GOBIERNO DE ESPAÑA MINISTERIO DE CIENCIA E INNOVACIÓN

CSIC Consejo Superior de Investigaciones Científicas

INIA Centro Nacional Instituto de Investigación y Tecnología Agraria y Alimentaria

CREATION OF A SPANISH POULTRY BREEDS CRYOBANK: OPTIMIZATION OF FREEZING PROCEDURE AND INFLUENCE OF ENDOGENOUS AND ENVIRONMENTAL FACTORS ON SPERM CRYORESISTANCE

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It is estimated that 30% of animal species including birds, mammals, reptiles, amphibians and fish will become extinct in the coming decades. In addition, the percentage of species classified as "Endangered" will be significantly increased.

There are currently around **1300 species of threatened birds** in the world.

The main causes of threat:
. Climate change
. Intensive farming
. Fragmentation and destruction of habitat
. Poaching
. Introduction of invasive species
. Indiscriminate use of agricultural pesticides
. Pollution
. Inbreeding
.Genetic hybridization

Threatened fowl (Gallus species):	**Causes of threat:**
<u>Wild fowl:</u> Indian red jungle fowl: *Gallus gallus murghi* Sri Lanka junglefowl: *Gallus lafayettii*	Habitat destruction, poaching, inbreeding and genetic hybridization
<u>Native domestic chicken breeds:</u> *Gallus gallus domesticus*	Intensive farming and commercial needs: use of lines of chickens with more profitability in egg and meat industry
	Restructuring of rural areas

* Half of the domestic poultry breeds are currently considered to be endangered. Most of them are native chicken breeds

The cryo-banking has great potential for application in *ex situ in vitro* conservation:

- Wild ancestor species: Scientific use as animal model on health and reproduction research activities (e.g. sperm from Indian red jungle fowl show a great resistance to the toxic/contraceptive effect of glycerol; it could be useful to study the glycerol effect on sperm and chicken's sperm storage tubules).
- Threatened breeds: Basic biological research into physiology, diet, reproduction or climatic tolerance at the physiological and genetic level.
- Native breeds with unique characteristics or traits: They could also help with the identification of specific genes involved in natural disease, parasite control, thermal stress.
- Pure lines with unique productive traits: Integration of their valuable genetic material into different production systems through artificial insemination.

Spermatozoa are the most accessible sex cells and are currently the main type preserved in the majority of genetic resource banks: non invasive and less expensive method available.

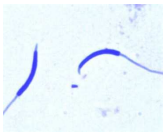
Sperm cryopreservation is the most feasible method in birds as cryopreservation of oocyte or embryo is not possible because of large size, high lipid content and polar organization.

Sperm banks allow to have precious germplasm for long period of time and/or to restore genetic diversity by mean artificial insemination.

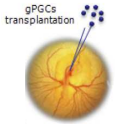
Alternative methodologies using primordial germ cells and ovarian tissue may be too invasive for at risk populations. Currently they are not sufficiently effective and are too costly for large programs of genetic conservation.

Cryopreservation of eggs cannot be used because
of the characteristics of the megalecithal egg

Germplasm and tissue banks: use to transfer genomes through insemination and transplantation.



Artificial insemination with frozen-thawed sperm



Transplantation of cryopreserved PGCs

Allo-transplantation of frozen-thawed or vitrified /warmed ovarian tissues

Chicken sperm cryopreservation – Scientific milestones:

1940 – Schaffner makes the first attempts to freeze rooster semen.

1949 – Polge et al. discover the cryoprotective action of glycerol for chicken sperm freezing.

1955 – Allen et al. confirmed the findings about contraceptive effect of glycerol after artificial insemination.

1960 – Lake designed a glutamate based extender taking into account the seminal plasma composition.

