before the final LN_2 plunge. The frozen straws were then stored in liquid nitrogen tanks until further evaluation for cryo-survivability. Two temperature thawing conditions either at 50℃ for 12 seconds or at 37℃ for 3 minutes were tried and compared. The thawed samples were evaluated thereafter for post thaw sperm motility characteristics (CASA MOT) and progressively motile sperm (CASA PMOT).

Cryopreservaton of Native pig Boar Semen ejaculates

Slow cooling tolerance of native pig spermatozoa before freezing

Finding an appropriate semen extender solution for Philippine native pig semen is a necessary pre-requisite for boar semen processing and its eventual transport for utilization in far flung areas. The effect of two shipment extender solutions in maintaining the quality of the semen from the site of origin to its final destination was determined. The sperm motility responses were assessed thru CASA in all samples that were subjected to the slow cooling phases of temperature dropdown in preparation to cryopreservation. Semen ejaculates from Boar #1 and Boar #2 collected from a native pig institutional farm were diluted at a 1:4 dilution ratio with an equal temperature of 32±1℃ in **BTS** semen extender solution labelled as **group A** sample and/or in **Minitube M-III** labelled as **group B** sample (as per personal communication with the technician from the farm origin). Group A semen samples for boar # 1 and #2 consisted of four (n=4) experimental trials whereas group B semen samples for boars #and #2 consisted of three (n=3) experimental trials. All of the processed semen contained in 50mL capacity conical tubes were packed in a polystyrene box with gel ice pack that was placed inside which was separated by a corrugated cardboard wall to avoid direct cooling of the samples thus prevent chilling injury. The samples originated from the Marinduque State College native pig farm in the island province of Marinduque via various modes of transport by land and sea.

After overnight of transport duration time between 23-24 hours, the samples were delivered to the Philippine Carabao Center Reproduction and Physiology Section Laboratory. Due to the absence of a data logger during the transport of the samples, the temperature was determined and recorded upon delivery in the laboratory. After checking for the pH, the delivered samples were evaluated for sperm motility using the CASA machine (CASA IVOS II, Hamilton Thorne, USA). Starting with the initial recorded temperature of 20-22℃ upon arrival of the samples, the CASA sperm motility (CASA MOT) and progressively motile sperm (CASA PMOT) values were recorded representing

treatment 1. Afterwards, the samples were placed in the cold room set up described previously for them to gradually cool to achieve a temperature reading of 15-16℃ herewith referred to as treatment 2. When the desired temperature was achieved, the CASA MOT and CASA PMOT were again determined and recorded. Then, the samples were then subjected to a centrifuge spin at 550g for 3 minutes to harvest the sperm precipitate/pellet and replace the semen extender with the Cooling extender solution. The Cooling Extender (**CE**) solution was initially added into each of the two boar semen precipitate samples from each of the two shipment extenders at 15-16℃ to attain an initial sperm concentration of $500x10^6$ sperm per mL. The **CE:sperm mixture** in all samples were again allowed to cool in the horizontal type refrigerator to attain a pre-freeze temperature of 5-6℃ herereto referred as treatment 3. As soon as the desired cool temperature was achieved, the CASA MOT and CASA PMOT for each samples was determined and recorded.

Cryogenic temperature freezing thru LN_2 vapor cooling of the Boar semen samples #1 and #2

The boar semen samples that were initially resuspended in the Cooling Extender (**CE**) were finally added with an equal proportion of the the Freezing Extender (**FE**) solution to achieve a final sperm concentration of $250x10^6$ per mL in preparation for the cryopreservation procedure. The whole sperm mixture was loaded in pre-sterilized 0.5ml straws (IMV, France) sealed on the other end of the plastic straw with sealing PVC powder and finally arranged horizontally on top of a metal tray. Cryopreservation was performed in a simple polystyrene box as previously described. The frozen straws were then stored in liquid nitrogen tanks until further post thaw sperm motility evaluation. Thawing of the straws at 45℃ for 30 seconds was done followed by sperm motility (CASA MOT) and progressively motile sperm CASA PMOT evaluation. To validate further the viability of the frozen spermatozoa, fluorescence microsocopy was used after staining with differential staining fluorescence probes SYBR: PI Live/Dead Sperm^{TM} Viability Kit.

