# UNIVERSITÀ DEGLI STUDI DEL MOLISE

**Department of Agricultural, Environmental and Food Sciences**

International Ph.D Course in:

**AGRICULTURE TECHNOLOGY AND BIOTECHNOLOGY**

(CURRICULUM: Welfare, biotechnology and quality of animal production)

**(CYCLE XXX)**

Related disciplinary scientific section: AGR/20 (Zoocolture)

PhD thesis

# Semen cryopreservation as a tool for preserving genetic biodiversity of avian and rabbit species: national cryobank launch

**Coordinator of the Ph.D Course:** Prof. Giuseppe Maiorano

**Supervisor:**
Prof. Nicolaia Iaffaldano

**Ph.D candidate:**
Marsia Miranda-153768

ACADEMIC YEAR 2016/2017

# INTRODUCTION

The conservation of genetic diversity in livestock species can be related to biological, social, cultural and economic aspects. Animal genetic resources and animal management systems are an integral part of ecosystems and productive landscape in Italy, Europe and all over the world. Local breeds can be considered as a part of the history of some human populations as well as important materials from a scientific point of view. To date there is an urgent need of a specific conservation program for these breeds because of the limited number of their individuals and their risk of extinction. Although the local breeds are usually not competitive for production traits, they may carry valuable features such as disease resistance or distinctive product quality. The higher economic value of typical productions compared to conventional commercial products and the growing consumer preferences towards food quality, could strongly support the need to launch conservation programs of livestock biodiversity. In this regard, efficient *in situ* and *ex situ* conservation strategies are obligatory tools in order to implement an appropriate action for the conservation of livestock biodiversity. Conservation of genetic diversity across and within farm animal breeds is an insurance for the future. A broad genetic base is crucial to deal with future changes in environment, consumers demand for animal products and animal production systems. A wide genetic variation is also a safeguard against sanitary outbreak and natural disasters, in the maintenance of agro-ecosystem diversity and conservation of rural cultural diversity. The conservation of livestock variability is also a crucial element in order to preserve and valorize specific nutritional and nutraceutical properties of animal products.

*In situ* conservation strategies are effective in reaching all these objectives except for the safeguard against emerging diseases, political instability and natural disaster. In contrast, *ex situ* conservation strategy is the method of choice to safeguard farm Animal Genetic Resources (AnGRs) against natural calamities and it is a valuable option when socio-economic, cultural and ecological values linked to a breed, are missing or of no interest.

Therefore, the field of *ex situ* conservation is getting more attention with its wider application in the field of AnGRs management and conservation. To this aim, the cryopreservation technique is commonly used in the storage of genetic resources including oocytes, embryos, somatic cells, primordial germ cells (PGCs) and sperm. This technique offers important opportunities for the conservation and utilization of farm animal genetic

resources by the creation of a cryobank supported by government, institutional breeding organizations and private breeders.

Some European countries such as France, The Netherlands and others, outside of Europe such as America and Japan, have been involved in specific conservation programs for AnGRs and include poultry (e.g. chicken and turkey) and rabbit breeds.

Currently also in Italy there is an urgent need of national actions aiming at specific conservation programs for Italian poultry and rabbit breeds because of the limited number of their individuals becoming in risk of extinction. In the light of this, conservation and valorization projects of Italian poultry and rabbit breeds have been developing thanks to the financial support of regional and local public institutions. These projects are only based on the *in situ* conservation strategy. While recognizing that *in situ* strategy is a priority, the ideal conservation plan should consist in the integration of both strategies, *in situ* and *ex situ* (FAO, 2008; FAO, 2012). Regarding the *ex situ in vitro* technique, the semen cryopreservation represents a valuable tool to safeguard the genetic animal resources by cryobanks. This technology becomes particularly precious in poultry species for the reason that the semen cryopreservation in this species is the only reproductive procedure currently available because of the unique biological features of birds and in rabbit because cryopreserving sperm is easier and cheaper than obtaining embryos (Mocè and Vicente, 2009).

In this regard, the present doctoral thesis aims at the identification of reference procedures for the cryopreservation of poultry (turkey and chicken) and rabbit semen according to the "FAO guidelines - *Cryoconservation of animal genetic resources*" (2012).

The reference procedures will be implemented for the constitution of a sperm cryobank of Italian, turkey, chicken and rabbit breeds, that are not available at national level right now.

The following specific aims of the present doctoral thesis are reported in short below:

1. improvement of the cryopreservation success of both turkey (study 1) and chicken (study 2) semen packaged into straws;
2. development of effective protocol for rabbit semen (study 3) in order to reduce cryoinjuries during the freezing procedure and improve the sperm integrity after freezing/thawing

*To my family*

# AKNOWLEDGEMENT

The realization of this thesis was only possible due to the several people's collaboration, to which I desire to express my gratefulness.

I would like to express my deepest gratitude to my supervisor, Prof. Nicolaia Iaffaldano for her guidance, patience, motivation, and support.

I would like to express my gratitude to Dr. Michele Di Iorio for his help, encouragement, teaching and advice.

I would like to thank also my Slovak advisor Prof. Peter Chrenek for giving me the possibility to work in his laboratory, at the Animal Production Research Centre (NPPC Nitra) and his collaborators Dr. Barbora Kulíková, Dr. VašíčekYaromir and Dr. Andrej Baláži.

I would like to thank the General Coordinator Prof. Giuseppe Maiorano for his accurate organization of the Ph.D. course.

Many thanks to the president Dr. Michele Schiavitto of Italian National Rabbit Breeders (ANCI-AIA) for allowing me to use the Central Breeding Farm. I also thank Drs. Paola Principe for the technical assistance with semen collection and artificial insemination.

I would like to thank also Prof. Luca Romagnoli for help with the statistical analysis of data, and Amber Burchell and Francesca Di Nezza for the English revision.

Moreover, I would like also to thank all my PhD colleagues.

I also thank all my friends, who supported me during these years.

Finally, but not the least, I would like to thank my family, for their love, constant support and encouragement.

# EVALUATION REPORT OF DOCTORAL THESIS

Title of the thesis

| |
|---|
| **Semen cryopreservation as a tool for preserving genetic biodiversity of avian and rabbit species: national cryobank launch** |

Name of the PhD candidate

| |
|---|
| Marsia Miranda |

Name and affiliation of the thesis supervisor

| |
|---|
| *NICOLAIA IAFFALDANO – University of Molise* |

Name and affiliation of the referee

| |
|---|
| *PETER MASSANYI – Slovak University of Agriculture in Nitra, Slovak Republic* |

Evaluation:

**Overall clarity and organization of the thesis**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Clarity | ☒ | ☐ | ☐ | ☐ | ☐ |
| Organization | ☒ | ☐ | ☐ | ☐ | ☐ |

**Comments:**
Generally the submitted PhD. thesis is very well prepared, all parts are clear and the overall impression is very positive.
There are only some minor typo and grammar errors.
I has to be state that this PhD. thesis of a very good quality, the review is very well prepared, aim of the study are clear and real, all methods are acceptable and giving significant results. The role of supervisor and coordinator of the course (thesis) should be very highly appreciated.

**Clarity of the Objectives**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☒ | ☐ | ☐ | ☐ | ☐ |

**Comments:**
Objectives of the PhD. thesis are clearly established, very actual and fully in the scope of the scientific section.

**Novelty**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☒ | ☐ | ☐ | ☐ | ☐ |

**Comments:**
There are some new and very interesting outcomes that should be used in basic as well as applied science related to animal biology and biotechnology.

**Adequacy of the methods used and methodological rigor**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Adequacy of methods | ☒ | ☒ | ☐ | ☐ | ☐ |
| Methodological rigor | ☒ | ☐ | ☐ | ☐ | ☐ |

**Comments:**

Methods used in these studies are very actual and ensure significant results and possible clear conclusions.

**Relevance of Results and Conclusions**

| | Out standing | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Results | ☒ | ☒ | ☐ | ☐ | ☐ |
| Conclusion | ☒ | ☐ | ☐ | ☒ | ☐ |

**Comments:**
Results are well prepared, clear and finely described. As all results were submitted to scientific journals, underwent serious revision and were accepted for publication and/or were published no additional questions are necessary.

**Contribution to the advancement of knowledge**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☐ | ☐ | ☐ | ☐ | ☐ |

**Comments:**
All results are very significant and very useful for the development of knowledge mainly in the field of animal biotechnology/physiology and animal production and reproduction.

**Potential impact and applicability of the results**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Impact | ☒ | ☐ | ☐ | ☐ | ☐ |
| Applicability | ☒ | ☐ | ☐ | ☐ | ☐ |

**Comments:**
Results are useful for application in the field of animal biotechnology and creation of national bank launch. Also there is a significant relation to the field of animal biology and physiology.

**Adequacy of the references cited**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☒ | ☐ | ☐ | ☐ | ☐ |

**Comments:**

All cited references are actual and closely related to the topic of the thesis.

**Use this space for any other remark and comment**:

According to the aim of the study there are some question and/or ideas related to discussion:

- give comparison of strategies (conservation and maintain) also with some other animal species
- describe risk related to poultry production in China compared to other countries
- give the main targets/aims related to animal biodiversity management (mainly endangerous breeds)
- give in details: “safeguarding the biodiversity of poultry and also in the maintenance of the genetic variability through targeted actions” (P14)
- describe the effect of location/area on animal (rabbit) breeding and production (related to part 4.2)
- give clearly the aspects of global vs. local safeguards of biodiversity (P29)
- are there any request for owners of cryobank – state and/or private?
- describe the aspects of semen vs. spermatozoa cryoconservation (P37)
- give clearly the function of diluent, protectant and additive (P46)
- describe WHO/FAO criteria for semen/spermatozoa evaluation (Chapter 10)
- incorrect figure – 8.2; statement (P59) – pituitary?

**Overall evaluation**

Does this thesis satisfy the minimum requirements for the admission to the final exam?

| **YES** | | **NO** |
|---|---|---|
| The thesis can be submitted for the final exam AS IT IS | The thesis can be submitted for the final exam after MINOR REVISIONS (to be submitted within 30 days – No need for re-evaluation by the referee) | MAJOR REVISIONS are required. The thesis cannot be submitted for the final exam and should be re-evaluated by the referee, who will submit a new evaluation report and then re-submitted for final approval within 6 months |
| ☒ | ☐ | ☐ |

In the case NO or MINOR REVISIONS are required (i.e. the thesis can be submitted for the final exam), provide your overall evaluation

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☒ | ☒ | ☐ | ☐ | ☐ |

In the case MAJOR REVISIONS are requested, please provide your detailed comments and suggestions for improvements

| | Comments and suggestions for improvement |
|---|---|
| | NA |

Date and signature:

08/02/2018

*Péter Massányi*

# EVALUATION REPORT OF DOCTORAL THESIS

Title of the thesis

| |
|---|
| **Semen cryopreservation as a tool for preserving genetic biodiversity of avian and rabbit species: national cryobank launch** |

Name of the PhD candidate

| |
|---|
| Marsia Miranda |

Name and affiliation of the thesis supervisor

| |
|---|
| *NICOLAIA IAFFALDANO-University of Molise* |

Name and affiliation of the referee

| |
|---|
| Cerolini Silvia, University of Milan |

Evaluation:

**Overall clarity and organization of the thesis**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Clarity | ☒ | ☐ | ☐ | ☐ | ☐ |
| Organization | ☒ | ☐ | ☐ | ☐ | ☐ |

Comments:
The thesis has been very well organized in its contents. A complete overview of reproductive characteristics, conservation programs and semen cryopreservation technique is provided in poultry and rabbits. The research studies are also well organized and clearly described.

**Clarity of the Objectives**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☒ | ☐ | ☐ | ☐ | ☐ |

Comments : Research objectives have been described in details.

**Novelty**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☐ | ☒ | ☐ | ☐ | ☐ |

Comments :
The results provide very promising new procedures to improve the reproductive technologies in poultry and rabbits.

**Adequacy of the methods used and methodological rigor**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Adequacy of methods | ☒ | ☐ | ☐ | ☐ | ☐ |
| Methodological rigor | ☒ | ☐ | ☐ | ☐ | ☐ |

Comments :
Trials have been correctly planned, several factors of variation have been considered and assessed in order to collect the most significant results. Updated analytical techniques have been used for the objective assessment of semen quality.

**Relevance of Results and Conclusions**

| | Out standing | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Results | ☒ | ☐ | ☐ | ☐ | ☐ |
| Conclusion | ☒ | ☐ | ☐ | ☒ | ☐ |

Comments :
The presentation of results is very detailed and a comprehensive discussion is provided. Results are of interest for the animal production system and the conservation of AGR according to the ex situ in vitro technique.

**Potential impact and applicability of the results**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| Impact | ☒ | ☐ | ☐ | ☐ | ☐ |
| Applicability | ☒ | ☐ | ☐ | ☐ | ☐ |

Comments :
Results have the potential to improve in vitro semen storage and then a wider application or artificial insemination technique is expected in the animal production sector.
Results have also a positive impact in the development of sperm cryobanks for the conservation of animal genetic resources.

**Adequacy of the references cited**

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☒ | ☐ | ☐ | ☐ | ☐ |

Comments :
References are very consistent and exhaustive.

**Use this space for any other remark and comment**:

No other remark or comment.

**Overall evaluation.**

Does this thesis satisfy the minimum requirements for the admission to the final exam?

| **YES** | | **NO** |
|---|---|---|
| The thesis can be submitted for the final exam AS IT IS | The thesis can be submitted for the final exam after MINOR REVISIONS (to be submitted within 30 days – No need for re-evaluation by the referee) | MAJOR REVISIONS are required. The thesis cannot be submitted for the final exam and should be re-evaluated by the referee, who will submit a new evaluation report and then re-submitted for final approval within 6 months |
| ☒ | ☐ | ☐ |

In the case NO or MINOR REVISIONS are required (i.e. the thesis can be submitted for the final exam), provide your overall evaluation

| | Outstanding | Very good | Good | Sufficient | Poor |
|---|---|---|---|---|---|
| | ☐ | ☐ | ☐ | ☐ | ☐ |

In the case MAJOR REVISIONS are requested, please provide your detailed comments and suggestions for improvements

| | Comments and suggestions for improvement |
|---|---|
| | |

Date and signature:

Milan, 12 February 2018 Silvia Cerolini

# CONTENTS

# Part I: Overview on current status of poultry and rabbit farming and biodiversity conservation programs

# Chapter 1

## Analysis of poultry and rabbit farming

### *1.1 Analysis of poultry farming*

Today poultry farming is one of the most propulsive types of livestock production both in the world and in our country. The market orientation of the poultry meat sector is reinforced by the several advantages compared to others. Among these different characteristics, we can mention the affordability (price per kg of poultry meat is cheaper than pork, beef or lamb meat), convenience, absence of religious restrictions, health image (low fat content and good and balanced protein content). The most common type of poultry in intensive production systems, is represented by the domestic chicken (*Gallus gallus domesticus*), followed by the turkey (*Meleagris gallopavo*) and then, in a much smaller number, by ducks, geese, quails, pigeons, etc. Among these species, chickens and turkeys are the most common sources of poultry meat and eggs. Taking a longer view between 2010 and 2016, global poultry meat production raised from 98.067 to 115.192 million metric tonnes (mmt) reflecting on poultry consumption (OECD-FAO, 2016). Asia has been the largest producing country during 2016 with a production of 39.6 mmt, followed by North America (23.184 mmt), South America (21.578 mmt) and China (18.035 mmt) (figure 1.1).

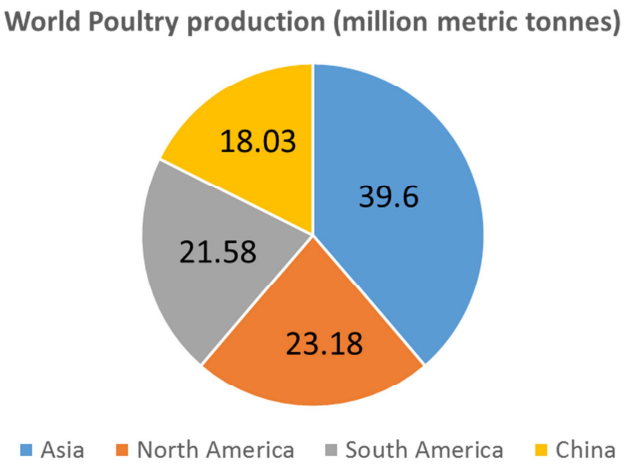



Figure 1.1. Global poultry meat production (Eurostat, 2016).

In European Union, poultry production corresponded to 14.400 mmt. Indeed six countries together, produced half of the EU's poultry meat: Poland (23 %), United Kingdom (18 %), France (16 %), closely followed by Germany and Spain (15 % for both) and Italy (14 %) (Eurostat, 2016). In addition, poultry meat is mostly from chicken (81 %) and turkey (14,8 %) while ducks account for only 3,6 %. The remaining 1,6 % is from other poultry species (figure 1.2) (Eurostat, 2016).

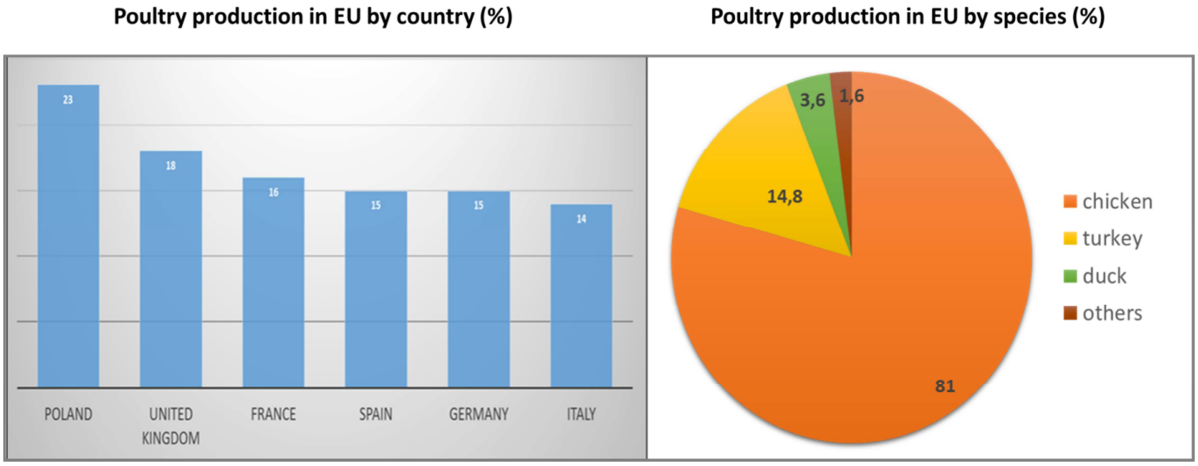



Figure 1.2. European Union poultry production (%) by country (on the left) and by species (on the right) (Eurostat, 2016).

Italian poultry meat production achieved the amount of 1.389.000 t. (+5,1 % than 2015) with an increase of +5,6 % for chicken meat (981.000 t.) respect to turkey meat production (+5,8 % or 331.000 t.) (Unaitalia, 2016).

World egg production reached a significant milestone by achieving 70 mmt (equivalent to 1,338 billion eggs). China and the United States (USA) top the list of world egg producers despite the outbreak of avian influenza on USA egg farms in 2015. In the European Union, eggs production has been concentrated mainly in seven countries (Ukraine, France, Spain, Germany, United Kingdom and Italy) that account for 75% of whole production. While in Italy the production achieved 12.900 billion eggs (+0,5% than 2015) (Unaitalia, 2016).

### *1.2 Analysis of rabbit farming*

Rabbits are a quick-breeding source of low-fat, high-protein meat and have long been enjoyed as a food by people around the world. The origins of the European rabbit *Oryctolagus cuniculus*, are difficult to pinpoint but date back 37 to 53 million years ago in Northern Spain (Dawson, 1967; Dalla Zotte, 2014). Today, the rabbit is reared systematically on a vast scale for meat production. However, rabbits can be produced also for other different markets: for breeding stock, as medical and educational lab animals, for pet food, as pet and show animals, and for their wool and skin. The four biggest world's producers of rabbit meat are China, Italy, Spain and France that contributed to almost three quarters of the world production (2.322.553 t) (figure 1.3) (Eurostat, 2015).


Figure 1.3. World's rabbit meat production (Eurostat, 2015)

The international trade of rabbit meat involves almost 50 thousands tons, equal to almost the 5% of the world production, to confirm that the principal part of the world's production is destined to the domestic use. The major commercialized volume is exchanged among few countries. In particular, the 50% of the imports come from 5 countries: France (1229.835 t), Hungary (248.6 t), Spain (286.07 t), Germany (5.45 t) and Belgium (31.284 t) (figure 1.4a) (Ismea, 2016).

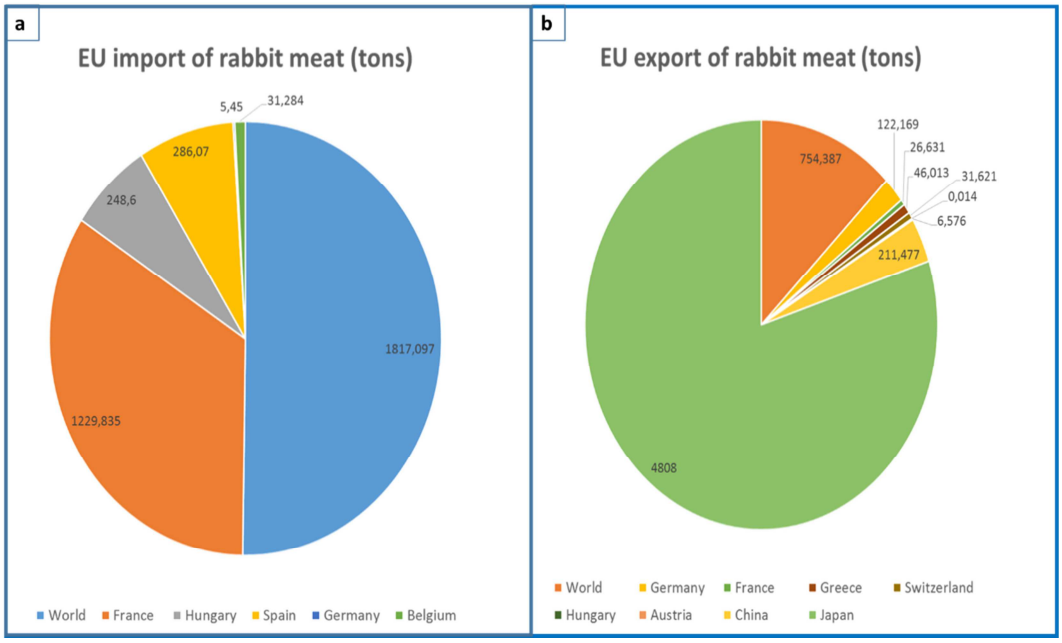



Figure 1.4. EU import (a) and export (b) of rabbit meat (Ismea, 2016)

Likewise, the exports are represented for the 80% by the Japan, for the 4% from China (211.477t), while Germany, France, Hungary, Greece and Austria contribute scarcely to export network for rabbit meat (figure 1.4b) (Ismea, 2016). An important role in the import-export is played by Belgium that is at the same time one of the principle countries that exports and imports rabbit meat, importing from China and East Europe and exporting part of its production in France and Germany (Ismea, 2016).

# Chapter 2

## Animal Biodiversity

According to the Convention of Biodiversity placed in 1992 at the United Nation Conference on Environment and Development (UNCED) in Rio de Janeiro, the term "biodiversity" (or biological diversity) can be defined as all forms of life including all species and genetic variants within them and all ecosystems that contain and sustain diverse forms of life including plants and animals. The convention was inspired by the growing concern all over the world for sustainable development and established three main objectives:

- Conservation of the biological diversity
- Sustainable use of its components
- A fair and equitable sharing of its benefits

Increased global use of highly productive breeds of farm animals has been associated with a loss of genetic diversity in most species (Özdemir *et al*., 2013). Assessing the risk status of livestock breeds is the first strategic priority in the Global Plan of Action for AnGRs and should be performed before the implementation of any conservation actions or programs, *in situ*, *ex situ* or both.