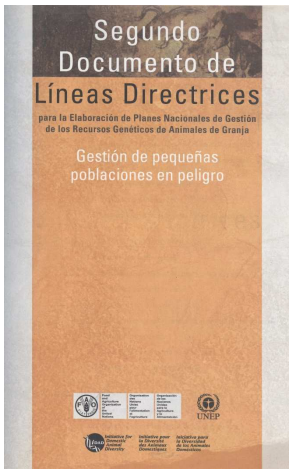
1968 – Lake used liquid nitrogen for successfully freezing chicken sperm.

1977 – Lake & Stewart improved the freezing procedure using a slow freezing rate, a reduction of temperature to -35 °C, before the semen is plunged into liquid nitrogen.

1999 – Tselutin et al. studied successful alternative cryoprotectants methods: DMA in pellets.

2005 – Blesbois et al. provided new insights about the role of sperm membrane fluidity and cholesterol content on the ability to survive cryopreservation.

2006 – Long et al. provided new insights about sperm bioenergetics and freezability.



FAO (Food and Agriculture Organization) and IDAD (Initiative for Domestic Animal Diversity) recommendations for sperm cryo-bank in an endangered breed of chicken:
(*Guidelines for conservation of **endangered small populations**. FAO, IDAD*)

Total number of cryopreserved doses: **6544**
Number of donors: **20 males** with proven fertility and proven sperm freezability
Number of doses/male: 327
Number of sampling/male: 66 (5 doses / sample)*
Duration: 154 days*(3 collections/week – 5 months for creation a cryobank)

* No realistic for most native breeds ("at least Spanish breeds"):

Total number of cryopreserved doses: **6544**
Number of donors: **20 males** with proven fertility and proven sperm freezability
Number of doses/male: 327
Number of sampling/male: 164 (2 doses /sample)
Duration: 383 days (3 collections/week – 13 months for creation a cryobank)

In Spain there are **17 threatened native breeds of chicken**

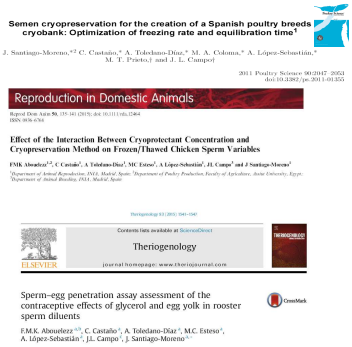
Different Spanish chicken breeds have been raised as part of a genetic resources conservation program since 1975.

Given the risks of epidemics such as avian influenza, the Spanish Ministry for Science and Innovation, via the Spanish National Institute for Agricultural and Food Research (INIA), supported the establishment of a germplasm bank in 2009 to guarantee the preservation of twelve threatened breeds.

INIA sperm cryobank is **very dynamic** due to frequent use of doses for experimental and conservation purposes. There is a constant update of cryopreserved doses through the latest biotechnological advances.

INIA's sperm cryobank **complements the *ex situ in vivo* conservation** program in the Avian Experimental Unit from INIA ("El Encín", Madrid) where unique populations of twelve Spanish chicken breeds are raised.

Spanish breeds of the conservation program are also useful **models for research activities** in reproductive and genetic biotechnologies, genetic and reproductive traits, animal welfare markers, free-range management, etc.



<u>Limits in the implementation of a chicken sperm cryobank</u>

Sperm frozen-thawed still shows very low and variable reproductive success rates following insemination with thawed semen which limits the use of gene banks.

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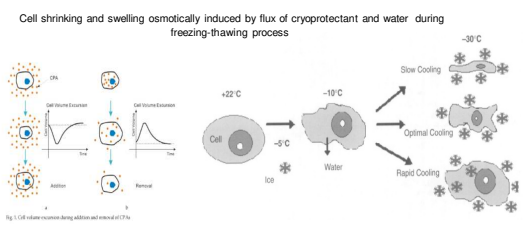
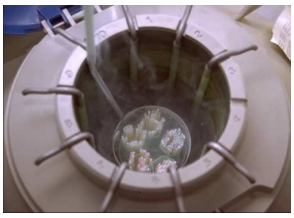
This is mainly due to the high sperm susceptibility to cryodamage during freezing-thawing process





Traits of rooster sperm determining high susceptibility to cryodamage

- Filiform with very low cytoplasmic content

- Very long flagellum (70-90 µm)

- Low sperm osmotolerance:
  Sperm are sensitive to anisosmotic events than occur during freezing-thawing process (extracellular hyperosmotic conditions and restoration of isotonicity).

