



Extended and Cryopreserved Semen of Banaba Native Chicken with Sugarcane Extract for Artificial Insemination

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ABSTRACT

The experiment was conducted to test the efficacy of sugarcane extract extender (40% SE+30% dH₂O (distilled water) + 30% EY-C (Egg-yolk citrate)) developed in our laboratory and semen cryopreserved with SE extender with 3% glycerol (SEG) as a cost-effective alternative in an *in vivo* fertility trial using a practical farm approach. The lactated ringers' solution (LRS) served as a control. The cryopreserved semen was thawed at room temperature (25-27°C) for 15-20 min for the artificial insemination. Thirty-six (36) 7.5 month old Dekalb White hens with an average weight of 1.65 kg were divided into three groups of 12 hens per group and housed individually in a completely randomized design (CRD). The groups: 1, 2 and 3 were artificially inseminated with LRS extended semen, SE extended semen and SEG cryopreserved semen respectively. A total of 284 eggs were collected and incubated, consisting of 95 (group 1), 89 (group 2) and 100 (group 3). The results showed that, fertility rates of LRS extended semen (60.04 ± 1.83%) and SE extended semen (56.94±2.62%) were significantly (p≤0.05) higher than the cryopreserved semen (25.56±3.22%). However, hatchability rates were statistically similar at 5% among the treatment means for the LRS (51.26±4.15%), SE (62.02±6.34%) and SEG (49.31±2.86%). The findings of this experiment suggest that sugarcane extract with 3% glycerol successfully produced fertile eggs and viable chicks. The extender showed no adverse effects on fertility or hatchability, indicating its potential as a promising medium for avian semen cryopreservation. The SE and SEG can be used for poultry propagation, breeding and conservation.

Keywords:

Extender,
Cryopreserved Semen,
Hatchability,
Sugarcane Extract,
Artificial Insemination.

INTRODUCTION

Artificial insemination (AI) is a potent and distinctive technique for achieving pregnancy in females of most farmed animals (Bustani & Baiee, 2021). The primary reason for using this technique in farm animals is to speed up the rate of genetic improvement by increasing the productivity of food producing animals including local chicken (Mittal *et al.*, 2019). This genetic improvement will ensure food security or meeting the soaring demand for food as native chickens possess unique adaptive traits that permit them to survive and reproduce under the harsh climatic, nutritional, and management conditions (Alebachew *et al.*, 2017),

In the poultry industry, AI is a valuable tool for efficient utilisation of superior males, save costs and improving the efficiency of reproduction and therefore increase profit

margin of farmers (Mohan *et al.*, 2018). The physiological challenges of avian semen make it susceptible to dilution and cryo-injuries, leading to membrane rupture, which in turn affects semen quality and fertilizing ability of sperm cells. A suitable dilution and freezing extender is crucial for post extension and post-thaw spermatozoa fertilizing ability (Thananurak *et al.*, 2017). The commonly used chemical extenders extenders such as Ravie and Lake extender, Beltsville, Raptac, N-methylacetamide (NMA), INRA 96 extender are costly, require careful preparation and are environment unfriendly.

Natural extracts from plant sources are known to maintain life because of relative high amount of medicinal compounds vital for health (El-Sheshtawy *et al.*, 2017; Karima *et al.*, 2025). The extracts are used in

conjunction with semen extenders for preserving animal semen. This extending and cryopreserving properties are associated with strong antioxidant capacity, essential amino acids, sugars, fatty acids and potassium which play key roles in oxidative damage, osmotic potential of cells and protect membranes from cryoinjury, membrane integrity and sperm survival during cryopreservation (Daramola *et al.*, 2016). Soybean lecithin and coconut water have been effective for conservation of sperm obtained from various species (Yodmingkwan *et al.*, 2016; Castro *et al.*, 2020; Esguerra *et al.*, 2020).

Researchers are exploring this potential health benefit of phytochemicals and the synergistic effects of these multiple fractions' compounds compared to the single purified active and toxic substances which are used in the extension and cryopreservation process.

Sugarcane extract (SE) as a natural product contains appreciable amounts of total sugars, minerals, vitamins, antimicrobial and cytoprotective effects and antioxidants for the maintenance of sperm motility and viability due to the sugar components in sugarcane water (Chinnadurai, 2017; Islam, 2017). SE is also easy to prepare, readily available, cheap, and eco-friendly in contrast to commercial extenders and/or cryoprotectants which are very expensive and environmentally unfriendly.