The biodiversity is commonly considered at three specific levels including:

-*Genetic diversity* which is the total number of genetic characteristics in the genetic makeup of a species. It is distinguished from genetic variability, which describes the tendency of genetic characteristics to vary and serve as a way for populations to adapt to changing environments. The accumulation of genetic differences between breeds and populations in domesticated animals, was strongly forced by farmers isolating and selecting them for favourable traits (Lush, 1994). Genetic variation is the basic material in animal breeding. However, diversity among breeds is probably reduced due to inter-crossing (Weigend *et al.*, 2013). Moreover, its loss will globally limit options for future genetic improvement programs.

-*Species diversity* refers to the number of species within a specific area. Within species the totality of all organisms of the same group, which live in a particular geographical area, and have the capability of interbreeding, constitutes a population. Populations contain genetic variation within themselves (intra-species diversity) and between other populations (inter-

species diversity). The existence of the population of a species in a geographically and environmentally distinct areas, is considered important in order to maintain diversity in the gene pool and to protect the species against events such as isolated epidemics of disease and predators that could exterminate entire populations.

-*Ecosystems diversity* is related to dynamic entities composed of the biological community and the abiotic environment, named "ecosystems" which are composed of abiotic and biotic components. The structure of each ecosystem is determined by the state of a number of interrelated environmental factors. Changes in any of these factors such as nutrient availability, temperature, light intensity, grazing intensity, and species population density, may result in detrimental effect with the consequent loss of biodiversity (Tacconi, 2000).

### *2.1. Risk status of animal breeds*

Over the years, many animal breeds have been lost. Modern intensive agriculture has encouraged many farmers to adopt uniform high yielding varieties of animal breeds (Pagiola *et al.*, 1997). The rapidly diminishing gene pool is a serious concern at this point in history due to climate change, urbanisation, changes in lifestyles and also, in the food habits and the emergence of new biology, notably biotechnology and genetic engineering (Floros *et al.*, 2010). Moreover, with the increase of marginal areas release and the start of industrial breeding, only a few breeds, have been selected and used for production.

As a consequence, many pure-breeds (including avian and mammalian species), are now at the risk of extinction. Since 2014, the percentage of national breed populations has increased to 57% and 61% for avian and mammal species respectively (FAO, 2015). A global total of 8822 breeds has been reported (FAO, 2015). 7761 are local breeds and 1061 are transboundary breeds. While 499 are regional transboundary breeds which are present in only one region and 562 are international transboundary breeds living in more than one region. Regional transboundary mammalian breeds are relatively numerous (more than 5% of the respective total number of breeds in the region) in Europe and the Caucasus region, Africa, and North America. In addition, Europe and the Caucasus region possess a large number of regional transboundary avian breeds. In particular among 1515 local chicken breeds at global level, 909 inhabit Europe and Caucasus region in contrast with local turkey breeds that are only 40 (figure 2.1). While as regards local rabbit breeds, 257 are present in the world, and only 197 are distributed in Europe and Caucasus region (figure 2.2.).

| Species | Africa | Asia | Europe & the Caucasus | Latin America & the Caribbean | Near & Middle East | North America | Southwest Pacific | World |
|---|---|---|---|---|---|---|---|---|
| Chicken | 133 | 307 | 909 | 88 | 33 | 15 | 30 | 1515 |
| Duck | 15 | 92 | 107 | 22 | 3 | 1 | 12 | 252 |
| Goose | 10 | 44 | 119 | 5 | 2 | 0 | 2 | 182 |
| Muscovy duck | 5 | 9 | 6 | 1 | 1 | 0 | 2 | 24 |
| Ostrich | 6 | 2 | 3 | 0 | 0 | 0 | 1 | 12 |
| Partridge | 2 | 8 | 2 | 0 | 0 | 0 | 0 | 12 |
| Pheasant | 0 | 7 | 5 | 6 | 0 | 0 | 0 | 18 |
| Pigeon | 7 | 13 | 35 | 7 | 8 | 1 | 2 | 73 |
| Turkey | 11 | 11 | 40 | 11 | 2 | 11 | 5 | 91 |
| Others | 39 | 30 | 31 | 12 | 2 | 14 | 0 | 128 |
| **Total** | **228** | **523** | **1257** | **152** | **51** | **42** | **54** | **2307** |

Figure 2.1. Number of reported local avian breeds in the World including chicken (in red box) and turkey (in blue box) breeds (FAO, 2015).

| Species | Africa | Asia | Europe & the Caucasus | Latin America & the Caribbean | Near & Middle East | North America | Southwest Pacific | World |
|---|---|---|---|---|---|---|---|---|
| Ass | 19 | 39 | 52 | 24 | 13 | 5 | 3 | 155 |
| Bactrian camel | 0 | 9 | 3 | 0 | 0 | 0 | 0 | 12 |
| Buffalo | 3 | 90 | 8 | 11 | 5 | 1 | 2 | 120 |
| Cattle | 178 | 242 | 375 | 141 | 39 | 17 | 32 | 1024 |
| Dromedary | 46 | 13 | 1 | 0 | 23 | 0 | 2 | 85 |
| Goat | 97 | 183 | 221 | 28 | 33 | 6 | 11 | 579 |
| Guinea pig | 4 | 0 | 0 | 13 | 0 | 0 | 0 | 17 |
| Horse | 40 | 138 | 379 | 85 | 14 | 22 | 25 | 703 |
| Pig | 55 | 218 | 190 | 60 | 1 | 12 | 15 | 551 |
| Rabbit | 11 | 16 | 197 | 18 | 7 | 8 | 0 | 257 |
| Sheep | 117 | 262 | 617 | 51 | 50 | 22 | 38 | 1157 |
| Yak | 0 | 25 | 2 | 0 | 0 | 1 | 0 | 28 |
| Others | 8 | 16 | 76 | 15 | 0 | 4 | 8 | 127 |
| **Total** | **578** | **1251** | **2121** | **446** | **185** | **98** | **136** | **4815** |

Figure 2.2. Number of reported local mammalian breeds in the World including rabbits (in green box) (FAO, 2015).

According to FAO (2015), there are seven different criteria to establish the risk status of a specific breed. Such criteria are summarized in figure 2.3. According to FAO, (2015), a total of 1500 breeds (17 % of all breeds including those that are extinct), are classified as being at risk. Among mammalian species, horse, sheep and cattle have the largest numbers of breeds at risk. However, rabbits (49 %) followed by horses (23%) and asses (22%) are the species that have the largest overall proportions of breeds at risk (FAO, 2015).

Moreover, there is a large number of breeds of which no risk-status data are available. This lack of data, seriously limits the effective prioritization and planning of the breed conservation measure. Among avian species, chickens have by far the greatest number of breeds at risk on a world scale. In addition, the proportion of avian breeds of unknown risk status, is even greater than for mammalian species (FAO, 2015).

| Criteria | Definition |
|---|---|
| **Extinct** | A breed is categorized as extinct if it is no longer possible to easily recreate the breed population. This situation becomes absolute when there are both no breeding males (semen) and breeding females (oocytes) nor embryos remaining. In reality extinction may be realized well before the loss of the last animal, gamete or embryo. |
| **Critical** | The total number of breeding females is less than 100 or the total number of breeding males is less than or equal to 5; Or the overall population size is close to, but slightly above 100 and decreasing and the percentage of females being bred pure is below 80%. |
| **Critical-maintained** | As for Critical, but for which active conservation programmes are in place or populations are maintained by commercial companies or research institutes. |
| **Endangered** | A breed is categorized as endangered if: the total number of breeding females is between 100 and 1000 or the total number of breeding males is less than or equal to 20 and greater than 5;or the overall population size is close to, but slightly below 100 and increasing and the percentage of females being bred pure is above 80%; or the overall population size is close to, but slightly below 1000 and decreasing and the percentage of females being bred pure is below 80%. |
| **Endangered-maintained** | As for Endangered, but for which active conservation programmes are in place or populations are maintained by commercial companies or research institutes. |
| **Not at risk** | A breeds is categorized as not at risk if: the total number of breeding females and males are greater than 1000 and 20, respectively, Or If the population size approaches 1000 and the percentage of females being bred pure is close to 100%, and the overall population size is increasing. |
| **Unknown** | Self explanatory, but also a call to action: find out!<br>If categorization of a particular breed is borderline, further consideration should be given to factors such as:<br>· degree of crossbreeding in the population<br>· reproductive rate and generation interval of the population.<br>Populations with low reproductive rates are at relatively greater risk than populations of high reproductive capacity of comparable size.<br>· special peculiarities and characteristics of the production<br>system (intensive, extensive, nomadic etc.)<br>· historic and current rates of decline in population numbers<br>· geographic isolation of the population or its concentration in one or a few locations that would place it at risk as a result of climatic, economic or political changes or disease outbreak. |

Figure 2.3.Criteria to establish the risk status of a breed: extinct, critical, critical-maintained, endangered-maintained, not at risk and unknown (FAO, 2015).

The majority of extinct breeds have mainly been reported among chickens whilst only a few cases among ducks, geese, guinea fowls and turkeys have been reported (figure 2.4) (FAO, 2015). Among mammalians, rabbits have got only one breed that results as extinct in Europe and Caucasus region (figure 2.5) (FAO, 2015).

| Species | Africa | Asia | Europe & the Caucasus | Latin America & the Caribbean | Near & Middle East | North America | Southwest Pacific | World |
|---|---|---|---|---|---|---|---|---|
| Chicken | 0 | 5 | 57 | 0 | 0 | 0 | 0 | 62 |
| Duck | 0 | 0 | 15 | 0 | 0 | 0 | 0 | 15 |
| Goose | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 |
| Guinea fowl | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Turkey | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| **Total** | **2** | **5** | **77** | **0** | **0** | **0** | **0** | **84** |

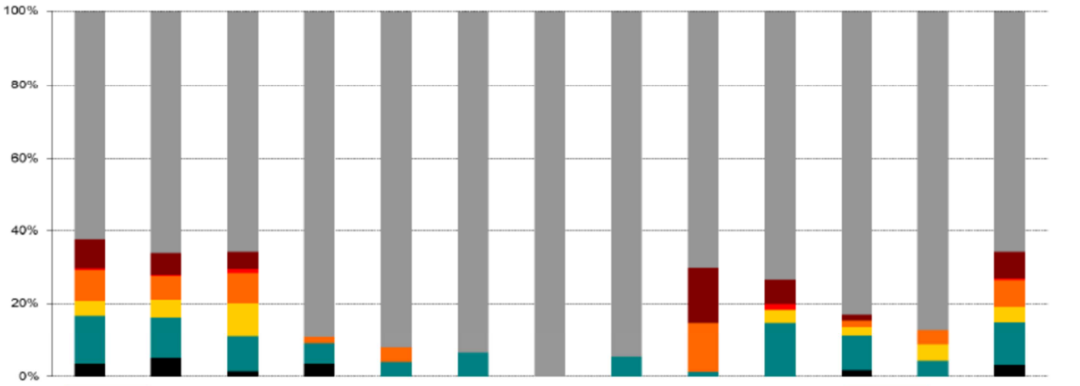
Figure 2.4. Number of extinct avian breeds in which chicken (red box) represents the majority of extinct breeds in contrast with duck, goose, guinea fowl and turkey (yellow box) (FAO, 2015).

| Species | Africa | Asia | Europe & the Caucasus | Latin America & the Caribbean | Near & Middle East | North America | South-west Pacific | International transboundar breeds | World |
|---|---|---|---|---|---|---|---|---|---|
| Ass | 1 | 0 | 3 | 0 | 1 | 0 | 0 | 0 | 5 |
| Buffalo | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Cattle | 20 | 19 | 119 | 20 | 0 | 1 | 2 | 1 | 182 |
| Goat | 1 | 2 | 16 | 0 | 0 | 0 | 0 | 0 | 19 |
| Horse | 6 | 1 | 67 | 0 | 0 | 8 | 1 | 0 | 83 |
| Pig | 0 | 15 | 91 | 1 | 0 | 0 | 1 | 0 | 108 |
| Rabbit | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Sheep | 5 | 6 | 146 | 0 | 0 | 1 | 2 | 0 | 160 |
| **Total** | **33** | **43** | **444** | **21** | **1** | **10** | **6** | **1** | **559** |

Figure 2.5 Number of extinct mammalian breeds among which rabbits (green box) have the lowest number of extinct breeds (FAO, 2015).

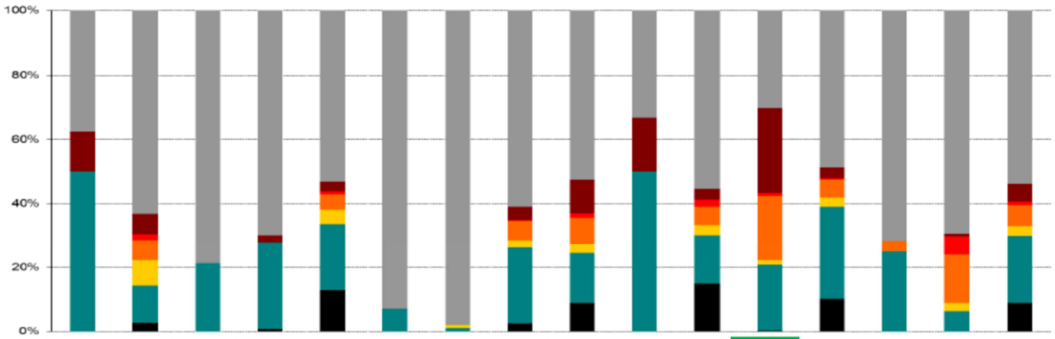
The regions with the highest proportion of breeds classified at risk, are Europe and Caucasus region. In particular, avian species percentage of risk is slightly higher than that for mammalian breeds (34% and 32% respectively). About the risk status of the world's avian breeds in 2015, it has been reported that of 1733 local chicken breeds, 1078 are classified as unknown, 143 are considered critical and 143 are defined as endangered (figure 2.6.). Among turkeys, 2 breeds are considered as critical and 2 breeds result extinct, while 96 local turkeys are defined "unknown". In contrast, of 317 local rabbit breeds, 96 are considered unknown, 84 are critical and 63 endangered (FAO, 2015) (figure 2.7).

In opposition to the global trend, in Italy the highest proportion of breeds at risk of extinction, is represented by chickens. Currently in Italy, there is a large number of chicken breeds found to be in critical status. The only data available about this species, have been reported by Zanon and Sabbioni, (2001) who evidenced that, 61% of Italian chicken breeds are extinct, 13.3% are endangered and 6.7% are involved in conservation programs.




| | Chicken | Duck | Goose | Guinea fowl | Muscovy duck | Ostrich | Partridge | Pheasant | Pigeon | Quail | Turkey | Other | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Unknown | 1078 | 194 | 137 | 49 | 23 | 14 | 12 | 17 | 52 | 44 | 96 | 20 | 1736 |
| Critical | 143 | 17 | 10 | 0 | 0 | 0 | 0 | 0 | 11 | 4 | 2 | 0 | 187 |
| Critical-maintained | 8 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 12 |
| Endangered | 143 | 19 | 17 | 1 | 1 | 0 | 0 | 0 | 10 | 0 | 2 | 1 | 194 |
| Endangered-maintained | 69 | 14 | 19 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 1 | 108 |
| Not at risk | 230 | 33 | 20 | 3 | 1 | 1 | 0 | 1 | 1 | 9 | 11 | 1 | 311 |
| Extinct | 62 | 15 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 84 |
| Total | 1733 | 293 | 208 | 55 | 25 | 15 | 12 | 18 | 74 | 60 | 116 | 23 | 2632 |

Figure 2.6. Risk status of the World's avian breeds including chicken (in red box) and turkey (in blue box) breeds, expressed in percentage (chart) and absolute (table) value (FAO, 2015).




| | Alpaca | Ass | Bactrian camel | Buffalo | Cattle | Deer | Drom-edary | Goat | Horse | Llama | Pig | Rabbit | Sheep | Yak | Other | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Unknown | 3 | 110 | 11 | 95 | 752 | 25 | 87 | 417 | 480 | 2 | 398 | 96 | 757 | 20 | 84 | 3337 |
| Critical | 1 | 11 | 0 | 3 | 43 | 0 | 0 | 29 | 95 | 1 | 24 | 84 | 50 | 0 | 1 | 342 |
| Critical-maintained | 0 | 3 | 0 | 0 | 13 | 0 | 0 | 2 | 13 | 0 | 15 | 3 | 7 | 0 | 7 | 63 |
| Endangered | 0 | 11 | 0 | 0 | 67 | 0 | 0 | 40 | 73 | 0 | 41 | 63 | 85 | 1 | 18 | 399 |
| Endangered-maintained | 0 | 14 | 0 | 0 | 61 | 0 | 1 | 17 | 27 | 0 | 24 | 5 | 43 | 0 | 3 | 195 |
| Not at risk | 4 | 20 | 3 | 37 | 294 | 2 | 1 | 160 | 140 | 3 | 108 | 65 | 443 | 7 | 8 | 1295 |
| Extinct | 0 | 5 | 0 | 1 | 182 | 0 | 0 | 19 | 83 | 0 | 108 | 1 | 160 | 0 | 0 | 559 |
| **Total** | **8** | **174** | **14** | **136** | **1412** | **27** | **89** | **684** | **911** | **6** | **718** | **317** | **1545** | **28** | **121** | **6190** |

Figure 2.7. Risk status of the World's mammalian breeds including rabbit breeds (in green box), expressed in percentage (chart) and absolute (table) value (FAO, 2015).

### *2.2. Animal biodiversity conservation programs*

Animal biodiversity conservation is recognized as an essential action and it has been widely promoted by many international Organizations. The Food and Agriculture Organization of the United Nations (FAO) has been fostering various actions since 1980; in 1992 the United Nations ratified the importance of genetic animal resources in Agenda 21 and in the Convention on Biological Diversity (CBD). In 1998 the report "State of the World's Animal Genetic Resources for food and agriculture" (SoW-AnGr) was drafted by FAO; European Association for Animal Production (EAAP) funded the European Farm Animal Biodiversity Information System (EFABIS) with the aim to merge the existing databases contributing to DAD-IS development in its present structure. In 2007 thanks to EFABIS project, the Domestic Animal Diversity Information System (DAD-IS) was created by FAO which provided the access to online data-bank collecting and displaying all information about origin, consistency and conservation program of around 14000 populations of different animal species classified by country (http://dad.fao.org/).

In 2013 in Italy, the Minister of Agricultural, Food and Forestry Policies (Mipaaf), published the "National Plan for the safeguard of Biodiversity in farm animals" (PNB), and the "Guidelines for conservation and characterization of farm animal genetic resources" (LGBAA). One of the purposes of PNB has been to support the introduction of a national strategy for biodiversity conservation in the agricultural sector, and it is capable of efficaciously reintroducing in the animal production system the majority of the native breeds with the consequence of benefiting the environment, sustainable agriculture and rural development.

In recent years, animal biodiversity management has become an important issue in the international scientific community because of changes of large-scale production systems (FAO, 2007). In North America, Europe, and China, about 50% of documented breeds are classified as extinct, critical, or endangered and local breeds have often been diluted by indiscriminate cross-breeding with imported stocks (FAO, 2007; Zanetti *et al*., 2010).

In particular in China, many livestock genetic resources even if under state protection disappeared at high speed (Li *et al*., 2002) and many indigenous breeds with low productivity, were replaced by imported or hybrid breeds which currently predominate in almost all of the animal husbandry (Liu *et al*., 2014). As regards poultry breeds especially chicken, highly productive commercial breeds were introduced into China in recent decades and crossbred with native breeds to meet local economic demands. Therefore, the number of locally adapted chicken breeds in China is declining due to crossbreeding with commercial strains (Jia *et al*., 2018).

A species or a population sample of a particular part of its genetic variation, can be maintained through specific conservation strategies. In procedural terms, three strategies are contemplated to carry on AGRs conservation: *in situ, ex situ in vivo* and *ex situ in vitro* (Woelders *et al.*, 2006; Blesbois, 2007; FAO, 2008):

*In situ conservation* consists in the utilisation of breeds within their production systems in the original and new diffusion areas. This method preserves both the population and the evolutionary processes that enable the population to adapt by managing organisms in their natural state or within their normal range. For example, large ecosystems may be left intact as protected reserved areas with minimal intrusion or alteration by humans. In Italy, an *in situ* conservation program was carried out by Veneto region where, four Venetian sheep breeds Alpagota (ALP), Brogna (BRO), Foza (FOZ), and Lamon (LAM), that survived extinction, were preserved in small farms located in mountainous and hilly areas and today, represent an important reservoir of genetic resources (Dalvit *et al*., 2009).

*Ex situ in vivo* consists of the maintenance of the living animals of the breed outside its production system (herds in protected areas, parks, zoos, experimental and show farms, research centers, hobby breeder farm). An example of this strategy, is provided by the National Center for the safeguard of Animal biodiversity that was created in Italy in Campania region, to preserve endangered local breeds including ass, cattle, bull, goat, horse, sheep and pig (Matassino, 2010).

*Ex situ in vitro* is based on the storage of genetic material in the form of haploid (semen and oocytes) or diploid cells (embryos, somatic cells) by cryoconservation, which allows an almost endless storage period. The first animal germoplasm collection launched in Italy was the "*Animal Germplasm Bank Giuseppe Rognoni*" with the aim to preserve animal biodiversity. Actually, the collection is constituted by 30.445 semen doses collected in 10 years from 98 animal donors of 4 livestock species: cattle, sheep, goat and pig.

To date, management of farm AnGRs is focused on a complementary strategy deriving from the combination of *in situ* and *ex situ* conservation (FAO, 2012). The current trend for *ex situ* conservation is to establish national gene banks. These are currently operational in different countries, including Brazil (Mariante *et al.*, 2009), France (Danchin-Burge *et al.*, 2006), USA (Blackburn, 2009) and the Netherlands (Woelders *et al.*, 2006).

# Chapter 3

## Conservation program for local avian breeds

### *3.1. Interest in local poultry*

In birds, the safeguard of biodiversity is recognized as an urgent need because the great level of standardization and specialization achieved in the poultry sector, was based on the exclusive global rearing of commercial strains (Ozdemir *et al.*, 2013). Poultry production constitutes one of the main sources of animal proteins in Italy and all over the world. The modern intensive poultry production system is characterized by very high bird performance and low cost of production. Genetic selection has continued to be one of the key elements in the constant progressive increase of productive performance; however, commercial strains require specific environmental conditions, bird management and feeding plans, and accurate biosafety measures. Other than financial benefits, genetic selection has determined some negative effects such as the reduction of animal biodiversity, with the consequent deterioration of animal wealth. Globally, many extinct poultry breeds have been listed and many others have been classified in different risk categories, from critical to endangered. Moreover, FAO has stressed the fact that in rural areas of developing countries the local breeds are more fitting than the global modern breeds (FAO, 2008). The genomic sequencing in chickens (Francham *et al.*, 2004) drew attention to the fact that commercial lines have been losing 90% of their alleles than native chicken breeds. The reduced genetic diversity in these lines limits the prospect of adaptation and produces new scenarios such as re-emerging of unknown diseases.

In such context, fast growing birds are not recommended, while slow growing birds with a high tendency to pasture, reveal a greater ability of adaptation (Bokkers and Koene, 2003; Lewis *et al.*, 1997). Local breeds have adapted to specific environments for thousands of years, and their potential as foodmakers or their genetic variability are still unexplored.

Therefore, many countries at global and European level, are focusing on the preservation and valorization of local poultry breeds including chicken and turkey as reported in figure 3.1. In this regard, some Italian universities are engaged in safeguarding the poultry biodiversity and also in the maintenance of the genetic variability through targeted actions.

Local breeds will be maintained in genetic research centers where the genetic variability will be ensured by specific mating plans in order to minimize inbreeding level of progeny. The final goal is to carry out coordinated and integrated activities at a national level, thus overcoming the present local fragmentation. Even now, a semen national cryobank for poultry is not available yet.