Statistical analysis

The data obtained for CASA MOT and CASA PMOT are expressed in percent mean± s.e.m. values. The Student's T test ($p<0.05$) was used to analyze the data when comparing the sperm motility parameters before and after cryopreservation. The data on CASA MOT and CASA PMOT sperm motility parameters during the three slow phases of cooling temperature

conditions from 20-22 ℃, 15-16 ℃ and 5-6 ℃ were analyzed thru ANOVA ($p<0.05$).

Fluorescence microscopy using Live /Dead™ Sperm viability kit

The viability of frozen thawed- boar spermatozoa was determined with the use of a commercially available fluorescence staining kit, SYBR/PI fluorescence probes (Live/Dead Sperm™Viability Kit Thermo Fisher Cat.#L7011). Following the prescribed procedure written in the manufacturer's manual one hundred microliter (100 µL) of the sample was stained with each of the fluorescence probes. Shortly thereafter, a stained smear sample was viewed with the recommended excitation and emission spectra for each of the fluorescence probes SYBR: PI used. Live spermatozoa with intact membrane that emitted the bright green fluorescence (SYBR green) in the sperm head was considered as live whereas damaged cells that was seen to absorb the red stain (Propidium Iodide) on the sperm head was confirmed as dead sperm.

Results

Cryopreservation of Native Pig Epididymal spermatozoa

There was a considerable reduction in the proportion of epididymal spermatozoa that survived the cryopreservation procedure (Table1). The mean ± s.e.m. values for sperm motility (CASA MOT) and progressively motile sperm (CASA PMOT) before cryopreservation was 71.75 ± 2.32 % and 44.0 ± 1.96%, respectively with a recorded post thaw motility (PTM) percent values that significantly decreased registering 30.1 ± 5.0% and 12 ± 3.0%, respectively after cryopreservation (Student's T-test $p<0.05$).

The two different thawing temperature with different exposure time conditions, 50 ℃ for 12 seconds and 37 ℃ for 3.0 minutes resulted in similar post thaw motility values averaging 30.0% for CASA MOT as shown in Table 2. Thawing of the frozen straws at 50 ℃ for 12 seconds resulted in numerically greater proportion of progressively motile spermatozoa (CASA PMOT) with a mean ± s.e.m. value of 13.8±3.5% but did not statistically differ with the obtained 10.0±4.7% for 37°C for 3 minutes (Student's T-test $p > 0.05$).

These results were found to be fairly low but manifested the cryopreservation potential of native pig EpS recovered from the cauda epdidiymis of post mortem testicles under the current local laboratory set up in the absence of a thermostatically controlled room solely dedicated for gamete cell cryopreservation activities.

Table 1. Sperm Motility (CASA MOT) and Progressively Motile Sperm (CASA PMOT) of Cryopreserved Native pig Epididymal Sperm

	Before Cryopreservation (% Mean ± s.e.m.)	**After Cryopreservation (% Mean ± s.e.m.)**
CASA MOT	71.75 ± 2.32[a]	30.1 ± 5.0[b]
CASA PMOT	44.0 ± 1.96[a]	12.0 ± 3.0[b]

[a,b] Mean ± s.e.m. percent values with superscripts within rows significanly differ by Student's T- test ($p<0.05$)

Table 2. Post thaw motility (PTM%) of frozen native pig epididymal sperm in two (2) thawing temperature and exposure time conditions

	50 ℃ for 12 seconds	**37 ℃ for 3 minutes**	**Average PTM**
CASA MOT (%)	30.3 ± 6.9[ns]	30.0 ± 7.7[ns]	30.1 ± 5.0
CASA PMOT (%)	13.8 ± 3.5[ns]	10.0 ± 4.7[ns]	12.0 ± 3.0

[ns] Values within rows do not differ by Student's T-test ($p \geq 0.05$)

Cryopreservation of Philippine Native pig boar semen ejaculates

Slow cooling tolerance of native pig spermatozoa before freezing

The semen ejaculated from two Native boar semen donors #1 and #2 in either of the two shipment semen extenders namely, in BTS as Group A samples and in Minitube MIII as Group B were recorded to have a pH reading that ranged between 7.2~7.4 and the proportion of live sperm ranged between 92-94%.