The experiment was conducted to test the efficacy of sugarcane extract extender (40% SE+30% dH₂O (distilled water) + 30% EY-C (Egg-yolk citrate)) developed in our laboratory and semen cryopreserved with SE extender with 3% glycerol (SEG) in an *in vivo* fertility trial using a practical farm approach to contribute to sustainable poultry semen preservation and poultry farming.

MATERIAL AND METHOD

The animal care and sample collection procedures were approved by the Institutional Animal Care and Use Committee of the University of the Philippines Los Baños (UPLB) with an assigned number CAFS-2018-006.

Semen Collection

Twelve (12) 29-month-old Banaba native roosters with an average weight of 2.24 kg were used in this experiment as semen donors. The abdominal massage method was used for semen collection (Burrows and Quinn, 1937). The pooling was done to remove the individual variability effects amongst the semen donor roosters.

Semen Sample Preparation

The practical farm approach was adopted for this experiment. The semen dilution was done at the farm using the sugarcane extender developed in Experiment I

(40% SE+30% dH₂O (distilled water) + 30% EY-C (Egg-yolk citrate)) and Lactated Ringers' solution as a control. This was to anticipate and mimic the real-life situation of the rural farm where the farmer may not have access to scientific tools and equipment hence semen motility characteristics were not assessed before artificial insemination (AI). The dilution ratio was 1 part of semen to 5 parts of extender for both extension media. The collected semen samples were pooled, and 2 mL of the pooled semen sample was transferred to two empty 15 mL conical tubes (1mL for each tube). 5 mL of each extender was slowly added to each tube (semen + extender) and gently mixed. The mixture was allowed to stand for 10-15 min for sperm cell adjustment.

The cryopreserved semen based on protocol developed in Experiment II (40% SE+30% dH₂O (distilled water) + 30% EY-C (Egg-yolk citrate)+ 3% glycerol) was thawed at 5°C for 25-30 min in the laboratory and motility parameters were assessed before sending it to the farm for the AI. This was to ensure that only motile and viable spermatozoa were used for the artificial insemination.

In Vivo Fertility Evaluation

To evaluate the fertility of the extended and cryopreserved semen, 36 Dekalb White hens were procured and divided into three groups of 12 hens per group in a completely randomized design (CRD). The hens were caged individually. Group 1 was inseminated with semen extended in Lactated Ringer's solution (control group); Group 2 with semen extended using SE (40% SE + 30% dH₂O + 30% EY-C); and Group 3 with frozen-thawed semen cryopreserved using SE + 3% glycerol. Insemination was done twice a week for two consecutive weeks. Egg collection started on the second day after the first insemination and continued up to four days after the final insemination. Fertility rate (%) = (Fertile Eggs / Incubated Eggs) × 100. Hatchability rate (%) = (Hatched Eggs / Fertile Eggs) × 100.

Statistical Analyses

All data gathered were first tested for normality and homoscedasticity using Shapiro-Wilk's test and Levene's test, respectively. Data satisfying these assumptions were analyzed using Analysis of Variance (ANOVA), and Tukey's LSD was used for post hoc comparisons at 5% significance. Percentage data were arcsine-transformed and analyzed using STATA Version 15.

RESULTS AND DISCUSSION

The fertility and hatchability rates of the extended and cryopreserved semen of the Banaba native chicken are presented in Table 1.

Table1. Fertility and hatchability rates of extended and cryopreserved semen of Banaba native chicken (n=4).

Parameter	Extended semen		Cryopreserved semen
	LRS	SE	SEG
Fertility, %	60.04 ± 1.83 ^a	51.26±4.15 ^a	25.56±3.22 ^b
Hatchability, %	56.94±2.62 ^a	62.02±6.34 ^a	49.31±2.86 ^a

^{a,b} Means in the same row with different superscripts differ significantly ($p < 0.05$).