### 3.2. Global plans for the conservation of avian genetic resources

In 1984 in North America, an international symposium and Workshop on Genetic Resources Conservation for California, made the recommendations for the establishment of the Genetic Resources Conservation Program within the University of California (UC/GRCP). These recommendations recognized that the avian genetic stocks developed in California, primarily chicken and Coturnix quail, had been valuable in research and in the commercial poultry industry. Moreover, continued conservation of such stocks, depended upon long-term support for live-bird maintenance and further research on cryopreservation technology. However, recognizing the drastic reductions in human and financial resources for avian genetic resources management, the UC/ GRCP established a cooperation with 5 countries of USA (Illinois, New Hampshire, Wisconsin, Michigan and Connecticut) and 2 countries of Canada (British Columbia and Ontario) convening a national Task Force in 1995 to address the problem. The Task Force included representatives from the many public institutions that reiceved poultry research programs, from private companies, that utilized poultry genetic stocks. In support of the Task Force's work UC/GRCP conducted an analysis of existing genetic stocks in North America, providing a detailed database about all extant stocks in the USA and Canada, including 268 chickens, 20 turkeys, 65 Japanese quails, and 8 waterfowls or gamebird accessions. From this analysis, it has been evidenced that many avian genetic stocks were lost and many others were at serious risk of extinction. Therefore an Avian Genetic Resources System (AVGRS), with strong leadership, but shared responsibility, was proposed as the most efficient and secure way to conserve genetic stocks and address the concerns raised in this evaluation effort. The Avian Genetic Resources System has been envisioned as a multilocational organization that served the avian genetic resources needs for the USA and Canada. The work of the Task Force in fact, was accomplished thanks to financial support from UC/GRCP and from grants of the National Science Foundation and the Agricultural Research Service from the USA side, whilst research and development programs of AVGRS, were supported by Canadian scientists. Conservation methods

employed in the AVGRS included live-bird maintenance and cryopreservation. Such methods provided a secure backup repository for privately owned lines or populations, either live birds or cryopreserved germplasm at the central or secondary centers on a fee basis. The conservation emphasis of the AVGRS was accomplished by live birds, gametes, DNA, and tissues including chicken, turkey, and Japanese quail as target species which are worthy of interest in agriculture for food production and for basic biological and biomedical research in USA and Canada. However, the AVGRS system considered also wild turkey, jungle fowl, and game birds, which were commonly raised in captivity (Pisenti *et al.*, 1999).

In **Japan**, the project for the Conservation and use of Animal genetic resources was initiated in 1985 and coordinated by the Ministry of Agriculture, Forestry and Fisheries (MAFF). The aim was to classify, identify and preserve a wide range of native animal breeds including chicken by *in situ* and *ex situ* conservation strategies. Indeed, the project was extended also to other species such as cattle, horse, pig, goat, rabbit and honeybee (Obata *et al.*, 1992). The activity was carried out at the *National Institute of Agro-biological Resources* in collaboration with the *Laboratory of Animal Germplasm Evaluation and Preservation* in Japan. The first step was to collect and maintain animal genetic resources in a rearing facility close to subsidiary gene bank. The majority of these resources was designated as "natural monuments in Japan", therefore they needed to be protected. In total, 677 animal genetic resources were collected. Subsequently, based on genetic and phenotypic analysis of each species, a database was established according to morphological, physiological and economic characteristics. Finally as part of the *ex situ* conservation program, a semen cryobank was established. The chicken semen cryobank included 4 breeds (*Tosa, Nagoya, Toutenku and Koeyoshi*), however cryopreservation protocol resulted as effective only for 2 of them (Obata *et al.*, 1992).

**In Africa** the program "*Fowls for Africa*" for the conservation of local chickens in South Africa was initiated in 1994 by the Agricultural Research Council and the Animal Production Institute (API). The objectives of this program were to conserve native fowls as a genetic resource and to promote their usage for household food security. The program was structured to avoid inbreeding and conserve the observed phenotypic differences and genetic variation within the different lines, including the *Black Australorp, Potchefstroom Koekoek, New Hampshire, Ovambo, Lebova-Venda* and *Naked Neck* chicken. Within the framework of this program, modern molecular technologies provided the opportunity to study genetic

diversity within populations and to identify potentially unique alleles for breed characterization. Among the DNA markers available, microsatellite markers, resulted useful for describing variation and genetic relationships in various farm animal species. In addition, phenotypic characterization of the chickens kept in the "*Fowls for Africa*" program, indicated significant differences that contributed to breed definition and production potential of the various chicken lines. For conservation of genetic resources of this program, it was essential to be able to make informed decisions based on the parameters that described the genetic diversity and population structure (Koster *et al.*, 2008).

### 3.3. European plans for the conservation of avian genetic resources

The French National Cryobank was initiated in **France** in 2003 by INRA (*Institut national de la recherche agronomique*) at the Research Center of Tours, Nouzilly. Target species were represented by three high genetically selected chicken lines (Y33, $R^+$, and B4/B4) together with the historical *Gauloise doreè*, one of the oldest free-range chickens in France. The three chicken lines were selected for different traits. The traits were chosen according to their importance in poultry production and their usefulness in basic research (Blesbois, 2007).

In particular, Line Y33 was involved in various research programs dealing with metabolism and muscle development (Ricard *et al.*, 1994; Le Bihan-Duval *et al.*, 1998; Berri *et al.*, 2001; Guernec *et al.*, 2003). Line $R^+$ was a model for the study of feed intake and metabolic efficiency (Gabarrou *et al.*, 1997; Bordas and Minvielle, 1999) characterized by defective mitochondrial metabolism affecting the spermatozoa (Morisson *et al.*, 1997). Line B4/ B4 belonged to a series of 12 lines that had different haplotypes of the chicken major histocompatibility complex (MHC) and was used as a model for research about diseases, including sarcomas and coccidiosis (Plachy *et al.*, 1989; Miller *et al.*, 2004). The aim was to construct a semen and blood cryobank under optimal quality and traceability conditions. Indeed, INRA research center was associated to four experimental units including: 1) an experimental unit specialized in avian disease (*Unite´ Expèrimentale-Physologie Aviaire et Parasitologie*), that organized the screening and remediation of diseases of strains of unknown health status; 2) an experimental unit for avian breeding where semen production and treatment occurred, *Unite´ Expérimentale- Station de Recherches Avicoles* (UE-SRA); 3) a research unit specialized in avian biology (UR-SRA,) that managed the technical procedures and ensures the freezing of samples and transitory storage;and 4) an

experimental unit (*Unite´ Expérimentale-Genètique Factorielle Avicole*, involved in conservation of genetic diversity) that maintains a female flock of the *Gauloise dorée* breed for further characterization and fertility test. In order to limit the possibility to loose the frozen samples, two geographically separate locations were established for the long-term storage of frozen semen. The first was closer to the site of semen collection and located at the EFS (French Blood Institute) of Tours. The second storage site was located near Paris at the primary site of the French National Cryobank of Domestic Animals (ACSEDIATE, Maisons-Alfort).

In **Spain** since 1975 different Spanish chicken breeds have been raised as part of a genetic resources conservation program (Campo and Orozco, 1982; Campo, 1998). Given the risks of epidemics such as avian influenza, the Spanish Ministry for Science and Innovation (MICINN), via the *Spanish National Institute for Agricultural and Food Research and Technology* (INIA), recently supported the establishment of a sperm cryobank to guarantee the preservation of poultry breeds. In particular, the project involved 12 Spanish chicken breeds (*Black-Barred Andaluza, Black-Red Andaluza, Blue Andaluza, Black Castellana, Buff Prat, White Prat, Red-Barred Vasca, Red Villafranquina, Birchen Leonesa, Blue Leonesa, White-Faced Spanish, and Black Menorca*) and 2 Spanish quails (*Castellana* and *Silver Castellana*) (Santiago-Moreno *et al.*, 2011). Semen donors were selected via the preliminary assay of sperm variables predictive of fertility considering quantitative and qualitative parameters such as sperm volume, concentration, motility, viability, morphological abnormalities and acrosome integrity (Santiago-Moreno *et al.*, 2009a).

**Slovak Republic** was the first country belonging to the Council for Mutual Economic Aid (CMEA), which launched the preservation of local chicken breeds. With growing popularity of international lines, hens traditionally managed in backyards and farm yards were recognised as genetic, cultural and historical heritage (Benková, 2002). The project was initiated in 1970 by the Research Institute of Animal Production in Nitra (NPPC) which proposed to preserve the genetic resources of three local chicken strains: *New Hampshire, Rhode Island Red* and *White Sussex.* The projects were realized by the Poultry Breeding Station in Ivanka at Danube (PRIID) where the first *ex-situ* conservation project in the form of isolated DNA was set up in 1982 (Romanov *et al.*, 1996). Currently the National gene bank in the Slovak Republic includes *Oravka* hen, for which DNA only is available as

genetic resource. The project is still ongoing by the NPPC Research Institute which is actively involved in the construction of a gene bank in Nitra.

In **Poland,** local breeds have been included in a conservation program by *in situ* strategy since 1972. Conservation projects have been realized by the National Research Institute of Animal Production (Romanov *et al.*, 1996). Currently, all stocks of laying hens involved in the conservation program constitute a "gene pool". In Poland, there is only one closed stock of each breed or strain, except for "Green-Legged Partridge hen (Zk)". The program was carried out at the Experimental Farm in Chorzelów and Agricultural University of Lublin (Zk and Pb). Studies on local breeds have been performed in the areas of ecology, physiology, molecular and quantitative genetics, and quality of poultry products (Cywa-Benko, 2002). Moreover, according to the Biodiversity Conservation Program in the Kurpie region, pure breed hens have been used. In particular, recently a growing interest, especially in "Green-Legged Partridge hen", has been noted among hobbyists.

Since 1950 in **Hungary**, poultry conservation programs have been implemented for several indigenous, native or adapted poultry breeds, including local chicken breeds, colour varieties of landrace turkey and guinea-fowl, local varieties of domestic goose and duck. AnGRs conservation has been supervised and partly financed by the Ministry of Agriculture and Rural Development and the Animal Breeding Directorate of the Central Agricultural Office (MGSzH). Hungarian poultry conservation has been coordinated and organized by the Association of Hungarian Small Animal Breeders for Gene Conservation (MGE) as a non-governmental organization (NGO), founded in 1997. The main result of the growing conservation network, has been the stabilized number of breeders in poultry elite stocks in the recent years, conserved as officially registered breeds. In 2005 MGE in cooperation with Animal Breeding and Nutrition (ATK) started a project named HU-BA (*Hungaricum poultry*) for the conservation, management and control of poultry production. Subsequently between 2010 and 2013, MGE has been involved in a bilateral project "CRYOBIRDS" with French researchers. Conservation programs of elite stocks, have been carried out by breeding institutions having several decades of experience, including: *Debrecen University* (Copper and Bronze Turkey; Mihók, 2004), *West-Hungarian University, Mosonmagyaróvár* (Yellow Hungarian Chicken; Kovácsné Gaál, 2004), *Szeged University of Science, Hódmezővásárhely* (Speckled Hungarian and Speckled Transylvanian Naked-neck Chicken;

Sófalvy, 2005), and last but not least the *Research Institute for Animal Breeding and Nutrition, Division of Small Animal Research* (*Gödöllő Poultry Gene Bank*).

The primary task of the Research Centre for Farm Animal Gene Conservation in Gödöllő, was the *in vivo* maintenance of Hungarian indigenous poultry breeds in nucleus populations. Then, in 2012 the project was expanded with the introduction of "cold rooms" for the preservation of poultry spermatozoa (300 sperm samples/species or varieties). Within the frame of the project, all registered traditional poultry breeds 7 chickens, 2 turkeys, 2 geese, 1 guinea-fowl and 1 duck breed with some colour varieties were included (Szalay, 2004). The laboratory possesses two sets of deep freezers as well as liquid nitrogen containers supporting these devices and a storage unit equipped with automatic filling system suitable for long term keeping of around 4600 samples.

In **The Netherlands** the "Gene Bank Foundation for Farm Animals" was established in 1992. The project aimed to support increasing number of economically important breed or lines at critical risk through an *in situ* or *ex situ* conservation program. The management costs, were sponsored by the private sector, while a structural contribution to the gene bank activities, were provided by the Ministry of Agriculture, Nature Management and Fisheries in the Netherlands. The first point of this project was the establishment of a program for *in situ* conservation of genetic resources characteristic for the Netherlands supported by Dutch Government. The second step was to establish and to manage a gene bank known as the "*Centre for genetic resources of The Netherlands*" (CGN)". The collections of farm animal genetic resources by *ex situ* conservation, included chicken, duck, goose followed by other species such as cattle, sheep, goat, horse, and rabbit that has been introduced only in 2014. Within the framework of this project, the main technology used for *ex situ* conservation program for rare breeds of all species mentioned above, was represented by semen cryopreservation. The ongoing project of CGN has focused on semen cryobank for avian species, while genetic material of other sources e.g. embryos and ovarian tissue, are going to be added to the collections for other species. The third activity linked with this project, was represented by the creation of a databank to collect all information relative to each rare breed for each species. With respect to avian species, currently the databank includes 27 local chickens, 3 local ducks and only 1 local goose. Over 100.000 doses of semen were stored. The objectives of the cryopreservation program in The Netherlands have been the following: 1) to store a sufficient amount of genetic material of all endangered breeds in the gene bank to be able to re-establish the breed, 2) to store a back-up of all (Dutch)

commercial lines or breeds in the gene bank and 3) to support small/critical populations by providing semen from the gene bank. Different stakeholders have played an important role in the cryopreservation program and both government and private sector have contributed financially. Currently CGN is continuously looking for improvement of efficiency and efficacy of conservation methods. In particular, it has been focused on the improvement of cryopreservation protocol for chicken semen, which could give good opportunities for future conservation of local breeds.

Recently, the *Ecole Polytechnique Federale* in **Switzerland** (Lausanne) in collaboration with Argentina, Morocco, Egypt and Colombia and 11 European countries (France, Spain, Portugal, Italy, Austria, Germany, Poland, Hungary, The Netherlands, Scotland and Sweden), has been involved in a EU-funded project of 4 years, named IMAGE (innovative Management of Animal Genetic Resources) (2016-2020). This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N$^{o}$ 677353 and has been coordinated by INRA institute supported by the *University of Natural Resources and Life Sciences* in Vienna. The aim is to enhance the use of genetic collections and to upgrade animal gene bank management mapping genetic collections all around Europe. For this purpose i) germplasm collections covering biological samples of reproductive material, and ii) genomic collections covering other biological samples (DNA, tissue) have been studied. The target species include chicken, and in particular cattle, sheep, goat, horse and pig. Among them, chickens have been genetically characterized in order to study the level of genetic introgression of rare characteristics from the chicken genebank.

In 2007 in **Italy,** under the coordination of the Catholic University of Sacred Hearth (UCSC) (Piacenza), the GLOBALDIV project was funded by the European Commission to improve the conservation, characterisation, collection and utilisation of genetic resources in agriculture in EU by *in situ* management. The project was realized in collaboration with Kenya, Brazil and three European partners The Netherlands, Germany and Switzerland. Within the framework of this project, 505 different European animal breeds were described. In particular, among poultry breeds: 26 chickens, 3 ducks, 3 turkeys and 7 geese were included.

| The project/ Institution involved | Countries involved | Avian species involved | Conservation program Strategy | Genetic resources |
|---|---|---|---|---|
| **Global plan for the conservation of avian genetic resources** | | | | |
| UC/GRCP | North America | Chicken<br>Turkey<br>Japanese quail | *In situ*<br><br>*Ex situ in vivo*<br><br>*Ex situ in vitro* | Living animals in native environment<br><br>Living animals<br><br>Semen –DNA-Tissues |
| Fowls for Africa (API) | Africa | Chicken | *In situ*<br><br>*Ex situ in vivo* | Living animals in native environment<br><br>Living animals |
| Conservation and use of Animal genetic resources in Japan (MAFF) | Japan | Chicken | *In situ*<br><br>*Ex situ in vitro* | Living animals in native environment<br><br>Semen |
| **European plan for the conservation of avian genetic resources** | | | | |
| The French national cryobank (INRA) | France | Chicken | *In situ*<br><br>*Ex situ in vivo*<br><br>*Ex situ in vitro* | Living animals in native environment<br><br>Living animals<br><br>Semen-DNA |
| Spanish National Institute for Agricultural and Food Research and Technology (INIA) | Spain | Chicken<br>Quail | *Ex situ in vivo*<br><br>*Ex situ in vitro* | Living animals<br><br>Semen |
| Research Institute of Animal Production in Nitra (NPPC) | Slovak Republic | Chicken | *Ex situ in vivo*<br><br>*Ex situ in vitro* | Living animals<br><br>DNA |
| National Research Institute of Animal Production | Poland | Chicken hen | *In situ* | Living animals in native environment |
| Gödöllő Poultry gene bank | Hungary | Chicken<br>Turkey<br>Goose<br>Guinea-fowl<br>Duck | *In situ*<br><br>*Ex situ in vitro* | Living animals in native environment<br><br>Semen |
| Center for genetic resources of The Netherlands (CGN) | The Netherlands | Chicken<br>Duck<br>Goose | *In situ*<br><br>*Ex situ in vivo*<br><br>*Ex situ in vitro* | Living animals in native environment<br><br>Living animals<br><br>Semen |
| IMAGE project | Switzerland +<br>11 European countries +<br>Morocco, Egypt and Colombia | Chicken | *Ex situ in vivo*<br><br>*Ex situ in vitro* | Living animals<br><br>Semen-DNA-Tissues |
| GLOBALDIV project | Italy +<br>3 European partners +<br>Kenya and Brazil | Chicken<br>Turkey<br>Duck<br>Goose | *In situ* | Living animals in native environment |

Figure 3.1. Summary table of global and European plans for the conservation of avian genetic resources by *in situ/ex situ* strategy.

# Chapter 4

## Conservation programs for local rabbit breeds

### *4.1. Interest in local rabbits*

The knowledge of rabbit breeds is widespread throughout the world and particularly in areas where traditional methods of production are still common. The domestic rabbit, belonging to the wild species *Oryctolagus cuniculus* (European rabbit), is one of a few domesticated mammals of Western European origin. Since the beginning of domestication harbour, a number of rabbit breeds created a huge diversity. Such diversity is still not completely known and represents a key to the deciphering of individual breed genesis. These breeds constituted a unique reservoir of genetic variability for rabbits (Carneiro *et al.*, 2011), which is important for dealing with future changes to the environment, evolving markets for animal products and progress in animal production systems. In addition, many native breeds have been treated as a part of cultural heritage.

The role of rabbit breeds selected for meat production, has been dramatically reduced (Jochová *et al.*, 2017). This is due to the fact that, the world production of rabbit meat is mainly based on commercial hybrid lines (mainly New Zealand White and Californian) and a few large breeds that were used for the development of paternal lines (Maj *et al.*, 2009). According to the FAO guidelines (FAO, 2007) most of rabbit breeds have been included in the national programs of genetic resources conservation. The value of these breeds has encouraged many conservation programs (summarized in the figure 4.1), based on *in situ* and *ex situ* strategies at global and European level as reported below.

### *4.2. Global plans for the conservation of rabbit genetic resources*

In 1989, the **Vietnam** Government and scientists applied many efforts to the conservation of animal genetic resources in Vietnam establishing the action plan for sustainable conservation of biological diversity. The aim was minimizing the risks in animal raising and maintaining a gene pool to create new breeds and to meet the demand for breeding new animal races in the future (MAFF, 1995). This conservation program included a wide range of species. With respect to rabbit breeds, the main issues were the rehabilitation and maintenance of local breeds to prevent their disappearance. Some local breeds such as *Vietnam Black, Vietnam Grey, New Zealand and California,* were involved in the conservation program. The main goal was the improvement of *in situ* conservation in native areas. However, this strategy resulted very complex and delicate because of higher susceptibility of rabbits to environmental conditions especially ambient temperature (Suc *et al*., 1996; Zeferino *et al*., 2011). The suitable temperature for rabbit production in Vietnam is 18-28 °C. Higher ambient temperatures can cause infertility in breeding rabbits (low sperm volume and concentration) and reduce feed intake with the consequent decrease of liveweight gain (Suc *et al*., 1996). Due to high sensitivity of rabbit, *in situ* conservation program for small farmers in Vietnam, was supported also by *ex situ* strategy. For each native breed a survey on physiology, genetic and economic related traits were used for collecting informative data to develop alternative stocks with characteristics of native breeds, for meeting a wide variety of market production condition. Moreover, to establish a systematic conservation for rabbit genetic material, semen, embryos, somatic cells and DNA samples were collected in a gene bank.

In 1994, **China** Government enlisted genetic resources preservation into the “National Economical and Social Development Plan” encouraging enterprises and individuals to take part in the conservation and scientific development of animal genetic resources. Subsequently, the State Council issued the "Regulations on Breeding Livestock Administration". The project focused on *in situ* conservation program by the creation of specific areas dedicated to livestock species at risk status. Additionally this program was supported by complementary of modern biotechnology such as embryo, sperm, DNA and other modern freezing conservation technologies and methods. The project covered a variety of livestock species. Among them only two breeds of native rabbit were included. The first breed of interest for Chinese Government, was represented by *Sichuan white* rabbit

characterized by a strong abilities of adaptation, fertility and disease-restack. This breed native of Sichuan Province, presented also the maximum lactation number per year (7) and the largest litter size (11). The second native breed of great interest, was *Wanzai* rabbit widespread in Wanzai country. *Wanzai* rabbit presented a black wool for which it was mentioned as “fire rabbit” or “wood rabbit”. The main characteristic of this breed was represented by the rapid growth capacity.

### *4.3. European plans for the conservation of rabbit genetic resources*

In **France** the first national cryobank was created in 1999 to safeguard the genetic diversity of rabbit breeds (FAO 1998; ERFP 2003; Blackburn, 2004). The project has been funded by the French Agricultural Ministry supported by twelve different organisations involved in genetic resources management including not only rabbits but also different mammalian species cattle, goat, sheep and pig followed by avian species as reported previously. The main partners involved, were the National Agriculture Research Institute (INRA), the Institut français de recherche pour l'exploitation de la mer (IFREMER), the Fondation pour la Recherche en Biodiversité (FRB), and five Livestock Institutes that deal with breeding programs in different species: *Institut de l'Elevage for ruminants*, *Institut Technique du Porc* – IFIP - for pig breeding, *Institut Technique de l’Aviculture* – ITAVI - for poultry and rabbit breeding, IFCE for horse and donkey breeding, SYSAAF - for poultry and fish breeding). In addition the project was cofounded also by the French federation for domestic breeds associations (Races de France), the federation for Animal Insemination Production Centers (*Union Nationale des Coopératives d’Elevage et d’Insémination Animale* - UNCEIA) and ultimately by a veterinary laboratory “*Association pour le Contrôle Sanitaire, l’Etude et le Développement de l’Insémination Artificielle et du Transfert Embryonnaire* (ACSEDIATE). Although the semen has been considered as the simplest and cheapest material to collect in the French national cryobank, for some species such as rabbits, the preservation of genetic resources has been ensured also by embryo freezing. From a genetic point of view, three main types of material to be preserved have been considered: *type I* which includes rare domestic breeds; *type II* which involves rabbits showing a particular genotype from high selected population, that are of scientific interest for research especially in biomedical and pharmaceutical field; *type III* which includes rabbits selected for meat and for fur production of high economic value.

The French national cryobank for rabbit consists of 1253 donors selected from 34 local breeds. Individual data related to selected French breeds, are managed by centralised information systems under the supervision of stockbreeding organizations.