Mean ± s.e.m. percent values of CASA MOT and CASA PMOT during the slow phases of cooling for Boar semen #1 and #2, respectively covering Group A and Group B samples is shown in Tables 3 and 4. Cooling the semen samples upon arrival from 20-22 ℃ to 15-16 ℃ as treatments 1 and 2, respectively, did not cause any significant reduction in the overall sperm motility and progressive motility values obtained. Such observation was reflective of the shipment extender's capability in maintaining the quality of the native boar semen in preparation for further processing entailed in the cryoprservation procedure. The cooling duration from 20-22 ℃ to reach 15-16 ℃ was achieved after two hours in the cold room set up. A sufficiently high proportion of live sperm was recorded averaging between 92-95% and 2-3%

sperm abnormalities that pre-qualified the samples for further cooling at 5-6 ℃ after the sperm precipitate in all samples were resuspended with the cooling extender solution.

There was an observed cooling effect in treatment 3 at 5-6 ℃ which was manifested by a slight decline in the proportion of motile sperm (CASA MOT) and progressively spermatozoa (CASA PMOT) for both boar semen #1 and #2. Boar semen #1 in Group B (Table 3) showed a mean ± s.e.m CASA MOT of 58.6±2.1% and CASA PMOT 53.1±2.2% which were significantly greater in proportion (T-test $p<0.05$) when compared with semen samples in Group A registering 49.0±5.5% CASA MOT and 40.55±4.5% CASA PMOT.

Boar semen #2 showed mean ± s.e.m. values of 53.9±4.3% for CASA MOT in Group A and 50.6±4.8% in Group B that were not statistically different by T-test at $p>0.05$ (Table 4). The proportion of progressively motile sperm (PMOT) in Group A and B for Boar #2 were 48.4±4.9% and 44.5±5%, respectively which did not significantly differ from each other by Student's T-test ($p>0.05$).

The pre-freeze sperm motility characteritics for boar semen #1 and # 2 either as Groups A and B established the cryopreservation potential of native pig spermatozoa and pre-qualified the samples for further cryopreservation.

Table 3. Sperm Motility (CASA MOT%) and Progressively Motile Sperm (CASA PMOT%) of Boar semen #1 Group A (n=4 trials) and Group B (n=3 trials) during the slow phases of cooling

	CASA MOT(%) Mean ± s.e.m		**CASA PMOT(%) Mean ± s.e.m**	
	Group A	Group B	Group A	Group
Treatment 1 20-22 ℃	89.03 ± 2.2[a,1]	85.1±0.7[b2]	75.53 ± 1.2[a]	72.7 ± 1.7[a]
Treatment 2 15-16 ℃	90.43 ± 2.0[a]	88.2 ± 0.9[a]	72.93 ± 2.2[a]	75.8 ± 3.0[a]
Treatment 3 5-6 ℃	49.00±5.5[b,1]	58.6±2.1[c,2]	40.55 ± 4.5[b,1]	53.1 ± 2.2[b,2]

[abc] Mean ± s.e.m values of CASA MOT and CASA PMOT with different superscripts within columns statistically differ (ANOVA, $p<0.05$)

[1,2] Mean ± s.e.m values of Group A and B with superscripts within rows for CASA MOT and CASA PMOT, respectively differ (T-test $p<0.05$)

Table 4. Sperm Motility (CASA MOT%) and Progressively Motile Sperm (CASA PMOT%) of Boar semen # 2 Group A (n=4 trials) and Group B (n=3 trials) during the slow phases of cooling temperature

	CASA MOT Mean ±s.e.m		**CASA PMOT Mean ±s.e.m**	
	Group A	Group B	Group A	Group B
Treatment 1 20-22 ℃	86.1 ±3.0[a]	88.5 ±2.4[a]	74.7 ±1.7[a,1]	70.6 ±0.44[a,2]
Treatment 2 15-16 ℃	87.2 ±2.5[a]	87.0 ±1.8[a]	73.9 ±3.3[a]	68.3 ±6.5[a]
Treatment 2 5-6 ℃	53.9 ±4.3[b]	50.6 ±4.8[b]	48.4 ±4.9[b]	44.5 ±5.0[b]

[ab] Mean ±s.e.m Values of CASA MOT and CASA PMOT with different superscripts within columns statistically differ (ANOVA, $p<0.05$)
[1,2] Mean ±s.e.m Values of Group A and B with superscripts for CASA PMOT within rows statistically differ (Student's T-test $p<0.05$)