- Very sensitive to oxidative stress:
  - Large number of mitochondria in the mid-piece (approximately 30) →
  → High energetic metabolism-oxidative phosphorylation: ↑ ↑ATP but also ↑ ↑ROS

- Rapid induction of acrosome reaction *in vitro*

- Susceptibility of the DNA to the cryoinjury: Sperm contains no cysteine residues and lacks the potential stabilizing effect of S-S bonds of mammalian sperm chromatin

Role of endogenous and environmental factors on rooster sperm cryoresistance

Endogenous factors:

Breed and individual: some breeds and individuals return the best freezing-thawing response unlike other.

Seminal plasma: The presence of seminal plasma may affect sperm response to freezing-thawing process, and thus removal of seminal plasma decreased the variability of the results and DNA fragmentation damages.

Environmental factors:

Photoperiod and temperature: these environmental factors have a strong influence on rooster housed under natural photoperiod and temperature conditions (e.g. native breeds in free-range).

Social interactions: roosters with or without female contact affect sperm quality variables.

Diet and housing systems.

Ectoparasites: the mites and lice causes stress by itching and skin irritation.

Role of endogenous and external factors on rooster sperm cryoresistance

Some breeds return the best freezing-thawing response unlike other. The pattern of certain amino acids of seminal plasma seems play a role on sperm cryoresistance and vary in a breed-specific way.

The presence of seminal plasma may affect sperm response to freezing-thawing process, and thus removal of seminal plasma decreased the variability of the results and DNA fragmentation damages.

PLOS ONE

RESEARCH ARTICLE

Seminal plasma amino acid profile in different breeds of chicken: Role of seminal plasma on sperm cryoresistance

Julián Santiago-Moreno[1]*, Berenice Bernal[1], Serafín Pérez-Cerezales[1], Cristina Castaño[1], Adolfo Toledano-Díaz[1], Milagros C. Esteso[1], Alfonso Gutiérrez-Adán[1], Antonio López-Sebastián[1], María G. Gil[2], Henri Woelders[3], Elisabeth Blesbois[4]

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Influence of Season on the Freezability of Free-Range Poultry Semen

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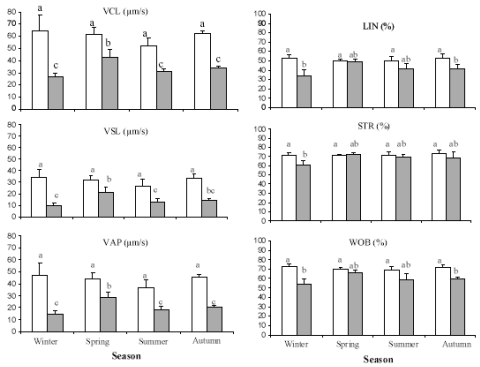

Fig. 3. Sperm movement characteristics (curvilinear velocity: VCL; straight-line velocity: VSL; average path velocity: VAP; linearity: LIN; straightness: STR; Wobble: WOB) in fresh (open bars) and frozen–thawed spermatozoa (grey solid bars) (means ± SEM). Different letters within and between seasons indicate significant differences ($p < 0.05$)

The percentage of spermatozoa showing progressive motility was higher in spring-collected sperm compared to winter-, summer- or autumn-collected samples. The curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) values of spring-collected sperm were also higher. In conclusion, spring would appear to be the best season for collecting and freezing the semen of free-range Mediterranean chicken breeds.