The mean fertility rates values were 60.04 ± 1.83 and $51.26 \pm 4.15\%$ for semen extended with LRS and SE extenders, respectively. The LRS extended semen was similar ($p > 0.05$) to SE extended semen. There was a significant decrease ($P < 0.05$) detected in the percent fertility of hens inseminated with spermatozoa from SEG extender ($25.56 \pm 3.22\%$) compared to those of the fresh groups (LRS: 60.04 ± 1.83 and SE: 51.26 ± 4.15). The results in this present study are lower than those reported in the previous studies (Abouelezz *et al.*, 2017; Mohan *et al.*, 2018; Thelie *et al.*, 2018; Shanmugam *et al.*, 2018; Salehi *et al.*, 2020).

Thelie *et al.* (2018) reported $98.3 \pm 1.1\%$ fertility rate using fresh semen of chicken with inseminating depth of 4 cm and AI dose of 200×10^6 spermatozoa per female. Abouelezz *et al.* (2017) also recorded fertility rate of 87.1% in extended semen for six consecutive intravaginal AIs at hours between 13:00 to 15:00 with inseminating dose of 100×10^6 spermatozoa per female. Similarly, Shanmugam *et al.* (2018), in a fertility trial using freshly collected semen conducted on Nicobari hens resulted in 90.4 ± 3.40 fertility rate with AI dose of 200×10^6 spermatozoa per hen, five times at two days interval.

On the other hand, Baguio and Capitan (2008) and Salehi *et al.* (2020) recorded moderate fertility of 76.4 and 73.85% of extended fresh semen for Philippine native chicken and Ross broiler breeder chicken, respectively. The variation or similarity of this present study and the previous studies are attributable to dose of spermatozoa, depth of insemination, method of AI, frequency of insemination and poultry species which are important factors that affect the success of artificial insemination (Lofti *et al.*, 2017; Masuodi *et al.*, 2019).

The mean hatchability values were 56.94 ± 2.62 and $62.02 \pm 6.34\%$ for LRS and SE, respectively. The mean values were statistically similar among the extended semen samples. The hatching rate determined based on fertilized eggs has not been influenced by extender types of the extended semen demonstrating that spermatozoa quality can affect fertility rate without impacting on the developing process in hatching stage. The finding of this report is in agreement with the study of Masuodi *et al.* (2019) who found that sperm quality did not have any influence on hatching rate. They opined that avian

species, incubator temperature, humidity, and eggshell's quality are some of the external factors which could affect the hatching rate.

The cryopreserved semen mean values were $25.56 \pm 3.22\%$ and $49.31 \pm 2.86\%$ for fertility and hatchability, respectively. The results in this report are higher than the results of Baguio and Capitan (2008), Seigneurin *et al.* (2013), Roushdy *et al.* (2014), Abouelezz *et al.* (2015), Shanmugam *et al.* (2018). Baguio and Capitan (2008) recorded fertility of 1.4% in cryopreserved semen containing DMSO from Philippine native chicken. Fertility rate of zero % for 11% glycerol, and 10 % EG, respectively in guinea fowl were reported when four different freezing methods were compared (Seigneurin *et al.*, 2013). Roushd *et al.* (2014) recorded zero percent fertility rate with frozen-thawed spermatozoa of Dokki-4 strain where glycerol and DMA were the permeating cryoprotectants. Again, Abouelezz *et al.* (2015) reported fertility values of 2.1 and 4.2% for straws and pellets, respectively with 11% glycerol concentration while Shanmugam *et al.* (2018) recorded $1.9 \pm 1.3\%$ fertility rate of cryopreserved spermatozoa with DMSO in Nicobari chicken. However, the findings in this present study are lower than those of Mehdipour *et al.* (2018), Masoudi *et al.* (2019) and Salehi *et al.* (2020). Hatchability rate of $49.31 \pm 2.86\%$ observed from frozen-thawed spermatozoa in the extender that contained 3% glycerol in this current study, is similar to the finding of Lofti *et al.* (2017), who reported an average hatching rate of 50% from frozen-thawed spermatozoa in a diluent containing 1- and 2-mM hyaluronic acid while Mehdipour *et al.* (2018) reported hatching rate of 48.11% by using Beltsville extender augmented with whole egg yolk.