In **Germany** the "National Programme for Conservation and Sustainable Use" was approved by the Conference of Agricultural Ministers in 2003. It was initiated by the working group of the German Society for Animal Production (*Deutsche Gesellschaft fuer Zuechtungskunde*, DGFZ) following the call of the Food and Agriculture Organisation of the United Nations (FAO) for National Reports on the Status of Animal Genetic Resources. The program was established to promote the *in situ* and *ex situ* conservation of different farm animals that were in critical status. The aim of the conservation program for animal genetic resources in Germany, was to enhance attractiveness of animal genetic resources for sustainable animal production systems by means of description, evaluation, documentation and breeding tests. This offered the opportunity to contribute to the conservation and use of agricultural grassland ecosystems and supporting the utilisation of animal genetic resources in nature and landscape protection areas, promoting cooperation at national, European and international level and exploiting the resulting synergies. The actions of the National Programme involved different species including rabbits followed by horses, cattle, pigs, sheep, goats, and avian species. The program focused on the monitoring of population data and the endangerment status of the indigenous breeds. The principal aim was to create a database of all organised animal breeding associations in Germany including approved insemination centres for each animal species and the biotechnology institutions. Hence, the database "Central documentation on animal genetic resources in Germany" (*Zentrale Dokumentation Tiergenetischer Ressourcenin Deutschland*, TGRDEU) was created, however only rabbits were not included. Local rabbits accounted numerous endangered breeds in Germany. Moreover, most of them, were reared at fancy farms managed by numerous local breeding associations that are represented by the National Association of German Rabbit Breeders (Zentralverband Deutscher Rasse-Kaninchenzuechter e.V., ZDRK). Local rabbit breeds were included into five categories. Totally, German rabbit breeds were 72 and were classified in according to their size, small (1.10-3.75 kg), medium (3.25-5.50 kg), large (over 5.50 kg) and their hair body, (short and long haired breeds). Advancement of conservation methods for rabbit breeds in Germany involved also the storage of semen and embryos.

In **Slovak Republic** the first "National gene bank" (*Genova Banka*) has been realized by the "*Research Institute for Animal Production in Nitra"* (NPPC). The maintenance and use of rabbit genetic resources are represented by cryopreservation of semen, embryos and DNA (Makarevich *et al.*, 2008). Currently, the project has given special attention to 5 local rabbit breeds: *Nitra rabbit, Zobor rabbit, Holic rabbit, Slovak Pastel Rex, and Slovak Greyblue Rex*. However, successful cryopreservation protocols have been designed for the first and the second breed only, whilst further studies are needed for finding optimal freezing protocols for the remaining Slovak rabbit breeds. Indeed, the project has been extended to other species including also other mammalians species such as cattle and sheep (Chrenek *et al.*, 2017).

In **Czech Republic** the conservation of rabbit genetic resources followed the European program RESGEN CT 95-060 which was coordinated by INRA institute. The aim was to provide a more comprehensive description of local rabbits and to evaluate ten of them at levels of both genetic diversity and zootechnical characteristics. The study of population and reproduction of the Czech national rabbit breeds, was performed on the base of the Central Herd Book of rabbits registered by Czech Association of Breeders since 2000. Within the conservation program, each breed was identified according to three characteristics: the population size, growth and fertility. The effective population size was evaluated on a number of rabbits in each breed according to the formula of Wright (1931). Whilst, fertility analysis was performed based on specific characteristics such as number of litters, litter size, number of weaned kits and number of registered kits.

The first semen cryobank in **The Netherlands**, for Dutch rabbit breeds was established in 2014 at the Netherlands Centre for Genetic Resources, (CGN). The project has been realized in collaboration with the Dutch Federation of Rabbits and Poultry (KLN) and the Dutch Rare Breed Survival Trust (SZH). Rabbit gene bank enclosed eight rare breeds which are part of a specific data base: *Beige, Deilenaar, Eksterkonijn, Gouwenaar, Havana, Hulstlander konijn, Sallander and Thrianta konijn*. Each breed has been tested by *in vitro* and *in vivo* assay. As reported by artificial insemination test, the frozen semen resulted in 50% pregnancies respect to fresh semen (60-80%) with a mean offspring of seven per litter.

| The project/ Institution involved | Countries involved | Rabbit breeds involved/ Type of material | Conservation program strategy | Genetic resources |
|---|---|---|---|---|
| **Global plan for the conservation of rabbit genetic resources** | | | | |
| Conservation of animal genetic resources in Vietnam | Vietnam | Vietnam black<br>Vietnam grey<br>New Zealand<br>California | In situ<br><br><br>Ex situ in vitro | Living animals in original environment<br><br>Semen<br>DNA<br>Somatic cells<br>Embryos |
| National Economical and Social Development Plan | China | Sichuan white<br>Wanzai | In situ<br><br><br>Ex situ in vitro | Living animals in original environment<br><br>Semen<br>DNA<br>Embryos |
| **European plan for the conservation of rabbit genetic resources** | | | | |
| The French national cryobank (INRA) | France | I: Rare breeds<br><br>II: breeds of interest for research<br><br>III: breeds selected for meat and fur production | Ex situ in vivo<br><br>Ex situ in vitro | Living animals<br><br>Semen<br>DNA<br>Embryos |
| National Programme for Conservation and Sustainable use (DGFZ) | Germany | German national rabbit breeds, 5 categories:<br>Small<br>Large<br>Medium size<br>Short-haired<br>Long-haired | In situ<br><br><br>Ex situ in vitro | Living animals in original environment<br><br><br>Semen<br>Embryos |
| Research Institute of Animal Production in Nitra (NPPC) | Slovak Republic | Nitra rabbit<br>Zobor rabbit | Ex situ in vivo<br><br>Ex situ in vitro | Living animals<br><br>Semen<br>Embryos |
| RESGEN CT 95-060 | Czech Republic + INRA (France) | Czech national rabbit breeds | In situ | Living animals in original environment |
| Center for genetic resources of the Netherlands (CGN) | The Netherlands | Beige<br>Deilenaar<br>Eksterkonijn<br>Gouwenaar<br>Havana<br>Hulstlander konijn<br>Sallander<br>Thrianta konijn | Ex situ in vivo<br><br>Ex situ in vitro | Living animals<br><br>Semen |

Figure 4.1. Summary table of global and European plans for the conservation of rabbit genetic resources by *in situ/ ex situ* strategy.

# Chapter 5

## Conservation programs for Italian poultry and rabbit breeds

### *5.1. Poultry genetic resources conservation in Italy*

Out of 2000 reported avian breeds, 9% (83% of which are chickens), were reported as extinct (FAO, 2007b). The majority of extinct breeds are reported from Europe.

The regions with a greatest proportion of avian breeds classified at risk are North America (79%), and Europe and Caucasus (49%). The global databank indicates that 195 poultry breeds (77% chickens, 9% ducks, 9% geese, 3% turkey) are enclosed in conservation programmes. At global level the preservation of poultry breeds, is ensured by 281 conservation programmes that include *in vivo* (194) and *in vitro* strategies (87). These activities are mainly concentrated in Asia, Europe and Caucasus and North America (Hoffman *et al*., 2009).

As concerns Italy, the number of native poultry breeds has suffered a dramatic decline. As reported by Zanon and Sabbioni (2001) of 90 rural poultry breeds (9 ducks, 11 guinea fowls, 53 chickens, 5 geese and 12 turkeys): 61.0% of these breeds are extinct, 13.3% are endangered and only 6.7% are involved in conservation programmes (Ceccobelli *et al*., 2013) that include only *in situ* strategy and are mainly concentrated in the Northern part of Italy.

In particular in Italy, birds safeguard biodiversity, is recognized as an urgent need because of the great level of standardization and specialization achieved in the poultry sector which is based on the exclusive global rearing of commercial strains (Marelli, 2008). Local breeds adapted to specific environments for thousands of years, and their potential as food makers or their genetic variability are still unexplored. Local poultry breeds may give an interesting different option to commercial lines, supplying high quality products of great interest for local and regional markets (De Marchi *et al.*, 2005). Poultry products, meat and eggs, obtained from native breeds show specific features (De Marchi *et al.*, 2005; Castellini *et al.*, 2006; Zanetti *et al.*, 2011), which distinguish them from standard ones; moreover, breeds can be reared in outdoor free range systems and even used to reintroduce agricultural activity in marginal rural areas. Chicken and turkey breeds still present in Italy, are reared only in fancy farms and the number of birds is very limited.

The inclusion of these breeds in a conservation program is essential for their safeguard and not loosing their peculiar traits, such as high adaptation to the environment and disease resistance, that are closely related to the agro-ecosystem resilience, both in biological and in applied management system (Bittante, 2011; Colli *et al.*, 2011). The activities included in conservation programs improve the characterization and the breeding management of the breed, then the presence and diffusion of the breed in the rural areas is promoted. Furthermore, the increased knowledge and size of the bird populations makes them suitable for innovative poultry productions, according to the increasing consumer demand for high quality food products and niche markets. In 2014 the *Registro Anagrafico delle Razze Avicole Autoctone* (Mipaaf -DM 19536, 1st October, 2014) was established with remarkable delay compared to other domestic animals. The Registro follows the effort of the national legislation in adopting international guidelines aimed to preserve AGRs in avian species and it includes 20 breeds of local chicken and 7 breeds of local turkey (figure 5.1).

Conservation and valorization projects of Italian poultry breeds have been regionally developed since 1990s thanks to the financial support of regional and local public institutions. These projects are only based on *in situ* conservation strategy and included the Italian chicken breeds from Veneto (*Ermellinata di Rovigo*, *Padovana*, *Polverara*, *Robusta lionata, Robusta maculata, Pepoi and Millefiori di Lonigo*), Piemonte (*Bianca di Saluzzo and Bionda Piemontese*), Emilia Romagna (*Modenese and Romagnola*), Marche (*Ancona*) and Toscana (*Valdarno, Livorno, Valdarnese Bianca and Mugellese*). Few projects have also considered turkey breeds such as *Ermellinato di Rovigo* (from Veneto), *Nero d'Italia and Brianzolo* (from Lombardia) (De Marchi *et al.*, 2005; Sabbioni *et al.*, 2006; Zanetti *et al.*, 2010; Ceccobelli *et al.*, 2013; Mosca *et al.*, 2015). In general, the purpose has been to promote *in situ* conservation of small populations at risk, or to perform the genetic and/or productive characterization of the breeds. In order to implement the *in situ* strategy, both the characterization of living population and the careful selection of breeders are essential activities to control the erosion in genetic diversity. Of special interest is the conservation program of the *Valdarnese Bianca* breed from Toscana. The breed was included in the "*Repertorio Regionale delle Risorse Genetiche Autoctone Animali della Toscana*" (LR 50/97) and is the only Italian breed which has been introduced into the "*Registro Anagrafico delle razze avicole autoctone italiane*" since 2005 (Mammuccini, 2006).

While, recognizing *in situ* strategy being the priority, the ideal conservation plan should consist in the integration of both strategies, *in situ* and *ex situ* (FAO, 2008; FAO, 2012).

Regarding the *ex situ in vitro* technique, semen cryopreservation is the only reproductive procedure currently available in avian species because of the unique biological features of birds and DNA is stored in sperm cryobanks (Blesbois, 2007). Cryopreservation has been studied in different poultry species since 1950s. However, it is still a matter of research studies because of the high cellular damage suffered by male gametes during the freezing/thawing process and the consequent severe reduction in fertility (Blesbois, 2007; Blesbois *et al.*, 2007, Cerolini *et al.*, 2007; Santiago-Moreno *et al.*, 2011).

In Europe, (France, The Netherlands, Hungary and Spain) several semen cryobanks have been created for local poultry breeds (Woelders *et al.*, 2006; Blesbois *et al.*, 2007; Santiago-Moreno *et al.*, 2011). Recently, the most feasible cryopreservation procedure has been implemented in order to identify the reference procedure to establish semen cryobank. In fact in birds, semen cryopreservation is not a standardized procedure and its success is still greatly variable and dependent on the species, inter-intraspecies variability and *in vitro* processing. In light of this, the present doctoral thesis aims to find a reference procedure for freezing turkey and chicken semen by implementation of the first semen cryobank of Italian poultry breeds for which *in situ* conservation strategy only has been realized. In this regard, the semen cryopreservation is a valuable tool for the conservation of animal biodiversity by facilitating the storage of gametes in a gene bank by *ex situ in vitro* strategy.

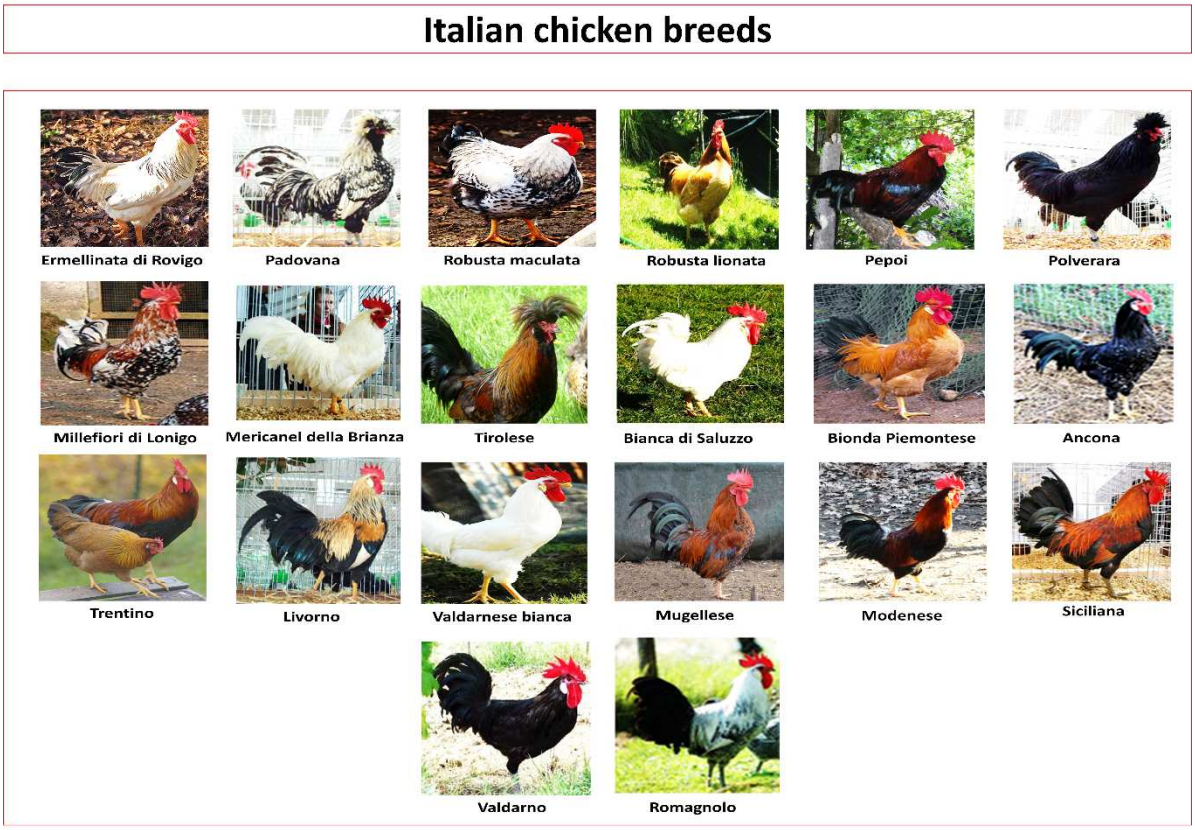




Figure 5.1. List of Italian chicken and turkey breeds (Mipaaf, 2014).

### *5.2. Rabbit genetic resources conservation in Italy*

A full sight of features of local rabbits in Italy, was provided by The "*Registro Anagrafico della specie cunicola*" created by "Italian rabbit breeders association" (ANCI), which promotes institutional activities (Rabbit Genealogic Reference and Rabbit Directory), under the supervision of MIPAAF, the development of Italian rabbit raising and the technical assistance to rabbit breeding farms. This document is directed by ANCI Central Office which is devoted to technical monitoring. The latter, is carried out through 36 Local Offices present in different regions and in cooperation with the Experts Directory in order to obtain a morphologic assessment of reproducers either at the registered breeding farms or at official exhibitions. Local rabbit breeds registered by ANCI are 43 and are classified according to the wool structure and their weight (light, medium, heavy line) (figure 5.2). Although, local rabbit breeds have been well identified by their profile sheet provided by ANCI, in Italy, there is a lack of data about their consistency. Among mammalian breeds, in fact, the "ItAnGRs" DAD-IS database, includes only cattle, goat, sheep, pig, ass and horse (http://dad.fao.org/). At global-level, erosion of farm animal genetic resources (AnGRs) is advancing at high speed and livestock genetic diversity is decreasing. Italy is an example of this decreasing trend. In order to counteract biodiversity losses, solutions are needed, both at global and local level (Pizzi *et al.*, 2016). Local breeds are old populations which arise in the past in specific geographical area characterized by a great adaptation ability and high disease resilience. According to Minister for Agriculture, Farming and Forestry 2008 (Mipaaf), the patrimony of local breeds has to be protected by active national conservation programs. The conservation program for local rabbit breeds will be in line with the development of a sustainable agriculture ensuring the safeguard of genetic resources and leading to the increase of farmers income. Likewise to the poultry species, the Italian projects for rabbit species are only based on *in situ* conservation strategy, whilst nothing has been done about the *ex situ in vitro* strategy. In light of this, the ANCI in collaboration with the University of Molise is going to create the first Italian semen cryobank in order to promote combined use of *in situ* and *ex situ* conservation strategies for the safeguard of rabbit breeds.

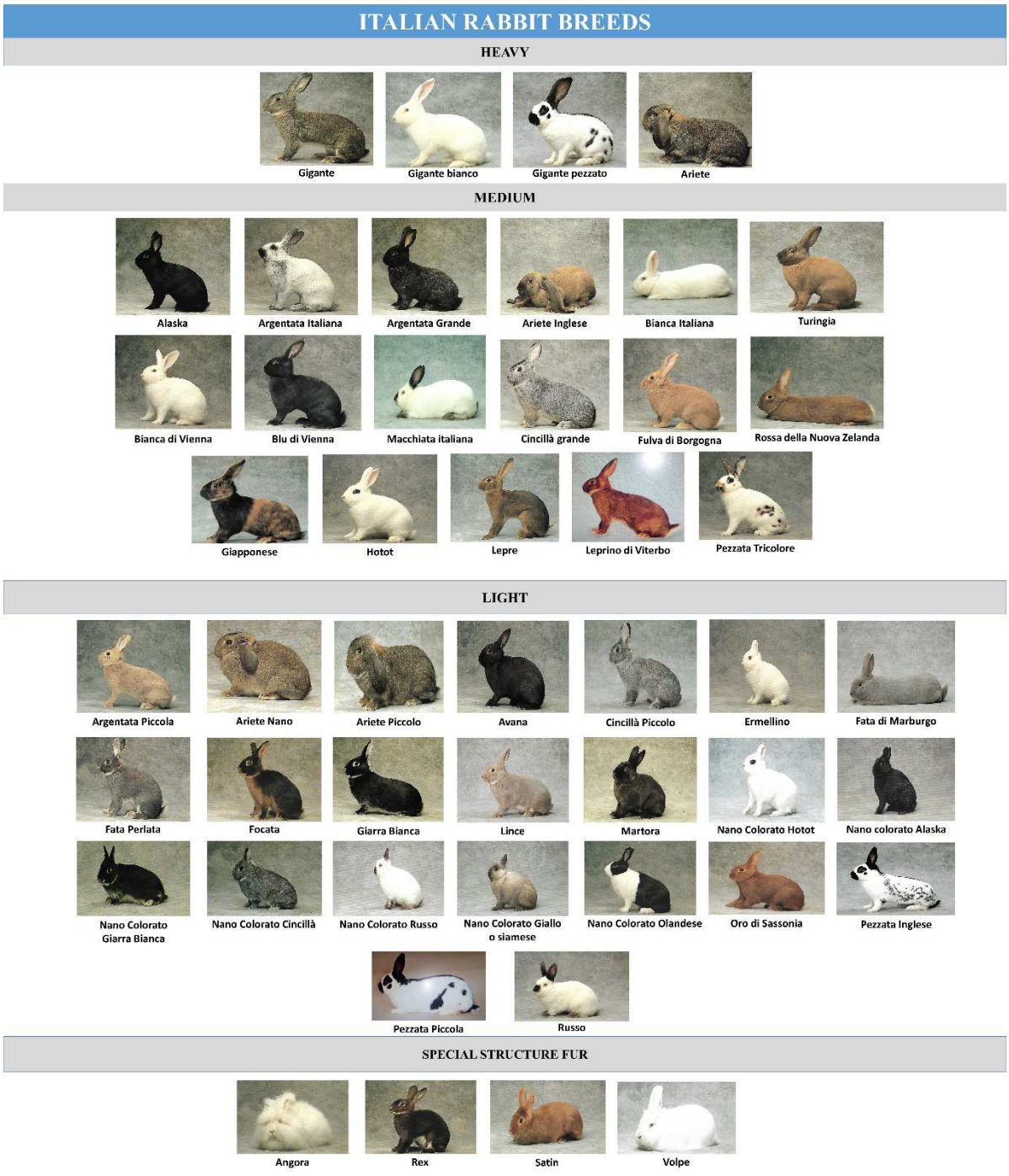



Figure 5.2. List of Italian rabbit breeds (ANCI, 2010).

# Chapter 6

## Ex situ in vitro conservation

### *6.1. Cryobank*

Conservation is only one of the four strategic priority areas of the Global Plan of Action for Animal Genetic Resources, and cryoconservation is one among several options available for conserving AnGRs. Ideally, gene banks should be established within the framework of a national strategy and action plan (NSAP) for AnGR (or similar national programme for AnGR management). FAO has developed guidelines on the preparation of NSAPs (FAO, 2009), which recommend that countries establish National Advisory Committees on AnGRs. One common reason for establishing a cryobank is to provide the possibility of recreating breeds or breeding lines if they are lost as the result of natural calamity. Storage of germplasm for this purpose, is typically long term, and does not involve frequent use of the stored material or necessitate regular updating of the collection (FAO, 2012). When reconstituting a breed from germplasm collections, significant attention must be given to the mating plan because it should be maintained high level of genetic diversity minimizing the consanguinity driven by mates relationship with a common ancestor (Comizzoli *et al.*, 2010).

A second potential purpose is to support *in vivo* conservation. Frozen semen, embryos and other biological substrates, can be used to minimize inbreeding and genetic drift in small managed populations. Moreover, the combination of live animals and cryopreserved germplasm can be a powerful tool in conservation (Hiemstra, 2011).

Material stored in a gene bank may also serve as a back-up of a breed that has been permanently lost. A fourth important use of cryoconserved material, is for the development of new lines or breeds, or for quickly modifying or reorienting the evolution or selection of a population. For instance, storage of original or extreme genotypes can provide the means to redirect the genetic trend of a selected population.

Finally, gene banks can serve as the primary source of material for scientists performing DNA research. Storing isolated DNA along with germplasm in a gene bank, can allow researchers to access genomic information more quickly and without potentially damaging the valuable germplasm. It can also provide access to genetic material from common sets of animals for genotyping research. Material stored in a cryobank includes embryos, oocytes,

somatic cells, primordial germ cells, spermatogonia, semen and epididymal sperm as follows.

Currently, the widespread use of **embryos** cryopreservation is limited to cattle, sheep, goats and rabbits (Prentice and Anzar, 2011). In contrast, this technology is not feasible for birds (Nandi *et al.*, 2016). The embryo cryopreservation has been a very useful tool for embryology since 1972 when the first successful cryopreservation of mouse embryos was performed (Whittingham *et al.*, 1972). This technology is the best method for the long-term preservation of valuable genetic resources from experimental and livestock animals. The use of cryopreservation is also essential for the widespread use of embryo transfer, which allows the exchange of genetics with reduced transportation cost, avoiding animal welfare problems and with a minimal risk of disease transmission. At present, millions of offspring have been born from cryopreserved embryos of more than 40 mammalian species (Saragusty and Arav, 2011). Embryo cryopreservation allows the conservation of the full genetic complement of both dam and sire and has tremendous opportunities for maintaining heterozygosity and population integrity, but it is more complex and costly procedure than semen cryopreservation (Mocè and Vicente, 2009). Moreover, a large number of embryos would be required for complete reconstruction of a population and are unlikely to be available from donor females of endangered breeds (Boettcher *et al.*, 2005). Two basic strategies have ruled the embryo cryopreservation field: the traditional slow freezing, also referred as conventional “equilibrium freezing” or “controlled slow freezing” and vitrification. In the slow freezing process, the embryo is placed in a hypertonic solution, while during vitrification is solidified in a water-based solution forming a glass-like amorphous vitreous state without ice crystal formation.