Cryogenic temperature freezing thru LN_2 vapor cooling of the Boar semen samples #1 and #2

The overall cryopreservation results of native pig spermatozoa of Boar #1 and Boar #2 are pesented in Table 5. The mean ± s.e.m. post thaw sperm motility (CASA MOT) percent values did not statistically differ between Boar #1 with 29.1±4.1% and for Boar #2 with 31.0±4.9% (Student's T-test, $p>0.05$). The progressively motile sperm (CASA PMOT) likewise resulted in mean ± s.e.m. values of 25.0±3.9% and 26.6±4.6% for Boar #1 and 2, respectively.

From all indications, the cryopreservation process resulted in significant differences in the proportion of motile and progressively motile sperm population before and after cryopreservation (T-test $p<0.05$). These cryo-survivability results were further confirmed thru fluorescence microscopy as depicted in Fig 1 that revealed sperm head emitting green fluorescence to indicate viability. The green fluorescence is clearly indicated in the intact plasma membrane's integrity as opposed to the dead spermatozoa that absorbed the Propidium Iodide stain showing a visible red sperm head due to the compromised plasma membrane.

To the best of our knowledge, the research outcomes obtained from study 1 and study 2 were found to be the first report in demonstrating the cryogenic tolerance and survivability of Philippine native pig spermatozoa. Moreover, the establishment of a cold room set up proved useful in attaining the required work environment during the optimization of the native pig spermatozoa

cryopreservation procedure in the absence of a thermostatically controlled cryopreservation room such as those found at the USDA genebank in Colorado and in NARO Genebank in Japan.

Table 5. Sperm motility characteristics (CASA MOT and CASA PMOT) before and after cryopreservation of Boar stud#1 and Boar stud #2 semen ejaculates

	CASA MOT		**CASA PMOT**	
	Before Cryopreservation	After Cryopreservation	Before Cryopreservation	After Cryopreservation
Boar#1	**53.1 ± 3.6[a]**	**29.1 ± 4.1[b]**	**45.9 ± 3.6[a]**	**25.0 ± 3.9[b]**
Boar#2	**52.3 ± 3.0[a]**	**31.0 ± 4.9[b]**	**46.4 ± 3.3[a]**	**26.6 ± 4.6[b]**

a,b Average ± s.e.m. CASA MOT and CASA PMOT values with superscripts within rows statistically differ by Student's T-test ($p<0.05$)