In contrast, Ansari *et al.* (2017), Mehdipour *et al.* (2018) and Salehi *et al.* (2020) reported higher hatching rates than those in this present study. Ansari *et al.* (2017), recorded hatchability rates ranging from 73.54 ± 1.39 to $84.06 \pm 1.11\%$ using modified BPSE extender with oral administration of D-aspartic acid to the experimental chickens. Similarly, Mehdipour *et al.* (2018) reported hatching rates of 74.07 and 83.33% for Beltsville extender supplemented with soybean lecithin 1%, and egg yolk plasma 20%, respectively.

Furthermore, Salehi *et al.* (2020) recorded 82 and 79% hatchability rates for Lakes' and Beltsville extenders, respectively with 3% glycerol inclusion rate.

The results showed that cryopreserved semen had statistically ($p < 0.05$) lower value for fertility compared with fresh extended semen. This finding is consistent with the findings of Abouelezz *et al.* (2017), Thelie *et al.* (2018), Shanmugam *et al.* (2018) and Salehi *et al.* (2020) who all reported inferior fertility of cryopreserved semen to that of fresh semen irrespective of diluent and cryoprotectant used. The reduced hatchability rate recorded in this study also agrees with the results of Salehi *et al.* (2020) whose cryopreserved semen showed significant reduction in hatchability when compared with that of fresh semen group. These results are not surprising because, it is well documented that cryopreservation has detrimental effects on the spermatozoa during the cryopreservation process. It is generally proven that fertility of frozen-thawed poultry semen is dramatically lower as compared to that of fresh semen (Çiftci and Aygün, 2018). This is because avian spermatozoa have a unique morphological and physiological features and membrane fluidity different from mammalian sperm which are susceptible to damage during freezing/thawing. The sperm heads of poultry have less cytoplasmic volume and a relatively long tail hence less transfer of cryoprotectants into the sperm cells for protection resulting in damage to the these long-tailed sperm cells during freezing/thawing. According to Çiftci and Aygün (2018), the ultrastructure of avian sperm cells are damaged, particularly the midpiece, mitochondria, and perforatorium/axial body during cryopreservation and thawing process. The sperm plasma membrane is made up of lipids, cholesterol, and proteins. The cholesterol in the plasma membrane plays a key role of maintaining membrane stability resulting in the regulation of many membrane properties and functions including fluidity and permeability to water and other lifesaving molecules. Also, plasma membrane with lower cholesterol concentrations and lower fluidity is associated with lower freezability tolerance of sperm cells in poultry. The membrane phospholipid content and fluidity of sperm cells also determine the tolerance level of sperm cells to cryopreservation and fluidity is linked to membrane cholesterol/phospholipids ratio (Çiftci and Aygün, 2018) and is a signal of spermatozoa freezability (Blesbois *et al.*, 2005).

Again, cryopreservation causes a significant reduction in the fertility of avian semen (Salehi *et al.*, 2020) due to high polyunsaturated fatty acids in the plasma membrane which exposes avian sperm to cold shock (Santiago-Moreno *et al.*, 2012) and lipid peroxidation (LPO) in the presence of reactive oxygen species (ROS) in the freezing medium (Çiftci and Aygün, 2018). As ROS increases with cryopreservation, the ROS causes damage to the sperm

plasma membrane resulting in sperm death during the freezing/thawing process.

According to Abouelezz *et al.* (2017), depending on the cryopreservation technique used, 30% to 67% of sperm die during the cryopreservation process. This was attributed to the mechanical damage that happened during the process of decreasing glycerol concentrations which was considered more injurious to rooster sperm fertilizing ability than toxicity of DMA and the freeze/thaw process.

Apart from cryopreservation effects on sperm quality, other factors are equally important for a successful cryopreservation and a subsequent success in AI (Çiftci and Aygün, 2018). According to Buss (1993), sperm cryopreservation success is dependent on: selection of proper extender; selection of the best cryoprotectant; determination of optimum freezing and thawing rates for maintenance of fertilization potential; and elimination of any ingredients harmful to fertility before insemination. Çiftci and Aygün (2018) stated that, the choice of cryoprotectant and its use during the process is a vital step in a successful poultry semen cryopreservation. It is generally accepted that glycerol is the least toxic cryoprotectant but with a corresponding contraceptive effect (Blesbois and Brillard, 2007). The contraceptive effect is attributable to a decreased acrosome reaction of sperm as sperm cells undergo acrosome reaction instantly after the supplementation of cryoprotectant (Çiftci and Aygün, 2018) and removal of the glycerol is recommended. Abouelezz *et al.* (2017) reported a negative effect of adding cryoprotectants to fresh semen on the fertilizing ability of the spermatozoa. In their earlier report, increasing glycerol concentration to 4% or more, resulted to nearly no fertilized eggs (Abouelezz *et al.*, 2015). Seigneurin *et al.* (2013) recorded no fertile eggs for glycerol and EG and it was stated that diluent and cryoprotectant influence fertilizing potential of spermatozoa. They further concluded that, fast freezing/thawing rates, and zootechnical adaptations of the conditions of AI led to consistent fertility rates of 65 to 70%. Shahverdi *et al.* (2015) stated that, egg yolk is effective and efficient against cold shock during freezing and thawing, but its contraceptive effects within the genital tract of the hen affects fertility rate.