**Oocytes** are large cells, with a low surface to volume ratio, surrounded by zona pellucida. Oocytes collected by *in vivo* pickup or at slaughter, can be frozen for extended periods of time for subsequent *in vitro* fertilization (IVF) to produce embryos. In the last ten years, considerable progress has been made in the cryopreservation of oocytes. For a long time, IVF rates with cryopreserved oocytes in humans and in other species, had been poor due to release of cortical granules, which makes the zona pellucida impenetrable to spermatozoa; and to the disintegration of the metaphase II spindle. IVF rates have improved since the introduction of intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992). In the most recent years, both slow-freezing and vitrification protocols, seem to be giving excellent results in humans, and both techniques are considered to work equally well (Porcu and

Venturoli, 2006), although there may be more enthusiasm lately for vitrified oocytes (Jain and Paulson, 2006). Fewer data are available from livestock than from humans. This is due to species-specific problems, but also to less incentive to develop and use cryopreservation methods for oocytes in livestock species than in humans. Viable oocytes have been recovered after freezing and thawing in a great number of animal species, i.e. cattle, pigs, sheep, rabbits, mice, monkeys, humans (Critser *et al.*, 1997), goats (Le Gal, 1996), horses (Hochi *et al.*, 1996) and buffaloes (Dhali *et al.*, 2000). Successes have been reported with post-thaw oocyte maturation, fertilization and embryo development in a number of species. Live-born young from embryos produced from cryopreserved oocytes, have been reported in cattle (Otoi *et al.*, 1995; Abe *et al.*, 2005) and horses (Maclellan *et al.*, 2002) as well as in several model species. Freezing the oocytes of avian and fish species has not been successful, mainly because of the large size, high lipid content and polar organization (vegetal and animal pole) of bird and fish ova (Blesbois, 2008). Oocyte banks would enlarge the gene pool, facilitate several assisted reproductive procedures, safeguard female genetics after unexpected death, and avoid controversy surrounding the preservation of embryos (Prentice and Anzar, 2011).

Cryopreservation protocols for **somatic cells** are relatively simple and do not require controlled-rate freezing equipment. This means that establishing the collection, is easy and cheap, and can make somatic cells a very attractive option for gene banking, especially for countries with many breeds and/or limited resources. Vietnam, for example, has adopted cryoconservation of somatic cells as a primary component of its AnGR conservation program (Thui *et al.*, 2013). However, the complexity and costs involved in using somatic cells are much greater than those for other types of cryopreserved material. Manipulation of somatic cells involves cloning and culturing after thawing (or prior to freezing), reprogramming the nuclei and collecting oocytes by ovum pick-up or from slaughtered animals. This technology, was used for a number of livestock species such as sheep, goats, cattle, water buffaloes, pigs, horses, mules, camels, deer and rabbits (Mehrabani *et al.*, 2016). After the maturation of the oocytes, and their enucleation, somatic nucleus is transferred into recipients of the same species. The use of nuclear transfer, means that the original mitochondrial genotype is lost. In addition, cloning involves some ethical issues, concerning about the welfare of cloned animals and the safety of their food products. Many countries in fact, have not yet approved the consumption of products from cloned livestock species. Somatic cells offer a convenient solution for collection and long-term storage of

AnGRs under adverse conditions with limited infrastructure. As such, cryobanks of somatic cells can serve as a back-up source of genetic material for regenerating at-risk livestock breeds.

Only recently the use of **primordial germ cells** (PGCs) as genetic resources, has been experimented in fish and in birds (Bednarczyk, 2014; Psenicka *et al.*, 2015;). In chickens, primordial germ cells usually migrate to the gonadal ridge via the blood stream between four and six days of incubation. During this migration stage PGCs, can be harvested from the blood of a chick embryo, and then cultured and transferred to other developing chick embryos, resulting in germline transfers (Etches, 2010; Nakamura *et al.*, 2016). In quail, male or female primordial germ cells have been successfully transferred into chick embryonic gonads, subsequently replacing the host germ cells (Ono *et al.*, 1996). Germline chimeras have also been reported, with host quail that have subsequently produced live offspring from the donor-quail germ cells (Kim *et al.*, 2005). Using germline transplantation, live offspring have been produced by surrogate birds from other avian species (e.g. pheasant) (Kang *et al.*, 2008). This research area is a promise for future cryoconservation systems. However, this technology needs more time to be recognized as conservation strategy for bird species for which only semen has been considered for establishing a cryobank.

**Spermatogonia** reside within the basal layer of the seminiferous tubules of the testis and have the capacity to give rise to spermatozoa. Beginning before puberty and continuing in the adult animal, spermatogonia undergo continuous replication, thereby, maintaining their number in a process known as stem cell renewal. It was shown in mice (Brinster and Zimmermann, 1994) that these stem cells, when isolated from the testes of donor animals, can be processed and used to repopulate another testis without evidence of immuno-rejection. Kimura and Yanagimachi, (1995) reported the development of normal mice from oocytes injected with secondary spermatocyte nuclei. Frozen–thawed testicular tissue from day-old chicks, has been transplanted into host chicks, resulting in the production of live offspring from the donor tissue sperm (Song and Silversides, 2007b).

Spermatogonia transfer could potentially be used to pass genetic material from one generation to the next, and together with cryopreservation, it offers a means of saving genes from male animals for future generations.

The use of **semen** for cryoconservation of genetic resources, is more advantageous respect to other substrates because its collection is very simple.
In many countries semen is used for artificial insemination procedure (AI). For a number of species such as cattle, small ruminants, horses and pigs, there are many AI centres with dedicated animal housing for semen collection and processing facilities. Such centres may facilitate the acquisition, storage and future use of semen. If dedicated facilities are not available, field collection may be an option; it may be the only means available for collecting material in areas where extensive livestock keeping is practised. Relative to other types of germplasm, semen has the disadvantage that only a single complement of chromosomes is preserved. If a breed is cryoconserved only in the form of semen, and all living animals belonging to the breed are lost, then the only means of reconstituting the breed is to inseminate founder females from another breed of the same species (or the female gamete in the case of other reproductive techniques). A series of backcrossings is then required to restore the breed to its (nearly) original genetic status. By using only the conserved semen (i.e. from the breed that is being recovered) in each generation, the percentage of genes from the founder breed decreases logarithmically, while the percentage of genes from the breed being recovered increases. This means that enough semen must be available to inseminate the required number of animals in the series of consecutive crosses. To restore the "original" genotype of the lost breed, at least four to five generations of backcrossing are required, depending on the level of purity desired in the restored population (Ollivier and Renard, 1995).

In such cases, the collection of **epididymal sperm** post-mortem, may be a good alternative for preserving genetic diversity. Epididymal sperm can be collected from a number of species, but species differ in terms of the numbers of insemination doses that can be produced per male. For example, rams have quite a high semen yield in terms of the number of doses per animal (Ehling *et al.*, 2006). In addition, epididymal ram sperm has been shown to have good freezability and good fertilizing ability in cervical and in laparoscopic inseminations.

### *6.2. Guidelines for establishing a sperm cryobank*

When building a collection of cryopreserved material, the following three principles should be considered (FAO, 1984):

1. conserve small amounts of germplasm from many donor animals rather than large amounts from few donors;
2. choose donors that are as genetically and phenotypically diverse as possible; and store the breeds as pure lines rather than gene pools, allowing the use of the unique combinations of traits and the flexibility of stocks combination.
3. duplicate the material and store the sets of samples at separate locations in order to reduce risks of loss.

The minimum number of donors to establish a cryobank is 25. In most rare breeds, the creation of a cryobank is used as a way to help farmers to have their animals reproducing in pure breed. In this case, it is assumed that a breed can be recreated just by using semen issued from 25 males that are not related (in order to keep inbreeding at a limited level). According to species, the number of semen doses for insemination of females is variable. For poultry and rabbit in particular, 40 doses of semen are necessary to recreate a breed within 6 generations .

According to FAO guidelines, before initiating collection activities, gene bank managers have to assess the various breeds and species in the country in order to establish their risk-status and their prioritization for conservation. This evaluation can be made on the basis of breeds's population size, potential genetic uniqueness, economic importance and/or cultural importance. In general, the decision about conserving a given breed, should be undertaken by the National Advisory Committee on AnGR (FAO, 2011b) or similar committee, in consultation with the wider stakeholder community. Nevertheless, gene bank managers and curators should be able to initiate the collection of germplasm from breeds that are in an extreme state of risk or critical to the country's livestock sector. Various quantitative measures can be used for deciding which breeds should be prioritized for inclusion in collections. In general, two primary factors to determine the conservation priority of a breed have to be considered:

1. level of risk of extinction;
2. conservation value;

1. **The level of risk extinction** of a given breed, is established according to the quantification of the current population size. As reported by FAO, (2015), there are six criteria (that have been previously described in chapter 2) to define an endangered breed. Although many countries undertake periodic censuses of livestock, even if it is possible to obtain only a general overview of the national livestock industry, there is a lack of accurate information about the status of breeds within species. In this regard, national program for the conservation of animal genetic resources, besides to be a national strategy and action plan for their preservation, could be the best solution to lacking availability of data on national breed populations. Gene bank managers in fact, in consultation with relevant national working groups on AnGRs, may initiate their own protocols for surveying and monitoring AnGRs in the same time, gaining information about the consistence of a specific breed.
2. **The conservation value of a given breed** instead, depends on specific characteristics. An important criterion for establishing conservation priorities is the "genetic uniqueness". Understanding the genetic history of particular breeds, or formally estimating genetic distances among breeds, could assist in determining breed uniqueness. In contrast, genetic variation gives to AnGRs the capacity to adapt and allows for genetic response to selection. Conserving the most genetically different breeds, is the most efficient way to conserve the diversity of a species. The adaptation of breeds to specific environments, is likely to be under some genetic control. Thus, the conservation of AnGRs that thrive in specific environments (e.g. those that are in some way harsh), may be important. Moreover, breeds that perform valuable environmental services with special cultural or historic values, are worthy of consideration for conservation. For establishing a cryobank, donors have to be subject to phenotypic and genotypic characterization as described below.

### *6.3. Phenotypic characterization*

Distinct breed is identified according to its external and production characteristics within a given production environment, taking in consideration not only the natural environment but also the management practice. According to the FAO guidelines, there are two phases or levels of phenotypic characterization.

The *primary characterization* refers to activities that can be carried out in a single visit to the field such as measurement of animals, morphological features, interviews with livestock keepers, observation and measurement of some aspects of the production environment, mapping of geographical distribution.

The *secondary characterization* is executed to describe activities that require repeated visits including the measurement of the productive capacities (e.g. growth rate, milk, eggs and meat production) and the adaptive capacities including resistance or tolerance to specific diseases of breeds, measured by immunological tests, in specific production environments. There are different types of breed populations that can be identified:

*Traditional populations*: mainly local; often exhibiting large phenotypic diversity, are managed by farmers and pastoralists at low selection intensity, but may be subject to high natural selection pressure; pedigree may be partially known; genetic structures are mainly influenced by migration events and mutations; population size is generally large (unless subject to erosion).

*Standardized breeds*: derived from traditional populations by a community of breeders based on a recognized list of "standard" breed descriptors; exhibit less phenotypic diversity as they are selected to meet minimum standards of phenotype; pedigree is partially known; genetic structure may be influenced by important founder effects; population size may be large or small.

*Selected breeds or commercial lines*: derived from standardized breeds or from traditional populations through the application of an economic selection and the use of quantitative genetic methods; breeders are organized for pedigree and performance recording, and selected animals are used across flocks or herds; inbreeding increases as a consequence of high selection intensity; molecular markers may be used, for instance for parentage testing and/or for the identification of genes controlling performance; population size is generally large.

*Derived lines*: arise from the use of specific breeding methods such as close inbreeding; highly specialized inbred lines exhibit low genetic variability; synthetic lines are derived from crossing standardized breeds or selected lines, and exhibit a high level of genetic variability; transgenic and experimental selected lines fall within this category; population size is generally limited, except for synthetic lines.

### *6.4. Genetic characterization*

To establish the relationship between breeds and measure the level of genetic variation (heterozigosity), phenotypic characterization, is accompanied by molecular genetic analysis. Global program for characterization of AnGRs, including molecular genetic characterization, was introduced in 1993 with the secondary formulation of FAO guidelines for measurement of domestic animal diversity (MoDAD) (FAO, 1993). So far, most studies (Groeneveld *et al.*, 2008) have analyzed highly polymorphic microsatellite markers, which consist of repeated sequences of 1 to 6 base pairs (bp). Variability is in terms of the number of repeated sequences observed. Microsatellites do not encode proteins and are thus, assumed to be selectively neutral. They are now available for most livestock species and have proven their value for studying variation within and across breeds. FAO and the ISAG–FAO Advisory Group on Animal Genetic Diversity, have proposed panels of 30 microsatellite markers for nine major livestock species (cattle, buffalo, sheep, goat, horse, donk, camelid, pig and chicken). The many existing datasets from completed characterization studies that have used FAO markers, allow new data to be compared with more breeds than any other microsatellite panel. Ideally, studies should characterize the target populations using all 30 markers. This approach yields more accurate data than using a subset of the markers, and offers more opportunity for comparisons with results from previous studies undertaken with various subsets of the 30 markers. Microsatellite markers, are particularly indicated for characterizing local breeds considering their genetic variability in a global context. However, the determination of the number of repeats has poor reliability across laboratories and integration of data can thus be problematic.

A novel marker, is represented by single nucleotide polymorphisms (SNPs). As the name indicates, a SNP is a DNA sequence variation that occurs through a change in the nucleotide at a single location within the genome of a species or breed. SNPs usually have only two alleles. Generally, they can occur throughout the genome and may represent either neutral or functional genetic diversity. A variety of methods, can be used for assaying SNPs, including approaches based on hybridization, selective polymerization, and post-amplification analysis. The large number of SNPs can allow a description of individual and breed relationships with unprecedented accuracy and has the potential to supplement or substitute pedigree data. In addition, these markers are less expensive than microsatellites. However, high density screen SNP requires an equipment that is very expensive and this aspect represents the limiting factor for full genome sequencing.

More recently, mitochondrial DNA (mtDNA) markers, have been introduced. These markers are almost exclusively maternally inherited and allow assigning animals, to a precise maternal lineage. They have been an important tool for identifying wild ancestors, localizing domestication centres and reconstructing colonization and trading routes (Bruford *et al.*, 2003; Ajmone-Marsan *et al.*, 2010; Groeneveld *et al.*, 2010). Although most studies have been performed using the hypervariable control region (D-loop) as mtDNA target, complete mtDNA sequences resulted more advantageous providing more information by establishing the relationship between haplogroups (Achilli *et al.*, 2008). However, this technique is very complex and sometimes, it requires the use of long-range polymerase chain reaction (PCR) amplifications and homologous primers complementary to their target regions which are very expensive.

### *6.5. Structural organization of a cryobank*

The size and capacity of a cryobank varies substantially from country to country, and depends upon the types and amount of equipment needed. The quantities of germplasm to be placed in a cryobank, are associated to the objectives, the range of species and breeds to be conserved, and the financial resources available for the conservation program.

At structural level, a cryobank for livestock species, is composed of a single- or multiple-use facility, such as the laboratory for germplasm acquisition, processing and cryopreservation that do not need to be necessarily located in the same physical place. The gene banking process comprises three main activities: 1) collection of the germplasm; 2) processing and freezing of the germplasm; and 3) storage of the germplasm. These operations can occur in the same place but within separate facilities. The collection is performed in animal holding facility (figure 6.1) while germplasm processing and freezing, occurs in a specific laboratory (figure 6.2). The repetition of these operations is executed more times for each donor to obtain sufficient amount of germplasm collected.

As first step, animals are transferred to the cryobank for holding. Then, all incoming animals are placed into quarantine systems based on all in/all out policy to prevent the spread of diseases among them. This is in accordance with standards for holding facilities of Organisation for Animal Health (OIE). Once the quarantine is lifted, the animals can proceed back and forth to a separate collection building, after an accurate sanitization.

In case of animals with questionable health, they always must be housed separately from healthy animals, avoiding direct contact with them or common use of some spaces such as the collection facility. As established by FAO guidelines, the cryobank presents determinate characteristics. First of all, it has to be located in a low animal density area and possess an appropriate ventilation system to maintain the right level of humidity. In addition, the animal holding building should be placed at the distance of 3 Km from other farms to prevent biological risk and at 1 Km from railways or main roads, in order to safeguard animals.

The installation must have a perimeter fence to prevent the entry of other non-authorized persons or other domestic or wild animals and loading bay for donor animals, has to be localized outside the perimeter fence. Moreover, each facility should be divided into two areas: the clean area where laboratories, instrumentations rooms, germplasm storage facilities are placed and the dirty area where are located the storage area for waste material and feed, buildings animals and collection pens.

Regarding the staff, it should work exclusively in the cryobank and not have contact with other farms or facilities that house animal species from which material is stored in the gene bank. The clothes and footwear used in the laboratories should be stored in the changing facilities and not used or otherwise taken outside.

The management of waste from the facility should be organized to reduce the risk of disease transmission. Storage facilities from which waste is loaded and transported away, must be outside the perimeter fence.

Carcasses of animals that accidentally die in the facility, should be taken away as quickly as possible and stored outside the perimeter fence until they are collected, keeping them away from wild or stray birds. Moreover, to protect animals from the risk of contamination, each facility should be protected from the entry of other species such as rodents and birds using rodenticides or installing traps or bird-proofing nests for birds.

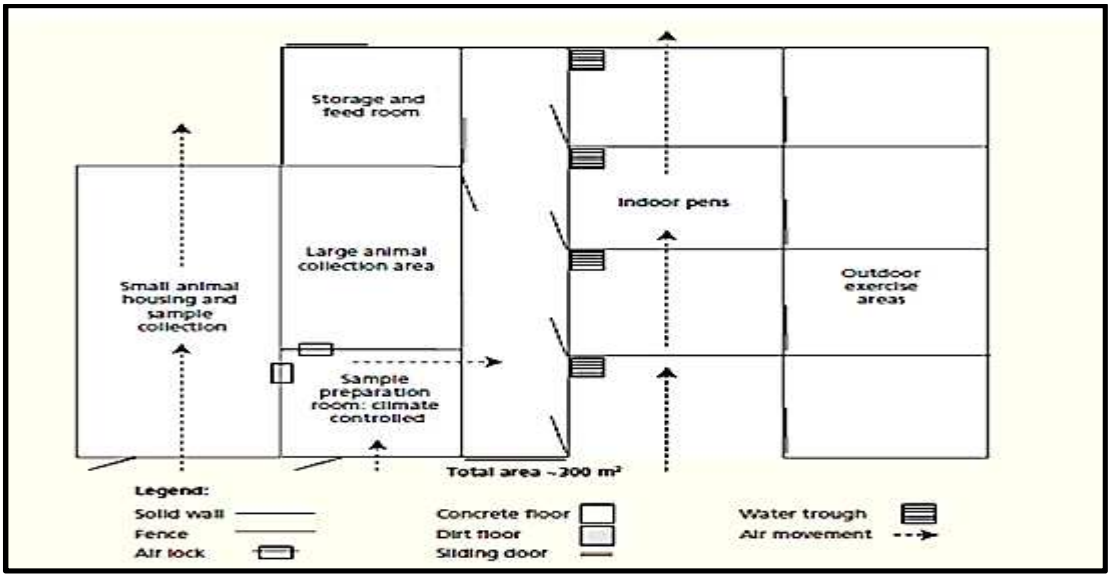



Figure 6.1. Example of the project about animal holding and facility in a cryobank according to the FAO guidelines (FAO, 2012).

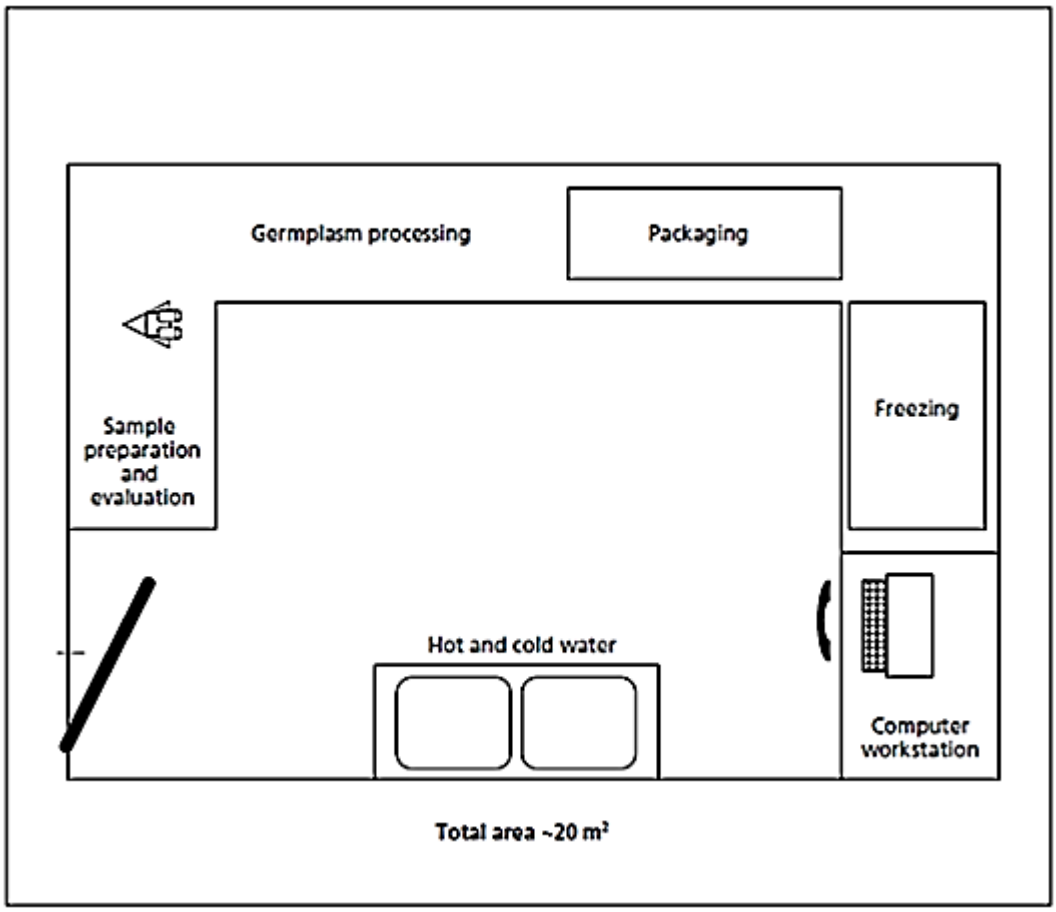



Figure 6.2. Design of a germplasm processing and freezing laboratory (FAO, 2012).