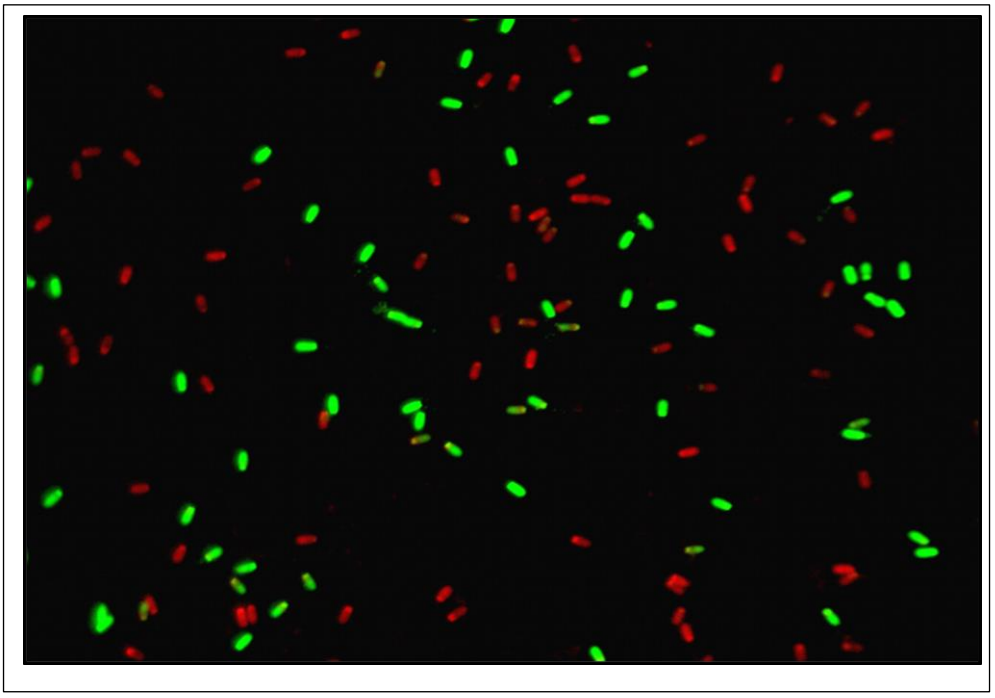

Figure 1. Sperm head emitting Green fluorescence shows LIVE spermatozoa after cryopreservation and Red stain sperm head means Dead sperm that did not survive freezing

Discussion

The cryogenic tolerance and survivability of Philippine native pig spermatozoa was successfully demonstrated irrespective of the male gamete germplasm source(s) either that of the cauda epididymal sperm (EpS) and/or native boar semen ejaculates. The scarcity in obtaining native pig spermatozoa for this basic research led to the exploratory use of epididymal sperm as an alternative gamete source while at the same time optimizing a workable freezing procedure under current local laboratory conditions that simulated the usual thermostatically controlled cryopreservation room in a temperature~and time~dependent manner.

The post thaw sperm motility (PTM) obtained for frozen thawed EpS were lower (30%) compared with the results of Kikuchi *et al.*, 1998 for frozen EpS of slaughterhouse derived testicles with an obtained PTM sperm motility of 44% but were comparable with the cryopreserved boar semen PTM values of 32% published by Granadozin *et al*., 2020. Epididymal spermatozoa although still devoid of the seminal plasma fluid have been reported to show a higher resistance to cold shock thus are a suitable alternative source of spermatozoa for cryopreservation (Bertol, 2016). Okazaki *et al.*, 2012 obtained a post thaw motility of 80% in frozen EpS of commercial pig breeds that were incubated for one hour (1h) at 37℃ in a thawing solution supplemented with 15% (v/v) seminal plasma resulting in improved conception rates after AI utilization. Epididymal sperm (EpS) cryopreservation in other farm animals have been likewise reported successful for nondescript/native goats with reported livebirths (Ocampo *et al*., 2021) and in high genetics goats (Blash *et al.*, 2000) and in the Indonesian white spotted water buffalo (Yulnawati *et al*., 2013).

The ejaculated boar spermatozoa bathed in seminal plasma are intrinsically motile exhibiting high metabolic activity until full depletion of energy reserves lead to shortened lifespan. In order to circumvent the aforementioned normal physiological phenomenon, various semen extenders that come in various concoctions either as short, mid and long term diluents have continuously evolved as diluents to reduce the metabolic activity of boar semen without compromising functionality or fertility. The beneficial effects of the two (2) semen extenders i.e. BTS and Minitube M-III proved purposeful in maintaining the integrity and/or quality of the semen during the overnight shipment across all samples registering overall sperm motility between 85-89% upon delivery at a holding temperature condition of 20-22 ℃.

These observations were in agreement with the findings of Zhou *et al*., (2004) who used BTS semen extender for the 24 hour storage of Harbin white boar semen at 20 ℃ temperature registering 89.3 ±4.4 percent sperm motility. Most semen extender solutions provide the nutrient requirement(s) such as glucose being a predominant source of energy for the metabolic support of sperm motility thru the glycolytic pathway. Medrano *et al*. (2005) reported that a 150mM glucose in combination with non-energy sugar substrate sodium lactate at 20mM concentration performed better in preserving the quality of boar sperm for 7 days at 17 ℃ when compared with the standard BTS containing glucose and 25mM sodium citrate. Other key components of semen extenders are the electrolytes and ions that contribute to sperm motility, while buffers help to neutralize the effect on pH of metabolic end products and antimicrobials prevent microbial contamination.

Further cooling at 15-16 ℃ of the semen samples #1 and #2 in either semen extenders did not cause any significant decline in the proportion of motile spermatozoa showing a CASA MOT value of ≥ 85% as opposed with the reported 40% sperm motility post cooling at 15 ℃ in BTS for commercial breeds of boar semen by Kaeoket *et al*. (2013) who concluded that long term extenders for cooling semen performed better than BTS before freezing.