Furthermore, Bagoio and Capitan (2008) stated that, the general reduction in fertility ability of frozen-thawed spermatozoa is a cumulative negative effect of processing, freezing, and thawing of semen samples used for artificial insemination. Processing and preservation of avian semen, which include semen dilution, cooling, freezing and thawing procedures have been observed to cause significant reduction in the fertility capacity of preserved semen (Bagoio and Capitan, 2008; Abouelezz *et al.*, 2017).

The low fertility and hatchability of frozen thawed spermatozoa observed in this present study could be partly as a result of the decline in viability and motility of the sperm. This is because the reduction in viability and motility of the cryopreserved semen were low compared to other studies may not have contributed significantly to these poor outcomes. The reasonable high sperm survival and motility may be due to synergistic protective effect of glycerol and egg yolk (Silva *et al.*, 2012) during chilling and freezing on one hand, while exerting their combined contraceptive effect on fertility (Shahverdi *et al.*, 2015; Çiftci and Aygün, 2018) on the other hand. Though removal of glycerol and egg yolk is recommended in cryopreserved semen (Buss, 1993; Çiftci and Aygün, 2018; Thelie *et al.*, 2018), the elimination of these materials in first trial of this experiment yielded no better fertility probably due to mechanical damage to the sperm cells during centrifugation.

The low fertility recorded in this experiment may be attributed to insemination dose/number of spermatozoa inseminated, depth of insemination, physical properties of the extender, method of AI, environmental conditions, zootechnical conditions, frequency of insemination, and poultry breed/strain (Shahverdi *et al.*, 2015; Lofti *et al.*, 2017; Masuodi *et al.*, 2019) as these are important factors affecting the success of artificial insemination. Thelie *et al.* (2018) reported a significantly lower fertility rate for inseminating dose of 100×10^6 spermatozoa/female compared with that of 200×10^6 spermatozoa/female. It was recommended in their study that AI dose of 200×10^6

spermatozoa/female should be used in cryopreserved semen for optimum fertility. Seigneurin *et al.* (2013) also found that freezing rate, thawing rate, cryoprotective agents and age had significant effects on fertility rate in guinea fowls. The study of Roushdy *et al.* (2014) reported that strain/breed of poultry, cryoprotectants and extender type significantly influenced the fertility rate of two local Egyptian chicken strains.

Semen processing, freezing, and thawing which are known to be associated with sperm structural, cellular, and molecular changes/damages and chemical alteration (Baguio & Capitan, 2008; Ansari *et al.*, 2017), which can lead to low sperm motility and death of spermatozoa may be implicated in this low fertility performance of the cryopreserved semen.

Notwithstanding the low fertility rate achieved with cryopreserved semen in this experiment, the fertile eggs produced showed the potential of freezing of Philippine native chicken semen. Moreover, normal chicks were hatched from some of the fertile eggs produced by cryopreserved semen. The number of chicks hatched from each treatment were 47, 43, 19 for LRS, SE and SEG, respectively and pictures of the hatched chicks are shown in Plate 1. This seems to be first time under Philippine environmental conditions of producing fertile eggs and hatching of normal chicks from cryopreserved semen of chickens using natural extender.