# Chapter 7

## Semen cryopreservation

Cryopreservation is the freezing of sperm at subzero temperatures, typically -196ºC. This temperature is the boiling point of liquid nitrogen, a common agent using in the freezing and storage process. At this temperature, all biological activities are stopped or paused until thawing. Sperm cryopreservation represents an important tool for the long-term storage of sperm, and can provide a year-round supply of paternal genetics from seasonal breeders. This technique is useful for freezing semen from genetically superior males for later use in AI of a large number of females, to transport semen for long distances, and for breeding programs such as cross breeding, genetic studies or conserving endangered animal species. Semen cryopreservation procedure consists of several critical steps such as semen dilution and the addition of the cryoprotectant. Semen dilution is performed by using specific extender whose composition is almost similar to that of seminal plasma and it is important because it provides nutrients and energy for sperm activities. The extender should be supplied with some nutrients needed for the metabolic maintenance of the sperm cell, control of the pH and osmotic pressure of the medium (Gadea, 2003), and in some cases with specific additives such as antibiotics (Morrell and Wallgren, 2014) and antioxidants (Bansal and Bilaspuri, 2011), which are able to inhibit microbial growth and oxidative stress respectively. In addition, the extender has to be supplemented with specific cryoprotectants (CPAs) which provide protection to spermatozoa and minimize the adverse effects of cryopreservation (Lemma, 2011). Sperm cryopreservation is considered a secure method for the *ex situ* preservation of biodiversity in different animal species (Blesbois *et al.*, 2007 a, b; Leroy *et al.*, 2011; Iaffaldano *et al.*, 2011; Zaniboni *et al.*, 2014) by facilitating the storage of their gametes in a gene bank. It can also be used to assist reproduction in infertile men and testicular tumors or prostate cancer patients (Marmar, 1998; Pesch and Bergmann, 2006). Semen cryopreservation has been widely used in the cattle industry, less used in other livestock species, such as pigs or sheep.

Generally, there are two freezing procedures: slow freezing (conventional freezing) which involves step-wise decrease in temperature and ultra-rapid freezing (vitrification or similar vitrification state), accomplished through the use of high cooling that solidifies the sample into a glass-like state, thus avoiding the formation of ice.

**-Conventional freezing** is the main technique to cryopreserve sperm cells by using liquid nitrogen vapours or programmable freezer and involves step-wise decrease in temperature. However, during this procedure the ice crystal formation could occur leading to deleterious effects on cell that can be prevented by the use of cryoprotectants (CPAs).

**Vitrification** is a cryopreservation that solidifies the sample into a glass-like state, thus avoiding the formation of ice. This is accomplished through the use of high cooling rates and high concentrations of CPAs (Liebermann *et al.*, 2002), that appeared not achievable on male gametes, due to their cytotoxic effect inducedby the high concentration. However, recent works on sperm cryobiology have demonstrated that sperm of some species can survive to a similar vitrification state achieved after ultra-rapid non-equilibrium freezing of semen excluding permeable CPAs (CPA-free vitrification; Nawroth *et al.*, 2002; Isachenko *et al.*, 2003, 2004).

**Semen cryopreservation** involves several steps, each one affecting sperm structure and function (Garner *et al.*, 1999; Bailey *et al.*, 2003): extension, cooling, CPA addition, freezing, and thawing (Bailey *et al.*, 2003). The semen cryopreservation process imposes numerous damages related to cell membrane (plasma and mitochondrial), acrosome and, in some cases to the nucleus also, with devastating consequences for sperm survival. The ice crystals formation inside of the cell, represents the main responsible of the injures of cellular membrane. During the process of cooling, freezing and thawing, the spermatozoa are subject to a series of drastic changes in their physical and chemical environment. The first change on spermatozoa occurs during cooling from room temperature to the near freezing point of water. A second change in the environment of spermatozoa takes place when liquid water is converted into ice (commonly referred as ice crystal formation). Finally, there is a spontaneous ice nucleation which usually occurs after the solution is supercooled at a temperature between -5°C and -15°C. Moreover, during cryopreservation, some metabolic changes affect sperm capacity to maintain ATP level, which is essential to support sperm motility (Long, 2006). In particular, these damages include loss of membrane selective permeability and changes in head plasma membrane fluidity (Buhr *et al.*, 1989; Canvin and

Buhr, 1989), swelling and breakage (Hammadeh *et al.*, 1999), and severe impairment of sperm motility and viability (Bailey and Buhr, 1994; Peris *et al.*, 2004, 2007).

The plasma membrane is the primary site of freezing injury to sperm and the principal damage that takes place during the freeze/thaw cycle (Hammerstedt *et al.*, 1990; Parks and Graham, 1992). As the temperature falls below 0°C, ice crystals form in the extracellular medium. Solute aggregates in the fluid portion and elevates the osmolarity of this unfrozen solution. Consequently, once the sperm are subject to this osmotic gradient, intracellular water diffuses out of the sperm, thus dehydrating the sperm or undergoing intracellular ice formation. The intensity of dehydration, depends on many factors such as the cooling velocity and membrane water permeability. At thawing, these processes occur in reverse as the extracellular ice crystals melt and water diffuses into the cell (Watson, 1995; Devireddy *et al.*, 2000; Bailey *et al.*, 2003; Morris *et al.*, 2006). Cells which have a severe loss of intracellular water and/or undergoing intracellular ice formation, are rendered osmotically inactive, due to the loss of cell membrane integrity. The ability of spermatic cells to cryosurvive, is species-dependent and is related to the freezing procedure applied (speed of freezing/warming, samples volumes, kind and concentration of CPA employed), the structural composition of sperm (i.e. different cholesterol/phospholipids ratio of membrane influencing membrane fluidity; different amounts of osmotically inactive water bound to several macromolecular structures such as DNA, histones, hyaluronidase; different molecular weight components in spermatozoa which affect the viscosity and glass transition temperature of the intracellular cytosol.

### *7.1. The cryoprotectants*

One of the most critical point during sperm cryopreservation is the cryoprotectant (CPA) addition. There are two types of cryoprotectants: permeating (P-CPAs) and non permeating ones (N-CPAs).

#### *7.1.1. Permeating cryoprotectants*

**P-CPAs** permeate the cell membrane increasing membrane fluidity. During this process, the CPA goes into the cell leading to water efflux and partial cellular dehydration, lowering the freezing point. This reduces the amount and size of intracellular ice crystals.

The paradox is that the permeating CPAs themselves can have a toxic effect on sperm (membrane destabilization, protein and enzymes denaturation) related directly to the concentration used and the time of cell exposure.

The main P-CPA sused are: dimethyl sulfoxide, acetamide, dimethylacetamide, ethylene glycol, formamide, methylformamide and dimethylformamide.

***Dimethyl sulfoxide (DMSO)*** is the most widely and successfully used low molecular weight or permeating CPA. It is an amphipatic molecule soluble in both aqueous and organic media and is commonly used as a solvent. The use of DMSO as cryoprotectant was first reported in the literature in 1959 by Lovelock and Bishop (1959) as an alternative to glycerol for the cryopreservation of human and bovine red cells. The optimal concentration for most cryopreserved cells is 5-10%. The ability of DMSO to scavenge oxygen free radicals particularly the hidroxy radicals, may also contribute to its effectiveness as a CPA. Another success factor is attributed to DMSO because of its ability to preserve fluidity of plasma and mitochondrial membranes at temperatures below 5°C. Presumably, this allows diffusion of both solvents and solutes through cell membrane and counteracts cell shrinking and swelling due to osmotic pressure changes during freezing (Swain and Smith, 2010).

***Acetamide*** (acetic acid amide, ethanamide) was a component of the cryoprotectant solution utilized by Rall and Fahy (1985) in their pioneering report of embryo vitrification. Permeability to acetamide in relation to other cryoprotectants has been examined in mouse oocytes and various stages of mouse embryos where it was shown, that its permeability increased sharply after the 8-cell stage.

***Dimethylacetamide (DMA)*** is a derivative of acetamide, which has been recently used as cryoprotectant for freezing sperm cell. Particularly in hare and rabbit sperm,it showed a cryoprotective effect that was lower than that observed using DMSO (Kodrowski *et al.*, 2009; Hall *et al.*, 2017). While recently, DMA achieved good results as cryoprotective agent in frozen semen from chicken and (Gliozzi *et al.*, 2011; Madeddu *et al.*, 2016; Mosca e al., 2016) turkey species (Iaffaldano *et al.*, 2009; 2011; 2016a).

***Ethylene glycol (EG)*** is the most often used CPA in embryo cryopreservation as it has the lowest toxic effect and it can rapidly diffuse into cell through the zona pellucida and the cellular membrane (Emiliani *et al.*, 2000). Rapid permeation and removal of EG from the cell and lower exposure time, increases protection from osmotic and toxic injury. Thanks to these characteristics, EG has been successfully used for the cryopreservation of equine (Mantovani *et al.*, 2002) and chicken spermatozoa (Seigneurin *et al.*, 2013; Mphaphathi *et*

*al.*, 2016). In addition, in our recent work EG improved the post-thaw motility of rooster semen compared to DMA, MA and DMF (Miranda *et al.*, 2018).

***Formamide*** is classified as amide, which is derivative of ammonia, and it includes two specific compounds ***methylformamide (MF)*** and ***dimethylformamide (DMF)***. These compounds offer an alternative to more conventional cryoprotectants such as glycerol and may create less osmotic stress for sensitive species/cells through their low molecular weight and low viscosity.

Both MF and DMF, are reported as adequate alternative cryoprotectants for use with goat canine and stallion sperm (Bezerra *et al.*, 2011; Mota-Filho *et al.*, 2011; Gibb *et al.*, 2013 respectively), yielding equivalent or better survival to that seen with glycerol. However, neither MF nor DMF provided efficient cryoprotection for mouse (Sztein *et al.*, 2001) and rabbit (Hanada and Nagase, 1980) sperm compared with other amides tested. DMF has also been used to successfully preserve sperm of different avian species including goose (Lukascewicz, 2002), guinea fowl (Varadi *et al.*, 2013) and rooster (Hanzawa *et al.*, 2010).

### *7.1.2. Non permeating cryoprotectants*

In order to mitigate the cryodamages caused by permeating CPAs which result toxic at high concentration, non-permeating cryoprotective substances (N-CPAs) such as proteins, or amino acids and sugars, acting mainly as osmoprotectants, could be added in freezing media (Blanco *et al.*, 2011). These substances are less toxic than P-CPAs at the same concentration, inhibit ice growth and help the sperm to stabilize its concentration of internal solutes under osmotic stress, reducing the amount of permeating CPAs needed (Iaffaldano *et al.*, 2016b).

The most common **N-CPAs** used include:

- sugars
- macromolecules or polymers
- other molecules

**Sugars** such as mono-, oligo- and polysaccharides, are used by various animal species in nature as a means for protection from cold climate (Fuller *et al.*, 2004). Monosaccharides dissolve in solution more readily than disaccharides and vitrify at lower temperatures. However, because monosaccharides are prone to non-enzymatic glycosylation which can result in protein interactions, are less preferred respect to di-and polysaccharides in many cryopreservation protocols.

The most common sugars that have been used as non permeating CPAs in sperm freezing protocols are: glucose, sucrose, trehalose and lactose.

***Glucose*** is used as energy source by gametes and is therefore included in most culture media in millimolar concentrations. As a result, glucose is present in most cryopreservation media as part of the basic solution though not commonly mentioned as a specific cryoprotective agent. It has been mainly used for mammalian semen for boar, rabbit and human species (De Los Reyes *et al.*, 2002; Iaffaldano *et al.*, 2014; Liu *et al.*, 2016). Recently it has been used also for semen of teleost fishes (Cierezko *et al.*, 2014; Nynca *et al.*, 2014; Iaffaldano *et al.*, 2016c).

***Sucrose*** is naturally occurring sugar found in systems able to tolerate freezing in nature and as a result, it has received great attention in cryobiology and has been used extensively in other cell systems because of its apparent unique properties. Often it has been used in combination with permeating CPA such as DMSO or DMA in different species. In avian species in particular, it has been shown that its combination with DMA (6-10%) reported the highest value of total motility in turkey sperm (Blesbois *et al.*, 2008).

***Trehalose*** is a natural alpha-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units which modulates sperm membrane fluidity by inserting itself into membrane phospholipids bilayer, thus rendering membrane more stable during freezing process. Recently, it has been used in a number of mammalian species such as boar (Athurupana *et al.*, 2015), bull (El-Sheshtawi *et al.*, 2015), ram (Dolti *et al.*, 2016), stallion (Da El-Badri *et al.*, 2017) and rabbit (Zhu *et al.*, 2017). Moreover, it has been tested for the cryopreservation of chicken semen alone or in combination with sucrose (Mosca *et al.*, 2016; Miranda *et al.*, 2018).

***Lactose*** is the disaccharide commonly found in milk and made up of one molecule of glucose and one of galactose. Lactose has been used as a cryoprotecting agent for bovine sperm and proved no different to other disaccharides tested (sucrose or arabinose), though all were better than the monosaccharides tested. Lactose was also used as a cryoprotectant with hybrid mouse sperm; although it did not preserve motility post-thaw as well as sucrose or trehalose, it did produce sperm with high fertilization capability (Sztein *et al.*, 2001). Recently, this substance was used as cryoprotectant also for semen of boar, lama and teleost fish (Silva *et al.*, 2015; Carretero *et al.*, 2015a; Vasconcelos *et al.*, 2015).

**Macromolecules or polymers** are often included into semen extenders though not required as additional protective additives in cryopreservation media. These compounds promote vitrification of a solution and thus may help to reduce toxicity allowing the concomitant decrease in concentrations of potentially harmful permeating agents. Macromolecules tend to increase viscosity of the cryoprotectant solution and also, for interactions through hydrogen bonding with water, thereby decreasing the propensity for ice crystal formation. Lipid solubility of the substance (which increases permeability) and hydrogen bonding (which decreases permeability) are the main critical factors. In general, permeability decreases as the molecular size of the substance increases (Best, 2015). Compounds that are less viscous, interact more with water (less self-interaction), vitrify at higher temperatures, reduce critical cooling rate by at least an order of magnitude, and penetrate cell membranes more readily (Wowk *et al.*, 1999; Best, 2015). Among them, ficoll, polyethylene glycol, and polyvinilpyrrolidone are included.

***Ficoll*** is a non ionic synthetic polymer of sucrose and it could also be classified as a polysaccharide. Ficoll is included as one of the several ingredients in various vitrification protocols for oocytes from several species including mouse, cat, pig, cow, monkey, humans (Jin and Mazur, 2014; Fernandez- Gonzalez and Jewgenov, 2017; Huang and Zhao, 2008; Zhou *et al.*, 2014; Yeoman *et al.*, 2001; Saragusty *et al.*, 2011 respectively). Further, it is also used for rabbit and human embryos (Makarevich *et al.*, 2008; Konc *et al.*, 2014). Additionally, the use of ficoll as non permeating cryoprotectant was extended also to sperm cell, reporting good results in rabbit semen (Kuliková *et al.*, 2015). Finally, in our recent work ficoll improved the *in vitro* post-thaw quality of rooster semen (Miranda *et al.*, 2018).

***Poliethylen glycol (PEG)*** even if it is also an alcohol derivative, this compound is a non permeating CPA (Swain and Smith, 2010). It has also been used to improve mouse oocytes vitrification over DMSO alone (Neil *et al.*, 1997) and in conjunction with other cryoprotectants to successfully freeze human oocytes (Liebermann *et al.*, 2003). In addition, mouse, bovine (Ohboshi *et al.*, 1997) and rat blastocysts (Kono *et al.*, 1988) have also been cryopreserved using polyethylene glycol as a protecting agent (Swain and Smith, 2010).

***Polyvinilpirrolidone (PVP)*** was the first N-CPA used to successfully cryopreserve 8-cell mouse embryos at -79°C for 30 min giving subsequently the first birth of mammal following cryopreservation. It is a viscous solution that allows for easier sperm micromanipulation by slowing or decreasing the motility of sperm for intracytoplasmic sperm injection (ICSI). However, in some reports PVP has been shown to cause significant damage to human sperm membranes that can be detected by transmission electron microscopy.

In addition, it has been associated with chromosomal abnormalities in pregnancy derived from ICSI embryos (Kato and Nagao, 2015). Recently, it has been successfully used for cryopreservation protocol of wild red jungle fowl (*Gallus Gallus murghi*) semen (Rakha *et al.*, 2017).

**Other molecules** used as N-CPAs are egg yolk and low density lipoprotein.

***Egg yolk*** has been used as non P-CPA characterized by high capacity to protect sperm cell from cryodamage during freezing process. This is due to the richness in low density lipoprotein (LDL) that confer the best resistance to cold shock and the best survival in sperm storage. Egg yolk has been widely used in semen freezing extenders and has proved its cryoprotective power especially for mammalian spermatozoa including boar (Hu *et al.*, 2006), dog (Bencharif *et al.*, 2008), buffalo (Akhter *et al.*, 2011), ram (Moustacas *et al.*, 2011), horse (Moreno *et al.*, 2013) and rabbit (Iaffaldano *et al.*, 2014) species. However, the use of egg yolk as a CPA has several drawbacks besides being a source of bacterial contamination (Moreno *et al.*, 2013) and some of its constituents could have detrimental effects on spermatozoa. Accordingly, the granules found in egg yolk were observed to reduce the respiration and motility of bull spermatozoa (Amirat-Briand *et al.*, 2004), and progesterone in egg yolk has been described as potentially responsible for the capacitation of spermatozoa and thus harmful for the preservation of spermatozoa during freezing (Bowden *et al.*, 2001; Moreno *et al.*, 2013). Therefore, the problems described above, could be overcome by adding extracted LDL to the extender rather than whole egg yolk (Iaffaldano *et al.*, 2014).

***Low density lipoprotein (LDL)*** appears in the soluble fraction of egg yolk called plasma. They are spherical molecules of about 35 nm in diameter and consist of a lipid core of triglycerides and cholesterol esters surrounded by a layer of phospholipid and apoprotein film (Moussa *et al.*, 2002) giving them a composition of about 87% lipids, 12% proteins (Anton*et al.*, 2003). The capacity of LDL to protect sperm against cryoinjury, is due to their ability of forming a film at the interface between the fatty acids and water, leading to the physical stabilization of the sperm membrane and are able to adhere to the sperm membrane causing influx of phospholipids and cholesterol. In addition, in some species such as bovines, it has been shown that LDL binding to seminal plasma proteins, inhibit sperm membrane destabilization, enhancing spermatic capacitation and reducing the detrimental effect of cholesterol and phospholipids, in sperm cells subject to cryopreservation (Manjunath *et al.*, 2002; Bergeron *et al.*, 2004).

Recently, LDL has been successfully used as cryoprotectant for sperm freezing protocols including rabbit (Iaffaldano *et al.*, 2014), dog (Prapaiwan *et al.*,2015) and ram (Loaiza-Echeverri *et al.*, 2015) species. In addition, LDL has been assessed for effectiveness as a cryoprotectant in the extender to improve the quality of frozen-thawed rooster semen (Shaverdi *et al.*, 2015).

# Chapter 8

## Reproductive system notes, semen production and storage in *Gallus Gallus* and *Meleagris Gallopavo*

### *8.1. Poultry male reproductive system*

Male reproductive tract in poultry species consists of three components: testes, epididymis, ductus deferens and phallus (figure 8.1). The testes have two functions, to produce sperm and the male hormone, testosterone. Grossly, the testes are either oblong or cylindrical in shape, smooth on the surface, and creamy-white or without color, although they may be partially or totally pigmented. The testes are very small and usually avascular. In a mature bird, the testes can vary in size and greatly enlarge during the breeding season. In birds with distinct breeding cycles, the testes atrophy after a period of active sexual stimulation. Microscopically, the testis consists almost entirely of tubular structures known as seminiferous tubules. Two types of cells line these tubules, spermatogonia cells and Sertoli cells (Cerolini *et al.*, 2008).

The spermatogonia cells proliferate and differentiate through definite stages of development to form sperm (figure 8.2). Spermatogonia initially multiply and grow to form considerably enlarged cells called primary spermatocytes. These cells then start a period of maturation in which the first maturation division forms secondary spermatocytes and the second maturation division forms the spermatids. Each spermatid develops into a spermatozoan. Sertoli cells are large cells interspaced between spermatogonia which extend from the base of the seminiferous epithelium to the interior of the tubules. Spermatids attach themselves to the Sertoli cells and some specific relationship seems to exist between the two cell types which cause the spermatids to change into active sperm.

Seminiferous tubules of immature males are small and lined by a single layer of cells. The mature testis has large irregular-shaped tubules with a multi-layered germinal epithelium consisting of cells representing all stages of spermatogenesis. This is what causes the testis to swell in size during the breeding season. During this phase, interstitial Leydig cells located between seminiferous tubules, produce testosterone which is responsible for a variety of secondary sex characteristics such as male sexual behaviour (including song),

feather form and color (if different from the female), and the development of a comb and wattles in some species.

The networks of seminiferous tubules (from the testis) unite in the epididymis and the contents, flow into and through the ductules, emptying into the ductus deferens. In birds, no capacitation of sperm is necessary for fertilization to take place (sperm taken directly from the ductus deferens can fertilize). No accessory sex glands are present, so seminal fluids are composed of secretions originating only from the testis, epididymis, and ductus deferens. During the erection an engorgement of the phallic structures in the cloaca can be made to pass through the epithelial lining and intermix with the seminal fluid.

Due to the anatomical variation of the phallic region in different birds, semen collection techniques are variable. There are two main varieties of phallus in male birds, the truly "intromittent" organ as seen in ratites (ostrich) and anseriforms (swans, geese) and the "non-intromittent" type which is present in Galliformes including chicken, turkey, quail and some passeriforms (figure 8.3). Their non intromittent phallus consists of folds and bulges that make contact with the female's cloaca at mating time (Coles *et al.*, 2007).

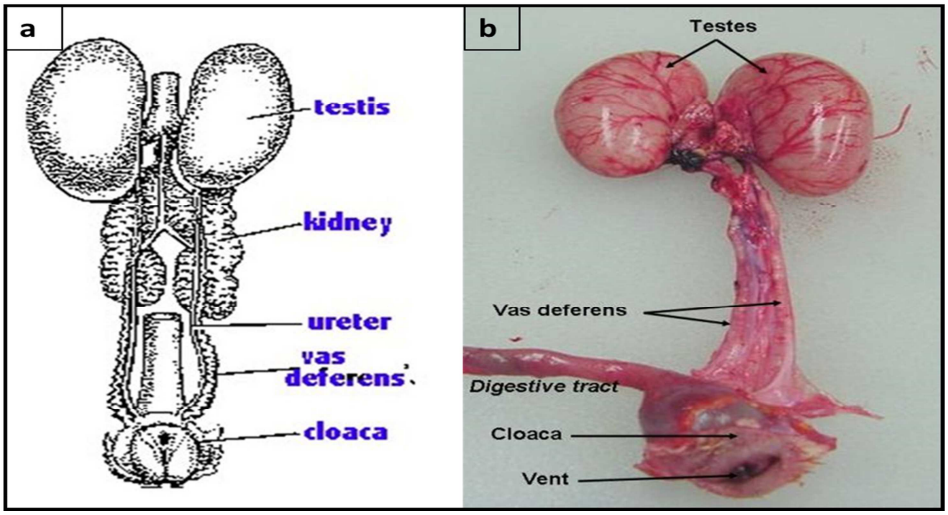



Figure 8.1. Illustration (a) and live picture (b) of ventral view of male reproductive system of avian species.

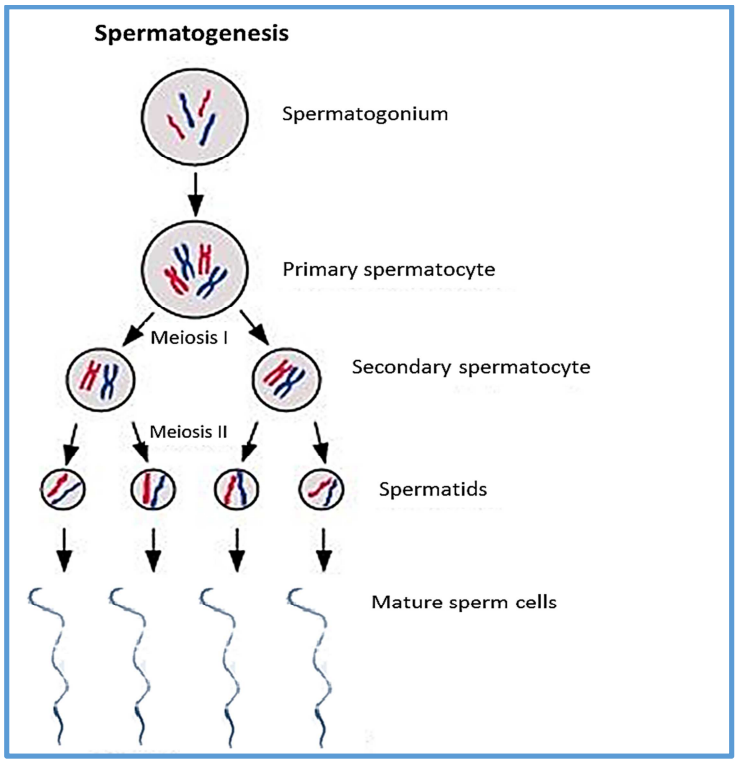



Figure 8.2. Development of spermatogonium into mature spermatozoa.