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Effect of the presence of hens on roosters sperm variables

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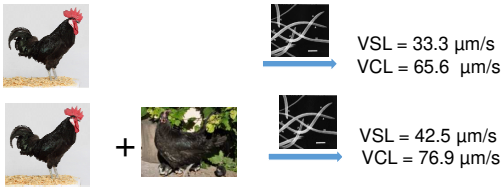
Sperm cryoresistance is usually related to the quality of the fresh sperm before freezing – which can vary according to social interactions. Compared to the no-female-contact group, the roosters living with hens showed significantly (P < 0.05) reduced percentages of non-progressive motile sperm and slow sperm, and significantly increased straight-line velocity (VSL) and curvilinear velocity (VCL) values.



2018 Poultry Science 97:4433–4441
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Access to pasture in an outdoor housing system affects welfare indicators and improves rooster sperm quality in two native Mediterranean breeds

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Outdoor housing system with access to pasture improved the motility of fresh sperm in roosters, but had no overall effect on sperm cryoresistance.

However, semen of the Red-barred Vasca breed with access to pasture did show a higher percentage of progressive motility (P<0.05), after freezing-thawing, than did that of the birds of the same breed with no such access.

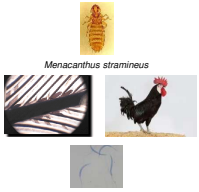
In conclusion, access to pasture improves fresh sperm motility. Although overall offered no cryoprotective benefits, in the Red-barred Vasca birds the access to pasture appears to have a certain protective role during freezing-thawing process.

AIDA 2015 XVI Meeting on Animal Production Vol. 2, pp: 405-407

RELATIONSHIP BETWEEN PLASMATIC TESTOSTERONE AND SPERM QUALITY WITH PARASITISM LEVELS BY LICE *(MENACANTHUS STRAMINEUS)* IN AUTHOCTONOUS SPANISH CHICKEN BREEDS

ABSTRACT Parasitism alters sperm quality by inducing stress processes or immunological disorders, which make affect spermatogenesis. In order to evaluate the hypothesis about the immunosuppressive effect of gonadal steroids (males with high testosterone levels would have increased Parasitism susceptibility), our study was conducted assessing testosterone plasmatic concentration and sperm quality in three Spanish chicken (Blue Andaluza, White-faced Spanish, Black Castellana) with different parasitism levels by *Menacanthus stramineus*. In our results, significant differences in sperm quality ($P < 0.01$) and plasmatic testosterone concentrations ($P < 0.05$) between breeds were observed. Negative correlation between parasitism levels and sperm quality was found ($P < 0.001$). In conclusion, high parasitism level by lice determines a reproductive cost in males, manifested in decrease in semen quality.

Keywords: Spanish chicken breeds, *Menacanthus stramineus*, sperm quality, plasmatic testosterone



Parasites cause stress (itching and skin irritation) and impose costs on sperm production – decreases semen volume and sperm concentration. It is suggested that the decline in the fresh sperm quality could affect sperm cryosurvival after freezing – thawing process.

Limits in the implementation of a sperm cryobank

Low reproductive success rates following insemination with frozen-thawed semen.

Only the male genome (Z chromosome) is conserved because in birds the female is the heterogametic sex.

IMAGE
Innovative Management of Animal Genetic Resources

To develop and implement methods and tools to improve the sperm cryoconservation is a priority: i) develop new combinations of cryoprotectants in the chicken ; ii) develop new additive supplies in order to better protect the plasma membrane and cellular compartments of sperm and to get higher and less variable results of fertilizing ability

IMAGE project has explored species specific mechanisms, to develop species specific methodologies and/or alternative methods notably for cryopreservation of female reproductive material (ovarian tissue).

In IMAGE project, alternatives to gametes, primordial germ cells, were considered for cryobanking, as they may provide alternative means to store the genome from both parents.

PLOS ONE

RESEARCH ARTICLE

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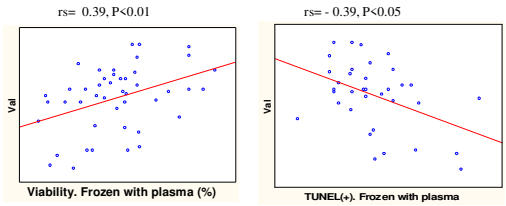

Correlation between sperm viability (percentage of sperm with intact membrane) and valine in seminal plasma of roosters (left). Correlation between sperm TUNEL + (percentage of sperm with DNA fragmentation) and valine in seminal plasma of roosters. rs, Spearman rank correlation coefficient.