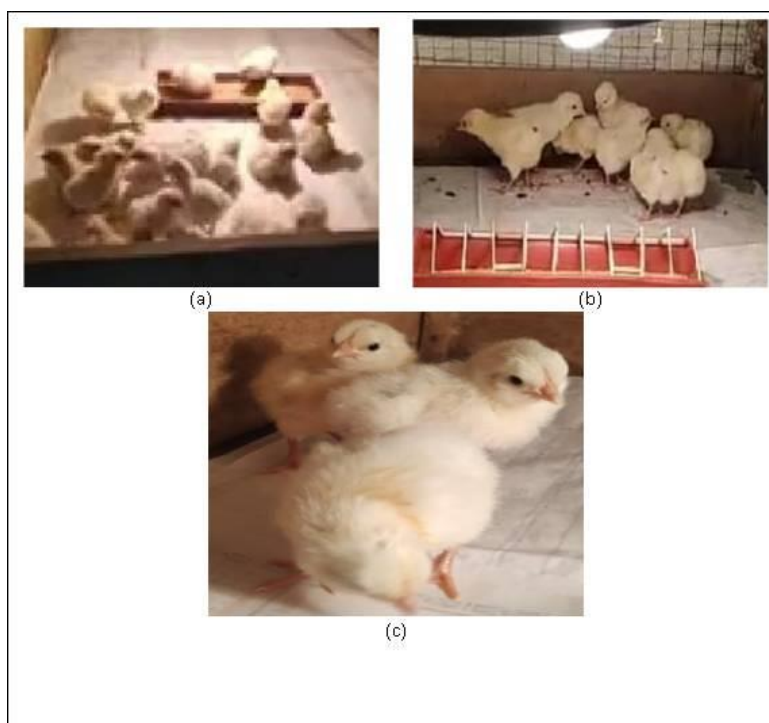


Plate 1. Chicks hatched using (a) LRS extended semen, (b) SE extended semen, and (c) cryopreserved semen.

CONCLUSION

The cryopreserved semen from Banaba native chicken using sugarcane extract with 3% glycerol successfully produced fertile eggs and viable chicks. The extender showed no adverse effects on fertility or hatchability, indicating its potential as a promising medium for avian semen cryopreservation. It can therefore be concluded that, SE and SEG can be used for poultry propagation, breeding and conservation. However, SEG protocol needs to be optimized to enhance poultry semen preservation and contribute to ecological poultry farming.

REFERENCE

Abouelezz, F. M. K., Castaño, C., Toledano-Díaz, A., Estes, M. C., López-Sebastián, A., Campo, J. L., & Santiago-Moreno, J. (2015). Effect of the interaction between cryoprotectant concentration and cryopreservation method on frozen/thawed chicken sperm variables. *Reproduction in domestic animals*, 50(1), 135-141. DOI: <https://doi.org/10.1111/rda.12464>

Abouelezz, F. M. K., Sayed, M. A. M., & Santiago-Moreno, J. (2017). Fertility disturbances of dimethylacetamide and glycerol in rooster sperm diluents: Discrimination among effects produced pre and post freezing-thawing process. *Animal reproduction science*, 184, 228-234. DOI: <https://doi.org/10.1016/j.anireprosci.2017.07.021>

Alebachew, H., Dedecha, D., & Tadesse, Y. (2017). Utilization, collection and conservation of animal genetics resources in relation to indigenous breeds. *World*, 1(1), 15-21. DOI: <https://10.11648/j.wjfst.20170101.13>

Ansari, M., Zhandi, M., Kohram, H., Zaghari, M., Sadeghi, M., & Sharafi, M. (2017). Improvement of post-thawed sperm quality and fertility of Arian rooster by oral administration of d-aspartic acid. *Theriogenology*, 92, 69-74. DOI: <https://doi.org/10.1016/j.theriogenology.2017.01.014>

Baguio, S. S., & Capitan, S. S. (2008). Motility, livability and fertility of cock spermatozoa as influenced by day of collection, dilution and cryopreservation. *Philippine Journal of Veterinary Medicine*, 45, 109-117.

Blesbois, E., & Brillard, J. P. (2007). Specific features of in vivo and in vitro sperm storage in birds. *Animal*, 1(10), 1472-1481. DOI: <https://doi.org/10.1017/S175173110700081X>

Blesbois, E., Grasseau, I., & Seigneurin, F. (2005). Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. *Reproduction*, 129(3), 371-378. DOI: [10.1530/rep.1.00454](https://doi.org/10.1530/rep.1.00454)

Acknowledgement

This work was supported by the DA-BAR (Department of Agriculture–Bureau of Agricultural Research) through the DA-BIOTECH program-funded project titled “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.