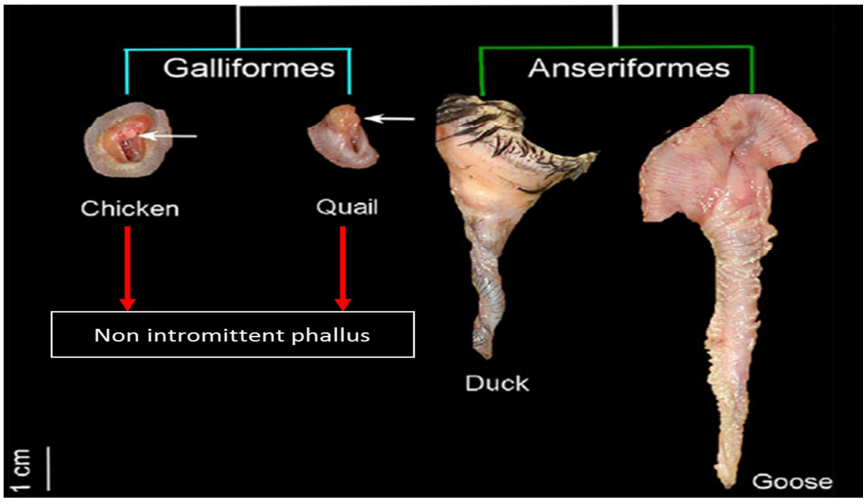



Figure 8.3. Two varieties of phallus in male birds. Chicken and Quail present the “non intromittent phallus” consisting of folds and bulges that make contact with the female’s cloaca at mating time in contrast with Anseriformes (duck and goose) in which phallus is more evident.

### *8.2. Poultry female reproductive system*

The female bird's reproductive tract (figure 8.4), consists of the left ovary and the left oviduct because of the degeneration of the right part in embryonic stage after hatching.

The ovary has two functions, to produce the ovum and the female hormones, estrogen and progesterone but also testosterone. The ovary consists of two parts, the medulla and cortex. The medulla contains connective tissue, nerves, smooth muscle, and blood vessels. The cortex covers the medulla and contains oogonia. Oogonia are precursor cells which form oocytes and eventually the ovum (counterpart to sperm). By the time of hatching, oogonia stop multiplying and begin to enlarge. They are now called primary oocytes. Within the ovarian cortex of the adult hen, several hundred primary oocytes may be visible to the naked eye. About 12,000 are visible microscopically. Few of these, start the stage of rapid growth. Primary oocytes visible on the ovary are often termed follicles, which pertain to the primary oocyte and its membranous covering. Grossly, in the ovary of a very young, immature bird, follicles are not evident. The ovary is flattened and may resemble a piece of fat. It may contain smooth grooves or folds which make it seem "brain like" in appearance. The ovary of an older immature female has a fine granular surface which resembles cobblestone. This is consistent with very early follicular development. The ovary of a mature bird has a grapelike cluster of small, but prominent follicles which are easy to identify. As the breeding season approaches, several of the follicles enter a phase of rapid growth and maturation, becoming yolk-filled follicles just before ovulation. After sexual activity, the ovary goes into a resting phase where it becomes diminutive in size again. However, a large number of follicles remain larger than as all appeared in the prenuptial phase. Such ovary is described as mature, but inactive. In those follicles which undergo rapid growth during sexual stimulation, yolk material accumulates and the primary oocyte gradually grows to full size. The avian primary oocyte is the largest cell in the animal kingdom. In the domestic fowl, its final weight is about 20 g. After reaching its full growth, the primary oocyte completes two maturation divisions, the first of which form the secondary oocyte and the first polar body (figure 8.5). This occurs about two hours before ovulation. This division is meiotic division which produces the secondary oocyte which has only one half the normal numbers of chromosomes. Ovulation occurs next. This is when the follicle splits at one end (this area is called the stigma) and the secondary oocyte is engulfed by the oviduct. The second maturation division, forming the ovum and second polar body occurs in the oviduct. Probably penetration by the spermatozoan before this division can be completed.

Penetration occurs about 15 minutes after ovulation. Since it must occur before the secondary oocyte, it is covered by albumen and this step presumably occurs in the infundibulum. Fertilization is the actual fusion of the male and female pronuclei (chromosomes).

The oviduct of the mature hen comprises five morphologically and functionally distinct small segments, i.e infundibulum, magnum, isthmus, uterus and vagina (figure 8.6). Furthermore there is a narrow band of utero-vaginal junction (UVJ) located at the cranial/anterior end of vagina, which is also morphologically distinct from the vagina and uterus. It possesses a unique ability to store sperm for a prolonged period in the oviduct inside sperm storage tubules (SST) located in UVJ and infundibulum where sperm can be stored and survive for few days to few weeks after single artificial insemination (AI) or natural mating. Nowadays, the advantage of prolonged sperm storage and survival in the oviduct of laying hens has been fully exploited and utilized in practical poultry production systems. This unique feature of prolonged sperm storage in the SST, enables laying hens to produce a series of fertile eggs following a single copulation event. Sperm storage function of SST is directly correlated with the fertility of laying hens.

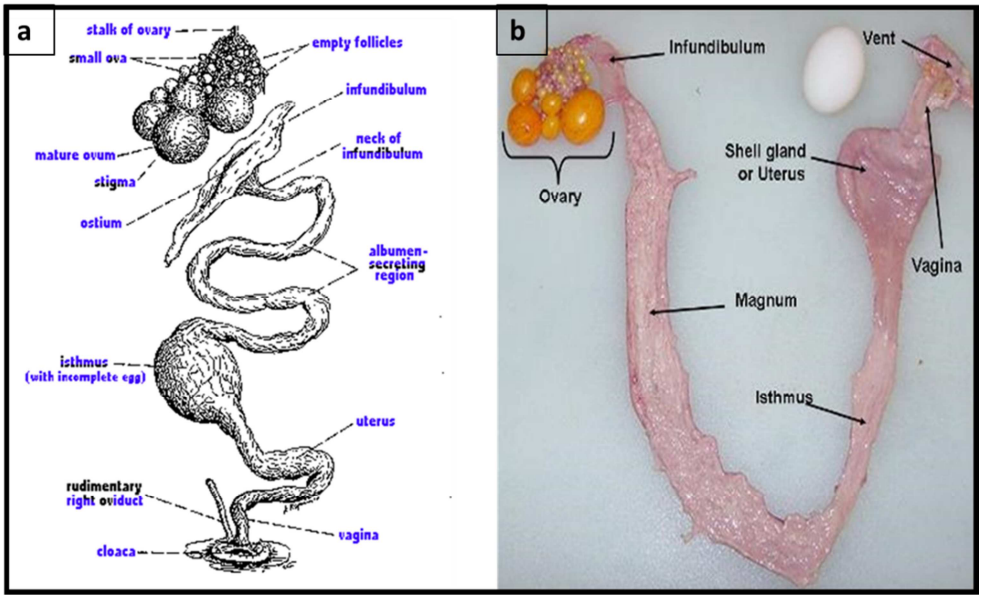



Figure 8.4. Illustration (a) and live picture (b) of ventral view of female reproductive system of avian species

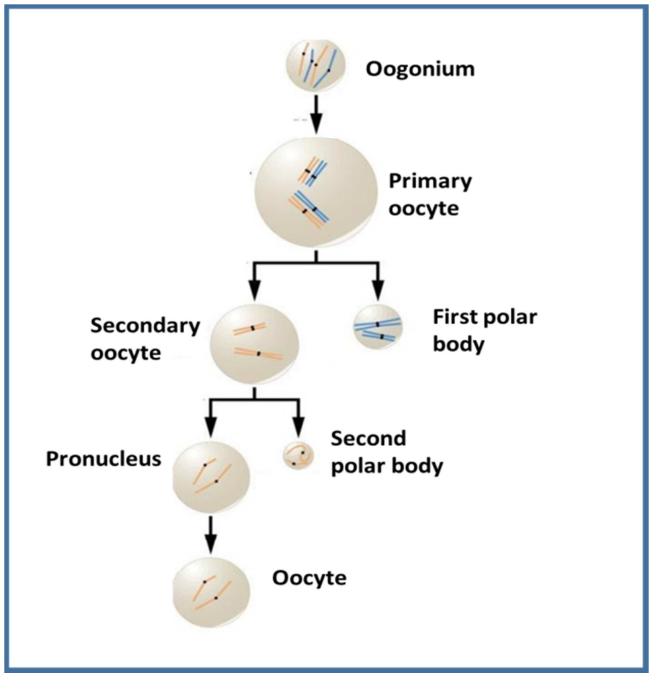



Figure 8.5. Development of oogonia into mature oocytes.

### *8.3. Poultry Semen*

**Semen** consists of two kinds of components: spermatozoa and seminal plasma. The concentration of sperm into seminal plasma corresponds to 3-8 and 8-14 billion sperm per ml for chicken and turkey respectively (Cerolini *et al.*, 2008). The colour is opaque and white. The pH is neutral and osmotic pressure is 325 mOsm/Kg. The volume is very exiguous respect to mammalian semen because of the lacking accessory prostate glands in male poultry reproductive system.

Generally, **avian spermatozoa** are specific cells devoted to the transfer of genetic information to the female gamete. The structure of avian sperm (figure 8.6) is more common to amphibians and reptile respect to mammalian sperm (Zaniboni and Cerolini, 2008). The head is cylindrical with a diameter of 0.5 µm and contains scarce amount of cytoplasm and a little acrosomal region. The head is occupied by the nucleus which is characterized by a filiform and slightly elicoidal shape in which the DNA is packaged through the chromatin that is the major component of chromosomes. Chromosome number (n) changes according to the species. The number of chicken sperm, compared to turkey sperm (n=41), is inferior

(n=39). Acrosomal region (deriving from the invagination of Golgi apparatus), is another important component, that covers the sperm head and contains hydrolytic enzymes which play a key role during oocytes fertilization (Mc Rorie and Williams, 1974; Morton, 1976; Hafez, 1984). Indeed, this last process if favoured also by acrosomal thorn similar to perforatorium of mammalian sperm. The latter, arises from nuclear membrane invagination and is penetrated by acrosome allowing the fusion between sperm cell and oocyte. There is also an intermediate tract placed between sperm head and the main tract of the tail. It consists of a central portion formed by axoneme containing 9+2 couples of microtubules and about 30 mitochondria, which are involved in sperm movement.

In particular, the microtubules are associated to the mobility of cytoskeleton of sperm cell while mitochondria provide the energy necessary to finance sperm moving.

The end portion is the tail which is long 90-100 µm (two times respect to mammalian sperm), and has a simple structure which makes avian sperm highly sensitive to cryodamages.

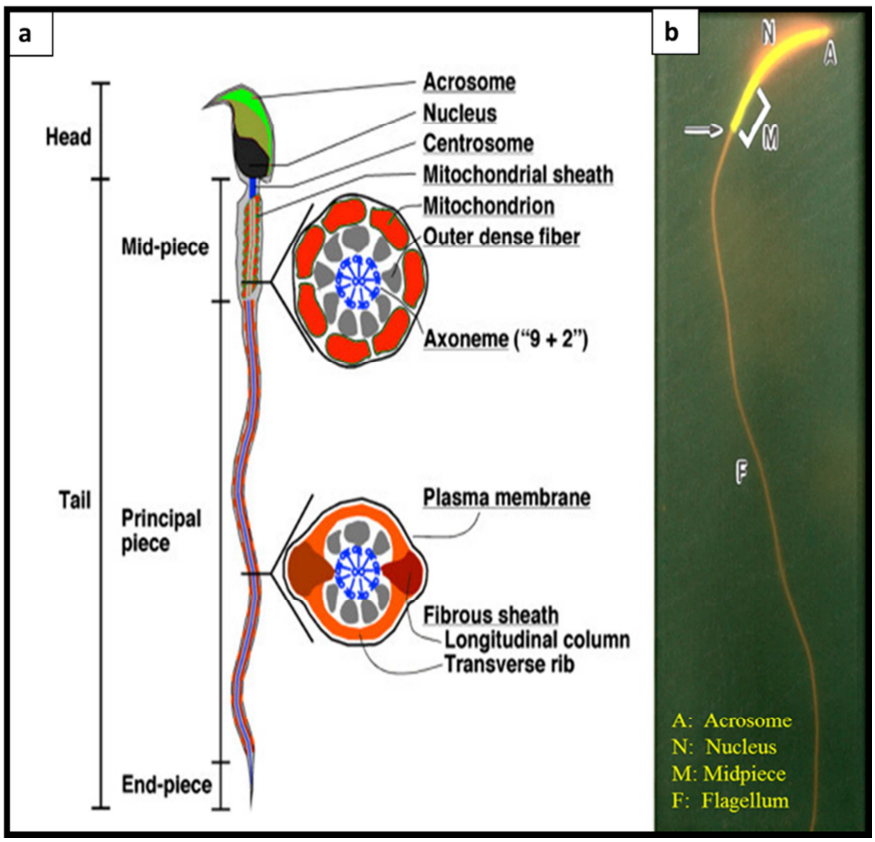



Figure 8.6. Structural components (a) and microscopic view of avian spermatozoa (b).

**Seminal plasma** is the acellular component of semen and is composed of different substances that are important for the sustaining of sperm cell (figure 8.7). Among these substances, sugars (glucose, fructose), aminoacids (glutamate), ions (calcium, magnesium) and lipids, are included. Seminal plasma is produced partly by Sertoli cells within seminiferous tubules of testis, and by epithelium mucosa from epididymis, deferent ductus and lymphatic pliers (Sturkie, 1986).

| Component | Concentration (mM) |
|---|---|
| Glucose | 0,18 |
| Chloride | 46 |
| Sodium | 145 |
| Potassium | 13 |
| Calcium | 1,4 |
| Glutamate | 75 |
| Lactate | 3,7 |
| Piruvate | 0,3 |
| Carnitine | 3,2 |
| Acetil- carnitine | 0,5-2,0 |
| Proteins (g/L) | 8 |

Figure 8.7. The composition of seminal plasma of poultry species

### *8.4. Semen collection*

Semen collection is performed by “abdominal massage” procedure described for the first time by Burrows and Quinn (1937). The technique involves restraining the male and gently stroking the back of the bird from behind the wings towards the tail with firm rapid strokes. The male responds with tumescence erection of the phallus (figure 8.8a), at which time the handler gently squeezes the cloaca expressing semen through the external papillae of the *ducti deferentis* collecting the semen into a container (figure 8.8b).

For turkeys, the technique is adapted by massaging the area around the cloaca before milking the semen (Lake and Stewart, 1978). Adaptations of this method were also made for species such as waterfowl which have penis-like copulatory appendages (Cooper, 1977), and non-domestic species which require additional restraint (Gee, 1995).

The volume of semen in one ejaculate averages 0.25 ml in the turkey, 0.50 ml in the rooster.

The proximity of the cloaca increases the likelihood of obtaining semen contaminated with feces, urates, and bacteria that are detrimental to semen quality. At the time of ejaculation in fact, semen should be pearly white, viscous and clean without contaminants (feces, urine and blood residues). However, this operation is easier in turkey than rooster. This is due to the fact that chicken semen is less viscous than turkey semen depending on the concentration (11 and 3-8 billion sperm per ml for turkey and chicken respectively) (Cerolini *et al.*, 2008; Donoghue and Wishart, 2000).

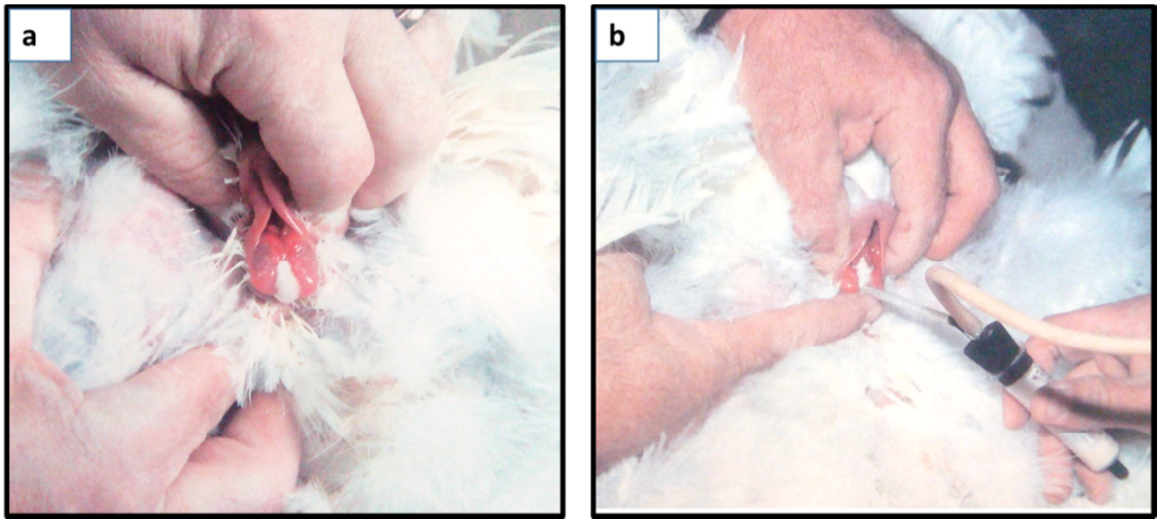



Figure 8.8. Abdominal massage procedure: the operator uses the index finger of the right hand to place pressure below the phallus (a); Pressure applied determines the flow of semen collected by aspiration (b).

### *8.5. Conservation methods for poultry semen*

The technologies available for semen storage are essentially: 1) hypothermic-liquid storage (refrigeration), which enables the storage of semen for up to 24 or 48 h at chilling temperatures around 5°C (Wishart, 2009); and 2) cryopreservation, whereby semen can be long-term stored at –196°C, the temperature of liquid nitrogen.

Notice that the semen cryopreservation is the more suitable technology for the *ex situ in vitro* conservation of genetic resources by the establishment of semen cryobanks.

**Hypothermic-liquid storage** enables the storage of semen at chilling temperatures around 5°C for brief time. This technology was applied for the first time in avian species such as chicken (Douard *et al.*,2004; Kotlowska *et al.*, 2007) and turkey semen (Wishart, 2009).

During liquid storage at reduced temperature, sperm metabolism is not completely arrested. Liquid storage at refrigerated temperatures allows to store semen for up to 6 h in turkeys and 24 h in chickens resulting in fertility levels comparable to freshly inseminated semen.

**Sperm cryopreservation** in birds has a great potential of application in different fields including optimization of the artificial insemination practice and selection, breeding at different levels in the poultry industry, disease prevention and the *ex situ in vitro* conservation of genetic resources by the establishment of semen cryobanks. The application in the last mentioned field is of particular interest, because sperm cryopreservation is the most feasible method for *ex situ* management of avian genetic resources since it is not possible to cryopreserve embryos or oocytes mainly because of their larger size, high lipid content and polar organization (Bellagamba *et al.*, 1993; Blesbois, 2007; Zaniboni *et al.*, 2014). The preservation of poultry semen in frozen state has been the subject of intense scientific interest beginning approximately in 1951 by Polge with the discovery of glycerol as a good cryoprotective medium. Despite the fact that this scientific breakthrough was accomplished with rooster semen (Polge, 1951), the overall fertility rates with frozen/thawed poultry semen are highly variable and not reliable enough for use in commercial production or preservation of genetic stocks.

However, despite the good progress made in the cryopreservation of semen in cattle, this preservation method has not been so successful in avian species. This is because of the high cost of preparing and storing frozen ejaculates compared to the market price of day-old chicks, and also low semen quality and consequently the fertility levels achievable with frozen/thawed spermatozoa (Blesbois, 2007; Iaffaldano *et al.*, 2011). The poor fertilization rates obtained for avian as opposed to mammalian species are attributable to the unique morphological characteristics of avian spermatozoa, such as their filiform shape, long tail and condensed nucleus, which makes them more susceptible to freezing damage (Donoghue and Wishart, 2000; Long, 2006).

In addition, membrane damage induced by cryopreservation, results in impaired sperm transport and survival in the female reproductive tract with the consequent decreased duration of fertility that has been correlated with the number of spermatozoa in sperm storage tubules at the utero-vaginal junction (Pierson *et al.*, 1988; Tajima, 2013).

Moreover, avian spermatozoa are generally recognized to be more sensitive to cryopreservation compared with mammalian spermatozoa. The fertility rates in fact of

cryopreserved poultry sperm are dramatically lower than any of the domestic mammalian species (Long, 2006; Zaniboni *et al.*, 2014; Santiago Moreno *et al.*, 2016).

In particular, chicken and turkey spermatozoa are characterized by very high proportions of long chain polyunsaturated fatty acids (PUFA) mainly n-6 and n-9 which make them more susceptible to lipid peroxidation. Between the two species, turkeys sperm are more susceptible than chicken spermatozoa according to the ratio of polyunsaturated to saturated fatty acids being 0.9 and 1.1 respectively (Ravie and Lake, 1985; Surai and Blesbois, 1998). Indeed, high concentration of PUFA makes sperm membranes highly sensitive to peroxidative degradation which is considered to be a major cause of fertility loss during avian sperm storage (Cerolini *et al.*, 1997; Zaniboni and Cerolini, 2009). During this process, reactive oxygen species (ROS) are capable of chemically altering virtually all major classes of bio-molecules (e.g. lipids, proteins, nucleic acids) with concomitant changes in structure and function (McCall and Freiz, 1999; Zaniboni and Cerolini, 2009). Against these molecules, avian semen, contains a complex antioxidant system, including non-enzymatic antioxidants vitamin E, vitamin C and glutathione as well as antioxidant enzymes glutathione peroxidase and superoxide dismutase (Surai, 1999). However, the antioxidant activity level is variable among different species including chickens, turkeys, guinea fowls, geese and ducks. In particular, as reported by Surai and Blesbois (1998), the lowest antioxidant activity was recorded in turkey sperm followed by chicken, guinea fowl, goose and duck.

Over a number of years, the preservation of poultry semen in a frozen state has been the subject of much investigation. Moreover, the successful cryopreservation of chicken sperm could play an important role in preserving and transferring valuable genes to future generation and it will also assist in the conservation of the male gametes of endangered indigenous breeds (Makhafola *et al.*, 2009).

A variety of semen cryopreservation protocols involving different cryoprotective agents (CPAs), packing methods and freezing and thawing rates, have been developed in different poultry species. However, the quality of avian sperm after freezing/thawing procedures and relative fertilization rates remain highly variable.

Semen cryopreservation involves several steps, each one affecting sperm structure and function (Garner *et al.*, 1999; Bailey *et al.*, 2003): extension, cooling, CPA addition, freezing, and thawing (Bailey *et al.*, 2003). Deleterious effects are the result of osmotic stress, and temperature changes produced during cooling, freezing and rewarming, ice

crystal formation being one of the main biophysical mechanisms of sperm death (Swain and Smith, 2010).

A principal challenge for the survival of cells during cryopreservation, is the lethality of the intermediate temperature zone (−15 to −60°C), which is crossed twice during the cryogenic cycle, as cells are cooled and rewarmed (Gao and Critser, 2000; Blanco *et al.*, 2011; Iaffaldano *et al.*, 2016b). Thus, the cryopreservation process causes numerous negative effects including damage to cell membranes (plasma and mitochondrial) and, in some cases, to the nucleus with devastating consequences for sperm survival (Blesbois, 2007). Following cryopreservation, metabolic damage may affect levels of adenosine triphosphate (ATP), which is essential for sperm motility (Long, 2006; Iaffaldano *et al.*, 2016b). For this reason, many researchers turned their attention to develop effective freezing protocols for the cryopreservation of avian semen, to reduce cell damages induced by freezing and thawing processes.