Results suggest that the presence of certain aminoacids (e.g. valine) protect chicken sperm membrane and DNA to cryoinjury.

Effect of supplementation of valine to chicken extender on sperm cryoresistance and post-thaw fertilization capacity

B. Bernal,[*,†] N. Iglesias-Cabeza,[*] U. Sánchez-Rivera,[†] A. Toledano-Díaz,[*] C. Castaño,[*] S. Pérez-Cerezales,[*] A. Gutiérrez-Adán,[*] A. López-Sebastián,[*] P. García-Casado,[‡] M. G. Gil,[§] H. Woelders,[#] E. Blesbois,[‖] and J. Santiago-Moreno[*,1]

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Red Villafranquina
Lowest sperm DNA integrity after cryopreservation

20 roosters

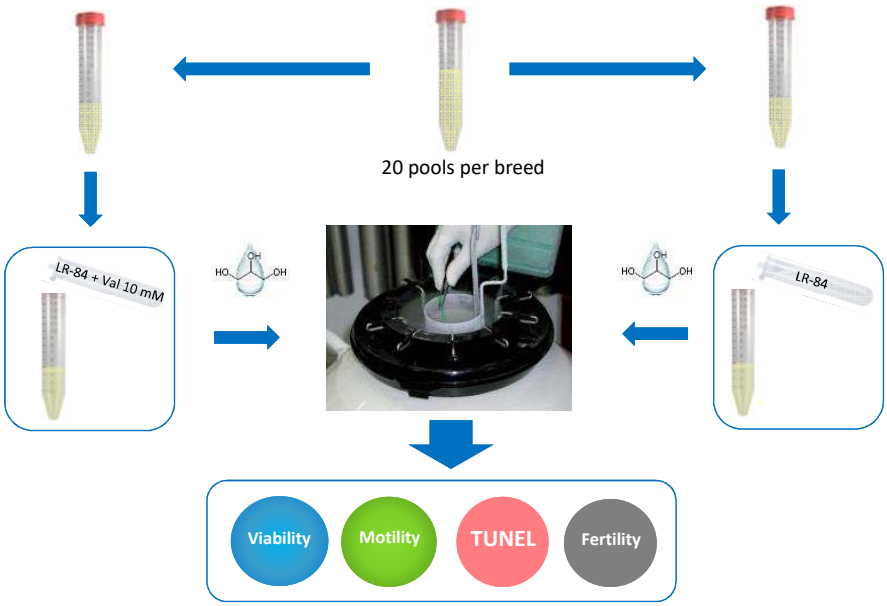
Pool

Quail Castellana
Highest sperm DNA integrity after cryopreservation

20 roosters

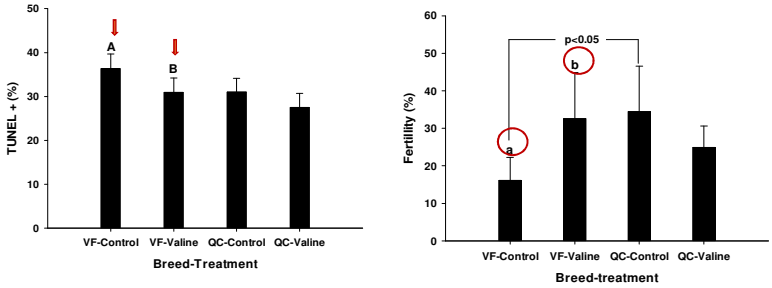
Pool

Experimental design



Effect of supplementation of valine to chicken extender on sperm cryoresistance

Valine effect on DNA integrity and fertilization capacity of frozen-thawed semen



- Valine addition increased the DNA integrity of cryopreserved semen (decreased post-thaw DNA fragmentation) in both breeds, with a significant effect in VF.
- Quail Castellana cryopreserved sperm control showed higher fertility rate than VF cryopreserved sperm control.
- Supplementing valine to the freezing extender doubled the fertility rate of VF compared with the control.