Burrows, W. H., & Quinn, J. P. (1937). The collection of spermatozoa from the domestic fowl and turkey. *Poultry science*, 16(1), 19-24. DOI: <https://doi.org/10.3382/ps.0160019>

Buss, E. G. (1993). Cryopreservation of rooster sperm. *Poultry science*, 72(5), 944-954. DOI: <https://doi.org/10.3382/ps.0720944>

Bustani, G. S., & Baiee, F. H. (2021). Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World*, 14(5), 1220. DOI: [10.14202/vetworld.2021.1220-1233](https://doi.org/10.14202/vetworld.2021.1220-1233)

Castro, C. H., Dichoso, G. A., Landicho, M. M., & Sangel, P. P. (2020). Honey or pineapple juice as extender components for Quezon native and Duroc boar semen at different storage temperatures. *Philippine Agricultural Scientist*, 103(4), 322-336.

Chinnadurai, C. (2017). Potential health benefits of sugarcane. *Sugarcane biotechnology: Challenges and prospects*, 1-12.

Çiftci, H. B., & Aygün, A. (2018). Poultry semen cryopreservation technologies. *World's Poultry Science Journal*, 74(4), 699-710. DOI: <https://doi.org/10.1017/S0043933918000673>

Daramola, J. O., Adekunle, E. O., Oke, O. E., Onagbesan, O. M., Oyewusi, I. K., & Oyewusi, J. A. (2016). Effects of coconut (*Cocos nucifera*) water with or without egg-yolk on viability of cryopreserved buck spermatozoa. *Animal Reproduction (AR)*, 13(2), 57-62. DOI: <https://doi.org/10.21451/1984-3143-AR724>

El-Sheshtawy, R. I., El-Badry, D. A., Gamal, A., El-Nattat, W. S., & Almaaty, A. M. A. (2016). Natural honey as a cryoprotectant to improve Arab stallion post-thawing sperm parameters. *Asian Pacific Journal of Reproduction*, 5(4), 331-334. DOI: <https://doi.org/10.1016/j.apjr.2016.06.004>