The slow cooling phases in treatment 2 on the other hand yielded results that were in agreement with CASA Motility percentage values of 75-80% reported by Purdy *et al*. (2010) in semen ejaculates of composite pig breeds diluted at 1:4 ratio with Androhep Plus (Minitube of America, Verona, WI, USA) that were transported and cooled to 15 ℃ for 24h. The semen extender Minitube M-III is commercially marketed as a medium-term extender and is advertised to be packed with antioxidants, with a potent pH buffer system and broad spectrum antibiotics however, the exact component(s) remain undisclosed. Antioxidant additives in semen extenders such as reduced glutathione (Zhang *et al*., 2016) and L-cysteine (Chanapiwat *et al*., 2009) have been reported to have great beneficial effect on sperm quality.

Boar semen's sensitivity to chilling injury is intrinsic with its plasma membrane composition which is abundant with polyunsaturated fatty acids (PUFA) rendering it susceptible to cold shock. In addition, the low cholesterol: phospholipid ratio makes it more prone to plasma membrane destabilization in temperatures equal to or lower than five degrees Celsius (5 ℃) causing irreversible damage (Parks and Lynch, 1992; Parks and Graham, 1992).

An overnight holding temperature of 17 ℃ of diluted semen was confirmed (Casas and Althouse, 2013) to influence boar semen freezing cryopreservation outcomes by causing improvements in cryotolerance thru plasma membrane stabilization of the lipid architecture. Similar observations

by Purdy *et al.,* 2010 reported that maintaining cooled boar spermatozoa at approximately 16℃ during storage result in higher fresh and frozen-thawed boar sperm quality.

Lumerio (2019) stated that during the initial 5-6h of hours of travel a recorded temperature reading between 16-17℃ was monitored in the boar semen samples transported in a polystyrene box with a gel ice cooling material from the same native pig farm source in our research study. Although the delivered experimental samples had a holding temperature reading of 20-22℃ upon arrival, we hypothesized therefore, the likelihood for the samples to have reached a similar temperature in the first 5-6 hr of transport duration. Keeping the boar semen in extenders solutions between 15℃ and 20℃ is a necessary step that promotes the capacity of spermatozoa to withstand thermal shock that can be attributed to the phosphorylation of related protein such as the expression of heat shock protein70 HSP70 (Yeste, 2017, Jovicic *et al.,* 2020).

The slow cooling changes in temperature when the samples were in transit may have enabled the native pig spermatozoa to acquire thermo-resistance in preparation to the next pre-freezing steps. The effect was manifested when an overall pre-freezing sperm motility CASA reading upon reaching the cold 5℃ temperature was achieved averaging a score of 52-53% in treatment 3. The aforementioned values however, were comparably lower with the pre freezing motility obtained for the EpS (71.75%) in study 1 of this paper and for native Spanish Iberico native pigs with an obtained of 77-80% sperm motility at 5℃ namely, Entrepelado and Lampiño (Sancho *et al*., 2007).

Surprisingly, the post thaw motility (PTM) results for the Philippine native pig spermatozoa irrespective of the source i.e. EpS or ejaculated semen with CASA MOT values between 29-31% were found to be within the range obtained by Purdy *et al*., 2010 for frozen thawed commercial boar semen with a PTM score averaging between 19-47%. Spanish native Iberico pigs such as the Entrepelado and Lampiño boars semen were reported to have post thaw sperm motility of 37.6 ± 13.3%, and 35.6 ± 9.0%, respectively (Sancho *et al.,* 2007).

The CASA PMOT values that ranged between 25-26% for Boar#1 and #2 were greater in proportion when compared with the reported 9.9-13.1% progressively motile sperm of frozen thawed Japanese native pig Agu (Yamauchi *et al*., 2009). The viability of the frozen-thawed native pig spermatozoa was positively confirmed with sperm head emitting green fluorescence to substantiate the post thawed motile and progressively motile spermatozoa results assessed by the CASA equipment.

In conclusion, the cryogenic tolerance to freezing temperature of native pig spermatozoa was demonstrated with cryosurvivability outcomes in this

study in an optimized a workable boar semen cryopreservation which is timely and relevant amidst the ASF scare. Future research directions are currently aimed to determine the fertilizing potential *in vitro* (IVF research) of the cryopreserved samples deposited at the National Livestock Cryobank in the Philippines.

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