Conclusions:

- Supplementation of valine to chicken extenders can have a positive effect on DNA fragmentation and fertility rates of frozen-thawed sperm.
- This effect is breed-specific: valine had a positive effect on Red Villafranquina sperm, reducing DNA fragmentation and doubling the fertility rate; while, for the case of Quail Castellana, it did not affect DNA cryoresistance or fertility rate.
- Supplementation of valine to chicken freezing extenders shows a positive effect on DNA fragmentation and fertilizing ability of frozen–thawed sperm, with a better response in a breed considered as the lowest freezer in our conservatory.

MAIN PROTOCOL OF INDIVIDUAL CHICKEN SEMEN CRYOPRESERVATION

IMAGE
Innovative Management of Animal Genetic Resources

1. Collect individual semen of each male in plastic tubes containing 200 µl LPC diluent at 20-25 °C. LPC: Magnesium acetate (0.7 g), Sodium glutamate (5.0 gr), Potassium acetate (5.0 g), D-Fructose (8.0 g), BES (1 g), Polyvinylpirrolidone (10000: 3.0 g), NaOH 1N (4 mL). Diluent pH 7.1, Osmotic pressure 340 mOsm. **In case of semen with low expected fertility, add 10mM Valine**. This is the dilution 1.

2. Add LPC diluent at 20-25°C to each collected semen in order to reach a final dilution 1:1 (dilution 2) and then put the different diluted semen in a fridge/cold room at 4-5°C for 10min.

3. Mix each sample with a volume of LPC (at 4-5°C) diluent equivalent to two initial semen volume containing 22% glycerol (Dilution 3). So, the final semen dilution will be 1:3, and the **final glycerol amount 11%.**

4. **Equilibrate 10 m**in at 4-5°C with gentle shaking

5. Put the diluted semen in 0.5 mL straws previously identified per male, make a bubble at the top of the straws, seal the straws, transfer them in a programmable freezer. Freezing**: -7°C/ min from + 4°C to -35°C, then – 60°C/ min from – 35°C to 140°C**. Transfer very rapidly the straws to liquid nitrogen tank of storage.

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Massage technique described by Burrows and Quinn (1937) modified: Semen is aspirated by means of a device with tubes to avoid faecal and transparent fluid contamination.

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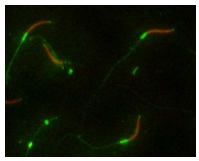
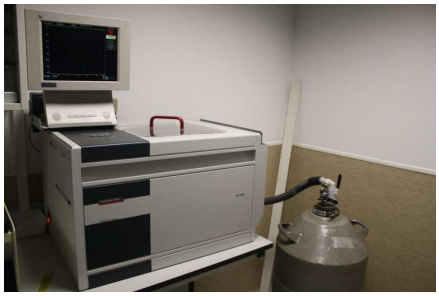
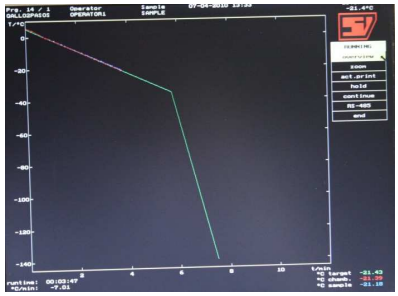


Two-step freezing method is associated with a higher percentage of motile spermatozoa after thawing, and with greater acrosome integrity ($P<0.05$), than the slow nitrogen vapour or rapid one-step methods: 10 min equilibration time. Two-step freezing : from 5°C to -35°C at 7°C/min, and then from -35°C to -140°C/min at 60°C/min (biological freezer unit) .