- El-Sheshtawy, R. I., El-Nattat, W. S., & Ali, G. A. D. (2017). Cryopreservation of cattle semen using coconut water extender with different glycerol concentrations. *Asian Pacific Journal of Reproduction*, 6(6), 279-282. DOI: <https://doi.org/10.4103/2305-0500.217343>
- Esguerra, J. P. M., Quimio, P. H., Undine, J. M., Dichoso, G. A., Junsay, C. A. L., Magpantay, V. A., & Sangel, P. P. (2020). Coconut Water with Either Tomato Juice or Garlic Extract as Extender Components for Paraoakan Native Chicken Semen at Different Storage Temperatures. *Philippine Journal of Science*, 149(1), 121-131.
- Islam, M. T. (2017). Sugarcane: Safety Concerns on its Cytogenotoxic Damage, While Hopes as a Source of Anticancer Agents. *Research Journal of Food & Nutrition*, 1(1), 10-12.
- Karima, M. R., Lawal, N. and Aderounmu, I. G. (2025). Assessments of Phytochemical and Nutritional Compositions of *Carica papaya* seeds *Citrullus lanatus* seeds, and *Syzygium aromaticum* buds. *Journal of Basics and Applied Sciences Research*, 3(3), 99-110. <https://dx.doi.org/10.4314/jobasr.v3i3.12>
- Lotfi, S., Mehri, M., Sharafi, M., & Masoudi, R. (2017). Hyaluronic acid improves frozen-thawed sperm quality and fertility potential in rooster. *Animal reproduction science*, 184, 204-210. DOI: <https://doi.org/10.1016/j.anireprosci.2017.07.018>
- Masoudi, R., Sharafi, M., & Pourazadi, L. (2019). Improvement of rooster semen quality using coenzyme Q10 during cooling storage in the Lake extender. *Cryobiology*, 88, 87-91. DOI: <https://doi.org/10.1016/j.cryobiol.2019.03.003>
- Mehdipour, M., Kia, H. D., Moghaddam, G., & Hamishehkar, H. (2018). Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. *Theriogenology*, 116, 89-94. DOI: <https://doi.org/10.1016/j.theriogenology.2018.05.013> Get rights and content
- Mittal, P. K., Madan, A. K., Sharma, V., Gottam, G. S., & Gupta, B. (2019). Cryopreservation of buffalo bull semen-restriction and expectation: A review. *International Journal of Current Microbiology and Applied Sciences*, 8(1), 1351-1368. DOI: <https://doi.org/10.20546/ijcmas.2019.801.144>
- Mohan, J., Sharma, S. K., Kolluri, G., & Dhama, K. (2018). History of artificial insemination in poultry, its components and significance. *World's Poultry Science Journal*, 74(3), 475-488. DOI: <https://doi.org/10.1017/S0043933918000430>
- Roushdy, K., El-Sherbieny, M. A., El-Gany, F., & El-Sayed, M. (2014). Semen cryopreservation for two local chicken strains as a tool for conservation of Egyptian local genetic resources. *Egyptian Poultry Science Journal*, 34(2), 607-618.
- Salehi, M., Mahdavi, A. H., Sharafi, M., & Shahverdi, A. (2020). Cryopreservation of rooster semen: Evidence for the epigenetic modifications of thawed sperm. *Theriogenology*, 142, 15-25. DOI: <https://doi.org/10.1016/j.theriogenology.2019.09.030>
- Santiago-Moreno, J., Bernal, B., Pérez-Cerezales, S., Castaño, C., Toledano-Díaz, A., Esteso, M. C., ... & Blesbois, E. (2019). Seminal plasma amino acid profile in different breeds of chicken: Role of seminal plasma on sperm cryoresistance. *PLoS one*, 14(1), e0209910. DOI: <https://doi.org/10.1371/journal.pone.0209910>
- Seigneurin, F., Grasseau, I., Chapuis, H., & Blesbois, E. (2013). An efficient method of guinea fowl sperm cryopreservation. *Poultry science*, 92(11), 2988-2996. DOI: <https://doi.org/10.3382/ps.2013-03166>
- Shahverdi, A., Sharafi, M., Gourabi, H., Yekta, A. A., Esmaili, V., Sharbatoghli, M., ... & Mostafayi, F. (2015). Fertility and flow cytometric evaluations of frozen-thawed rooster semen in cryopreservation medium containing low-density lipoprotein. *Theriogenology*, 83(1), 78-85. DOI: <https://doi.org/10.1016/j.theriogenology.2014.07.044>
- Shanmugam, M., Kumar, K. P., Mahapatra, R. K., & Laxmi, N. A. (2018). Effect of different cryoprotectants on post-thaw semen parameters and fertility in Nicobari chicken. *Indian Journal of Poultry Science* 53(2), 208-211. DOI: [10.5958/0974-8180.2018.00040.5](https://doi.org/10.5958/0974-8180.2018.00040.5)
- Silva, M. A., Peixoto, G. C. X., Lima, G. L., Bezerra, J. A. B., Campos, L. B., Paiva, A. L. C., ... & Silva, A. R. (2012). Cryopreservation of collared peccaries (*Tayassu tajacu*) semen using a powdered coconut water (ACP-116c) based extender plus various concentrations of egg yolk and glycerol. *Theriogenology*, 78(3), 605-611. DOI: <https://doi.org/10.1016/j.theriogenology.2012.03.006>
- Thananurak, P., Chuaychu-noo, N., & Vongpralub, T. (2017). Freezability and fertility of Thai native chicken semen in different diluents. *The Thai Journal of Veterinary Medicine*, 47(4), 551-556. DOI: <https://doi.org/10.56808/2985-1130.2869>

Thélie, A., Bailliard, A., Seigneurin, F., Zerjal, T., Tixier-Boichard, M., & Blesbois, E. (2019). Chicken semen cryopreservation and use for the restoration of rare genetic resources. *Poultry Science*, 98(1), 447-455. DOI: <https://doi.org/10.3382/ps/pey360>

Yodmingkwan, P., Guntaprom, S., Jaksamrit, J., & Lertchunhakiat, K. (2016). Effects of extenders on fresh and freezing semen of boer goat. *Agriculture and Agricultural Science Procedia*, 11, 125-130. DOI: <https://doi.org/10.1016/j.aaspro.2016.12.021>