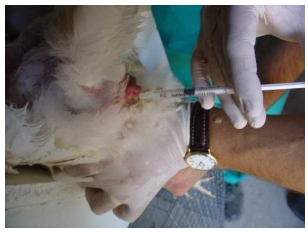
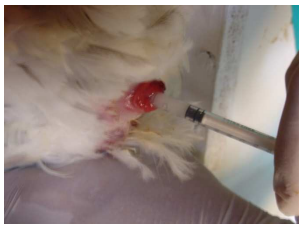
Thawing and insemination

1. Remove straws from liquid nitrogen. Plunge the straws for 3 min in water at 4°C. All the steps of thawing are at 4°C. Avoid any thermic shock.

2. Removing of the intracellular glycerol by serials dilutions in LC diluent (magnesium acetate tetrahydrated, potassium citrate monohydrate, sodium glutamate, D-fructose, sodium acetate, TES, NaOH 1N), each separated of 2 min. Centrifuge each sample for 15 min at 500g, 4°-5°C. Discard the supernatant (that now contain the glycerol) and replace it by an insemination diluent (e.g. L7.1, Lake and Ravie 1981).

3. Intra-vaginal insemination of a mean of 200 to 400 million sperm/female and with high caution since frozen-thawed semen is more sensitive to all variations than fresh semen and since the receptivity of the female is a key factor of success. The success also depends on the female fertility level. The insemination must be conducted in minimum 3 hours before or 3 hours after the daily lay in order to avoid opposite vaginal peristalsis.

4. Intra-vaginal insemination of a mean of 200 to 400 million sperm/female and with high caution since frozen-thawed semen is more sensitive to all variations than fresh semen and since the receptivity of the female is a key factor of success. The success also depends on the female fertility level. The insemination must be conducted in minimum 3 hours before or 3 hours after the daily lay in order to avoid opposite vaginal peristalsis.


Fertility of frozen-thawed semen using glycerol: influence of breed fertility level

	Mean Fertility Rate
Heavy meat type I99	53% (*Thelie et al., 2018, Poult. Sci.)*
Spanish native breeds	29% (*Abouelezz et al., 2015; Reprod Dom Anim*)

ALTERNATIVE PROTOCOL: The second recommended method of chicken semen cryopreservation is as simply as possible. It does not need a programmable freezer, and the internal cryoprotectant (DimethylFormamide, DMF) is not removed at thawing (Thananurak et al., 2020). This method is efficient for **highly fertile males**.

1. Collect with precaution individual semen of each male (Burrows and Quinn, 1937) at room temperature (mean 25°C).

2. At room temperature, dilute 1:2 (v:v) very rapidly the semen with BHSV-based diluent (5g glucose, 2.5g Inositol, 28.5g sodium glutamate, 0.7g magnesium acetate tetrahydrate, 5g potassium acetate, all of which were dissolved in 1,000 ml of double-distilled water, Schramm, 1991) supplemented with Serine 4mM and Sucrose 1mM (Thananurak et al., 2019; 2020).

3. Cool slowly the semen at 5°C by placing it in fridge with controlled temperature. When the semen reach 5°C, add another fraction of diluent prepared at 5°C (vol1, equivalent to initial semen volume 1) containing DMF (24%). The final semen dilution is 1:3 (v:v) and the final percentage of DMF in the diluted semen is 6%.

4. Load the diluted semen in 0.5 mL plastic straws sealed with PVP powders and equilibrate for 15 min at 5°C.

5. After equilibration, the filled straws are laid horizontally on a rack, 11 cm above the surface of LN2 (-35°C) for 12 min, then, placed 3 cm above liquid nitrogen vapor (-135°C) for 5 min, and subsequently immersed in LN2. Transfer the straws to a liquid nitrogen tank.

6. Thawing: Straws are thawed in a water bath at 5°C for 5 min. Intravaginal inseminations are then conducted rapidly with the same precautions as for the glycerol method.

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THANKS FOR YOUR ATTENTION

IMAGE Innovative Management of Animal Genetic Resources

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