The cryopreservation and storage of germplasm has long been valued for the indefinite preservation of genetic material, especially in cases of high-risk populations. An immediate need for this practice was identified for research using unique poultry lines (Long and Kulkarni, 2004). Today however, semen cryopreservation seems to be the only effective method of storing reproductive cells for the *ex situ* management of genetic diversity in birds (Blesbois, 2011; Kowalczyk and Łukaszewicz, 2015).

Therefore, successful semen cryopreservation has enabled the creation of semen banks for several wild and some poultry species (Saint Jalme *et al.*, 2003; Blackburn, 2006; Woelders *et al.*, 2006; Blesbois, 2007; Blanco *et al.*, 2009; Kowalczyk *et al.*, 2012). However, research efforts have not yet served to create a turkey and chicken semen cryobank in Italy.

### *8.6. Cryopreservation procedure*

Semen cryopreservation involves several steps affecting sperm structure and function such as extension, cooling, cryoprotectant addition, packaging system (pellets or straws), freezing and thawing (Bailey *et al.*, 2003; Iaffaldano *et al.*, 2016 a,b).

During **cooling step** avian spermatozoa are held at 5°C for a specific period of time depending on the extender and cryoprotectant used. In chicken the cooling time is around 20-45 min (Hanzawa *et al.*, 2010; Sasaki *et al.*, 2010; Santiago Moreno *et al.*, 2011). However, as reported by Bakst and Sexton (1979) and Mphaphathi *et al.* (2016), it is

possible that the cooling step duration could increase until 2 h. In other cases, the cooling step is not taken in consideration (Shaverdi *et al.*, 2013).

**Extension** is performed by addition of specific semen diluents (or extenders). Semen diluents are based on the biochemical composition of chicken and turkey semen (Lake, 1995). Glutamic acid, the most prominent anionic constituent of avian seminal plasma, became a standard component of diluents (Getachew *et al.*, 2016). There are many diluents available for poultry semen, both published recipes and commercially available products. Recently, the research has focused on defining the optimum diluent and improving storage systems addressing to the composition of seminal plasma and sperm metabolic requirements or through the use of antioxidant supplements (Zaniboni and Cerolini, 2009; Rosato *et al.*, 2012).

One of the most critical point of semen cryopreservation is the **cryoprotectant** (CPA) addition which protects sperm from cryodamages. There are two kinds of CPAs: permeating CPAs (P-CPAs) and non-permeating CPAs. P-CPAs are able to increase membrane fluidity through rearrangement of membrane lipid and protein and partially dehydrate the cell, lowering the freezing point and thus reducing the formation of intracellular ice crystals, one of the main biophysical mechanism of sperm death (Holt, 2000; Swain and Smith, 2010, Iaffaldano *et al.*, 2016; Mosca *et al.*, 2016). P-CPAs (DMA, DMSO, Ethylene glycol etc) could paradoxically have a toxic effect related to its concentration and the time of cell exposure causing sperm membrane destabilization and protein denaturation (Swain and Smith, 2010; Iaffaldano *et al.*, 2016b; Mosca *et al.*, 2016). N-CPAs (sugars, proteins, aminoacids) instead are generally large hydrophilic molecules without toxicity and able to act as osmoprotectants (Blanco *et al.*, 2011; Rosato and Iaffaldano, 2013; Iaffaldano *et al.*, 2016b). They lower freezing temperature of the medium and decrease the extracellular ice crystal formation. Moreover, the addition of N-CPAs to the freezing medium serves to offset the cryodamage caused by P-CPAs, which are toxic at high concentration (Iaffaldano *et al.*, 2016b; Mosca *et al.*, 2016).

The particularity of sperm from poultry species including chicken and turkey, is that freezing protocols developed for chicken are not effective on turkey semen. This is probably due to the difference in lipid composition of sperm plasma membrane.

Many research efforts have focused on developing freezing protocols for the improved cryopreservation of turkey semen reducing the cell damage caused by freezing and thawing.

The permeating CPAs mainly used in freezing protocols for turkey but also for chicken semen are: glycerol, DMSO and DMA at different concentrations. In particular, DMA and DMSO have been used as alternatives to glycerol that has to be removed before artificial insemination because of its contraceptive effect (Hammerstedt and Graham, 1992).

With respect to turkey semen, early studies examined the use of DMSO concentrations of 4% (Bakst and Sexton, 1979; Sexton, 1981). However, in our recent work, Iaffaldano *et al.* (2016a) reported that 10% DMSO was better than 4%. While DMA concentration of 8% worked better than 6% when cells were frozen at high cooling rates, by direct plunging of semen droplets into liquid nitrogen.

However, using 6% DMA in combination with trehalose or/and sucrose at a concentration of 0.1 M, Mosca *et al.*(2016) reported that trehalose had a positive effect on frozen-thawed rooster semen respect to sucrose and sucrose-trehalose combination. These results are in contrast with Blanco *et al.* (2011) who observed that the combined effect of the sucrose-trehalose on frozen turkey semen associated to DMA (6-24%), was better respect to trehalose alone (Blanco *et al.*, 2011).

In addition, other cryoprotectants have been successfully used for the cryopreservation of rooster semen. MA has been successful used to develop a procedure for freezing chicken sperm in Japanese and Korean breeds as well as DMF and DMA (Hanzawa *et al.*, 2010; Choi *et al.*, 2013), while EG and DMSO were found to be suitable for Venda cock semen from South Africa (Mphaphathi *et al.*, 2016).

Among the **freezing systems** assessed for the cryopreservation of avian spermatozoa, the pellet procedure is cheap, easily adaptable to field conditions, takes only a few seconds for cooling and warming. However, straws as a packaging system have benefits such as sperm traceability, and the safe transport of semen for breeding or storage in gene bank (Iaffaldano *et al.*, 2016a,b).

Once CPA added to a sperm suspension, a period time needed for the CPA to permeate the cells is the **equilibration time**. It varies according to the nature and concentration of the CPA and to the animal species (Fuller *et al.*, 2004) and occurs at low temperature (4-5°C). The optimal equilibration time depends on the CPA concentration in the freezing medium, its characteristics (i.e., molecular weight, affinity of chemical groups toward the sperm membrane), the temperature of the sperm when the CPA is added, as well as the structural composition of the sperm membrane, which influences sperm permeability and fluidity. Equilibration time for avian semen is highly variable according to different extenders and CPAs. As reported by Shaverdi *et al.* (2015), chicken semen was successfully frozen by

equilibration for 30 min at 5°C while Iaffaldano *et al.*, 2016a, equilibrated turkey semen at 4°C for 20 min.
In our recent work, chicken semen has been equilibrated at 5°C for 45 min (Miranda *et al.*, 2018). Moreover, in some cases this step is not required during freezing procedure for avian semen especially for chicken semen (Hanzawa *et al.*, 2010; Sasaki *et al.*, 2010; Choy *et al.*, 2013).

With regard to **freezing rate**, this parameter is intended as the distance of packaged semen from liquid nitrogen vapour. The liquid nitrogen vapours used in conventional freezing produce a step-wise decrease in temperature, whereas ultra-rapid freezing rapidly solidifies the semen sample avoiding ice crystal formation. In our recent work conducted in turkey, semen was frozen by exposure to liquid nitrogen vapour at different heights above the liquid nitrogen surface (1, 5 and 10 cm) for 10 min to give three different freezing rates (Iaffaldano *et al.*, 2016a). As result of this experiment, exposure of turkey semen at 10 cm returned the better post-thaw semen quality respect to the other heights.

The semen **thawing procedure** (temperature and time), is also crucial for the post-freezing quality of semen. During slow thawing (low temperature, long time), the small ice crystals formed during freezing start to melt, turning into large crystals (recrystallization) that are harmful to the spermatozoa (Watson, 1995). During fast thawing (high temperature, short time) the time for recrystallization to occur is limited and this increases the survivability of spermatozoa. Using the pellet procedure for freezing turkey semen, Iaffaldano *et al.* (2011), observed that thawing at a temperature of 75°C for 10 sec was better than 60°C for 12 sec, while when straws were used, thawing conditions of 50°C/10 s were more efficient than 4°C/5 min (Iaffaldano *et al.*, 2016 a). With respect to chicken semen instead, it was demonstrated that thawing temperature of 5°C for 1.2-2 min reported an increase of fertility nearly 80% (Sasaki *et al.*, 2010) in contrast with the thawing performed at 37°C for 30 s that produced a fertility rate of 33% (Santiago Moreno *et al.*, 2011).

### *8.7. Assessment of fresh and frozen semen quality in vitro and in vivo*

Evaluation of fresh and frozen semen quality is performed by ***in vitro*** and ***in vivo*** essay.

***In vitro*** assessment considers some specific parameters such as motility, viability, osmotic resistance, apoptosis, membrane integrity, ultrastructure. These settings will be illustrated in the chapter 10 in detail.

Evaluation of semen quality ***in vivo*** is performed by Artificial insemination (AI). AI is the manual transfer of semen into the female's vagina. Basically, it is a two step procedure: first, collecting semen from the male; and second, inseminating the semen into the female.

In poultry, depending on the objectives and goals of the farm or laboratory, there may be intervening steps such as semen dilution, storage, and evaluation (Bakst and Dymond, 2013). AI is the most widely used reproductive technology in the livestock industry. Its adoption in poultry species has increased in popularity, especially in turkeys that depend entirely on this procedure. Prior to semen collection, cocks need to be trained and this is achieved through abdominal and back massage for about a minute for 3 days, consecutively. The abdominal massage method is the most commonly used since it is non-invasive and has minimal stress on the cock. Vaginal insemination is commonly used for semen deposition as there are less risks of injury the hen. Preliminary stroking and massaging of the back and abdomen is required to stimulate the hen. This is followed by applying pressure to the left side of the hen's abdomen around the vent causing evertion of the cloaca hence protrusion of the vaginal orifice. An inseminator containing the semen, is inserted 2.5 cm deep into this opening for semen to be deposited (figure 8.9 a,b). As the semen is expelled by the inseminator, pressure around the vent is released, the oviduct can return to its normal position and draw the semen inwards to the utero-vaginal junction. Inseminators such as straws, syringes or plastic tubes may be used. During insemination, the volume of semen required per hen is about 0.1ml containing about 100 to 200 million sperms (Bakst and Dymond, 2013).

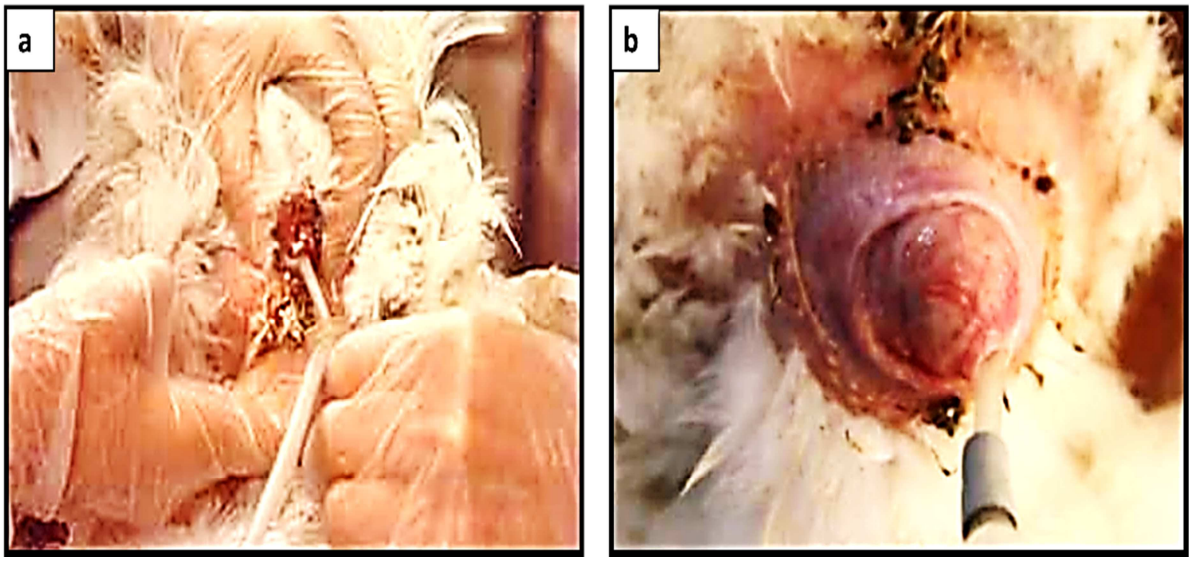



Figure 8.9. Artificial insemination in turkey (a) and chicken (b) hen.

# Chapter 9

## Reproductive system notes, semen production and storage in *Oryctolagus cuniculus*

### *9.1. Rabbit male reproductive system*

The reproductive organs of the rabbit male include testes, accessory sex glands, ducts and external genitalia (figure 9.1). The *testes* are the primary organs of male reproduction; they produce spermatozoa and hormones (androgens), which affect reproductive function and behaviour. The paired testes are ovoid structures measuring about 30-35 × 10-15 mm and weighing approximately 1.5-2 grams (g) (Cerolini *et al.*, 2008). The testes are essentially sacs of coiled tubules where the sperm are formed. This process, known as spermatogenesis, involves changes from a rather normal looking spheroidal cell, into the highly specialized spermatozoon, devoted to the transmission of genetic information to the ovum. Sperm development begins in the walls of the tubules, and as the spermatozoa develop, they move toward the center or lumen of the tubule (McNitt *et al.*, 2013).

In embryonic stage, testes lie in the abdominal cavity near the kidneys. They descend into scrotal sacs before birth through inguinal canals. Testes are maintained in their original position by spermatic cords. A spermatic cord consists of an artery, a vein and a nerve embedded in connective tissue. Testis, is covered by a connective tissue coat, known as *tunica albuginea*. Outside this tunica. there is the *tunica vaginalis* which is formed by two layers. Parietal layer lines the scrotum and visceral layer lines the tunica albuginea. Around the testis, between the two layers of tunica vaginalis, there is a narrow coelomic cavity filled with coelomic fluid. It allows sliding movements of testis. *Tunica albuginea* projects inwards as septa and divides the testis into many lobules. These lobules contain long, convoluted seminiferous tubules which are lined by the germinal epithelium. Germinal epithelial cells produce sperms by process of spermatogenesis.

Among the germinal epithelial cells, there are *Sertoli (sustentacular) cells*, which nourish the developing spermatozoa. Once the sperm reach the lumen of the tubule, they are transported through the tubule by fluid pressure. This transport takes the sperm to the top of the testis and out into the epididymis (McNitt *et al.*, 2013).

Connective tissue among seminiferous tubules contain *Leydig (interstitial) cells*. They secrete testosterone (male sex hormone) which controls secondary sexual characters. The Leyding cells are under the control of hormones from the anterior pituitary, which is located at the base of the brain. These controlling hormones, regulate the levels of the androgens in the blood, which, in turn, control spermatogenesis and sexual activity of the buck (McNitt *et al.*, 2013). The seminiferous tubules of a testis open into a network called *rete testis*, it opens into many fine ducts, called *vasa efferentia* merging into the epididymis.

The ducts through which the sperm move after leaving the testes, include the epididymides, deferent ducts, and urethra. The *epididymides* lie close to the top of the testes and function as a place for maturation of the spermatozoa. Spermatozoa that have not undergone a period of maturation in the epididymides are incapable of fertilizing eggs. The epididymides also serve as a place for storage of spermatozoa; fertile spermatozoa are recovered from epididymides after eight weeks of storage. The normal time required for movement of sperm through the epididymides is 8 to 10 days.

The epididymis is a long, narrow and highly convoluted tubule along the inner surface of the testis divided into three distinct parts:

1) *caput epididymis*: the anterior part of epididymis, is connected to testis through vasa efferentia. Caput epididymis is connected also to the dorsal abdominal wall by a spermatic cord;

2) *corpus epididymis*: the middle part of the epididymis, connecting caput epididymis and cauda epididymis;

3) *cauda epididymis*: the posterior part of the epididymis. The cauda epididymis is joined to scrotal sac by a short, thick, elastic cord known as *gubernaculum*.

The *deferent ducts* convey the spermatozoa from the epididymis to the urethra and also function to some extent in sperm storage. The accessory sex glands normally add their secretions to the semen at or near the junction of the deferent ducts and the urethra.

The *urethra* is the common passage for both semen and urine. It carries semen from the junction with the deferent ducts to the end of the penis, from which the semen is ejaculated into the female vagina. The bladder empties into the urethra just beyond the point of junction of the urethra and deferent ducts.

The external genitalia of the male include the penis, the scrotum and the prepuce.

The *penis* (copulatory organ) is an erectile organ that is used for insertion of the ejaculate into the female tract. It is normally flaccid and rests in the prepuce. It becomes rigid from constriction of the penile veins at the time of breeding. Since arterial blood continues to flow

into the organ, it becomes turgid and can thus penetrate the vulva and vagina of the doe. The erect penis is held forward along the abdomen. There is a sensitive tip on the penis, known as the *glans penis*. Stimulation of the glans penis by the vagina of the female (or by a properly prepared artificial vagina) results in ejaculation. This is due to a reflex contraction of the duct system that forces out spermatozoa stored in the deferent ducts and the last third of the epididymis. Fluids from the accessory glands flow into the deferent ducts and the urethra during ejaculation. After subsidence of erection, the penis is pulled back into the prepuce by muscular contraction. The penis is covered by a loose sheath of skin, the skin that hangs over the tip of the penis is known as *prepuce*. The tip of the penis covered by prepuce is called glans penis. The penis is composed of three longitudinal columns, namely, two columns of *corpora cavernosa* (upper) and one column of *corpus spongiosum* (lower) made by spongy tissue.

The *scrotum* consists of two relatively hairless sacs that contain the testicles. These function to protect the testes and to provide an area with a lower temperature than that of the body cavity, because spermatogenesis cannot occur at normal body temperature. Testes can move freely in and out of the abdomen and are not always found in the scrotal sacs (McNitt *et al.*, 2013).Associated with the male reproductive system of rabbit, there are the *accessory sex glands*, they include the prostate gland, bulbo-urethral glands (Cowper's glands), perineal glands and rectal glands. Their secretions are responsible for the production of seminal plasma. The functions of these secretes, include adding fluid volume to the ejaculate to facilitate sperm movement through the male and female reproductive tracts, providing nutrient substances, and buffers for rabbit spermatozoa. These secretions produce also a gelatinous plug to seal the female tract, and some substances that stimulate contractions of the vagina and uterus of the female facilitatingsperm movement through the tract.

Around the base of uterus masculinus, there is *prostate gland*, it opens into the urethra through many ducts and it secretes an alkaline fluid activating the spermatozoa and contributing to the main bulk of semen. Moreover, prostate gland secretes citric acid that enters in the Kreb's cycle to produce ATP. Posterior to the prostate gland, there is a pair of *Cowper's glands* whose secretions neutralize the urinary residue and vaginal acidity.

Behind the Cowper's glands, there are two *perineal glands*, they open into hairless perineal depressions, one on either side of the anus. Their secretions give a characteristic smell to rabbit. Two rectal glands are at the sides of the rectum. However, their function is still unknown.

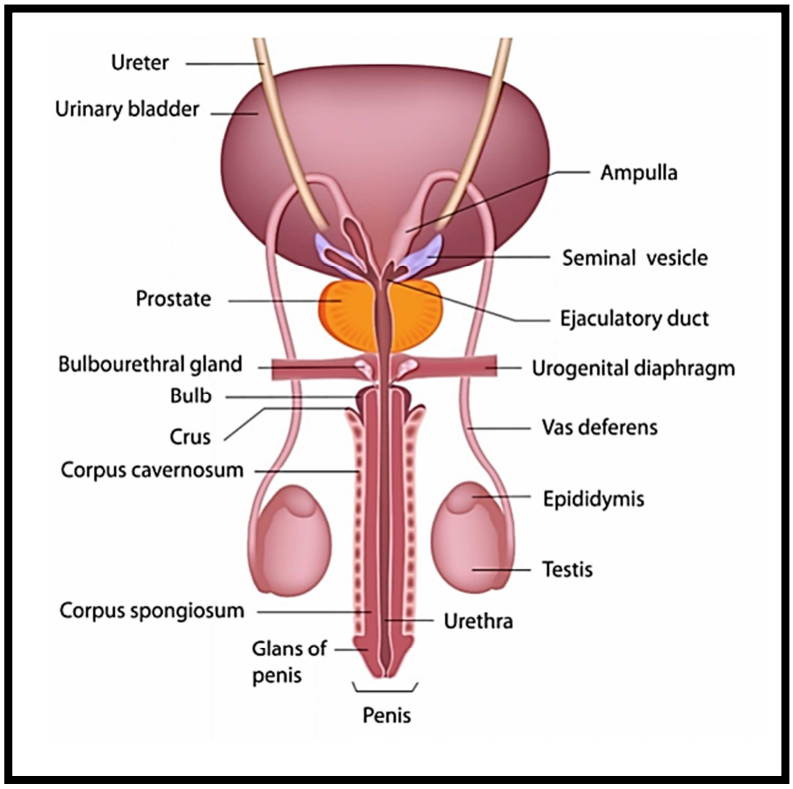



Figure 9.1. Rabbit male reproductive system

### *9.2. Rabbit female reproductive system*

The organs of female reproduction system include: the ovaries, oviducts, uterus, cervices, vagina, and external genitalia (Figure 9.2a).

The *ovaries*, the primary organs of reproduction, produce eggs, or ova, and hormones (primarily estrogens and progestins). They lie within the abdominal cavity, with one on each side, near the kidneys. The ovaries are ovoid structures with an extent of about 15 × 10 mm and a weight between 300 and 500 mg (Cerolini *et al.*, 2008), depending on the activity of the ovarian components. The middle portion, or medulla, of each ovary, is composed of connective tissue containing nerves and blood vessels. The outer layer, or cortex, contains the ova in various stages of development, as well as other types of tissue, including blood vessels, nerves, and muscle fibers. At the time of a doe's birth, thousands of undeveloped ova, are contained in the germinal epithelium layer of the cortex. From the time of puberty until death or the end of the reproductive life of the female, groups of these immature ova

undergo development and are shed (ovulated), or they degenerate. The oviduct is the site of fertilization, functions in a maturation process of spermatozoa known as capacitation, and is the location where early embryonic development occurs. *Oviducts* are thin tubes, characterized by willowy trend, of about 10-16 cm in length (Cerolini *et al.*, 2008).

The upper end of the oviduct is spread into the ostium tubae, which partially surrounds the *ovary*. On the edges of the ostium, tubae are numerous small projections known *fimbria*. These nearly cover the ovary. Beating of the fimbria, causes fluid movement toward the opening of the oviduct and, during ovulation, sweeps the ova into the oviduct (McNitt *et al.*, 2013).

The *uterus* is the organ in which the embryo and foetus normally develop and grow. It also provides muscular force for expulsion of the fetuses at birth. The uterus of the rabbit consists of two distinct horns, which are not connected to form entire body. Each horn of the uterus connects into an individual cervical canal, which opens into the common vagina (figure 9.2b). The cervices function as muscular plugs to keep the uterine horns closed except at the time of mating and parturition (birth, or kindling) (McNitt *et al.*, 2013).

The *vagina* is the site of sperm deposition at mating and acts as a channel for the young at parturition. It is long between 6-8 cm and presents longitudinal mucosal folds (Cerolini *et al.*, 2008).The *external genitalia* of the doe include the urogenital sinus, which is continuous with the vagina and is the chamber into which the urethra empties urine. The *external lips* of the urogenital sinus form the vulva, that can be used as an indicator of sexual receptivity of the doe. A doe with a moist red or pink vulva is much more likely to accept service than a doe with a pale, dry vulva. The *clitoris* is within the urogenital sinus, with the sensitive portion, the glans clitoris, projecting into the urogenital opening. Because the urethra opens into vaginal sinus posterior toward the place where sperm are layed, urination by the doe following breeding, does not necessarily interfere with fertilization.

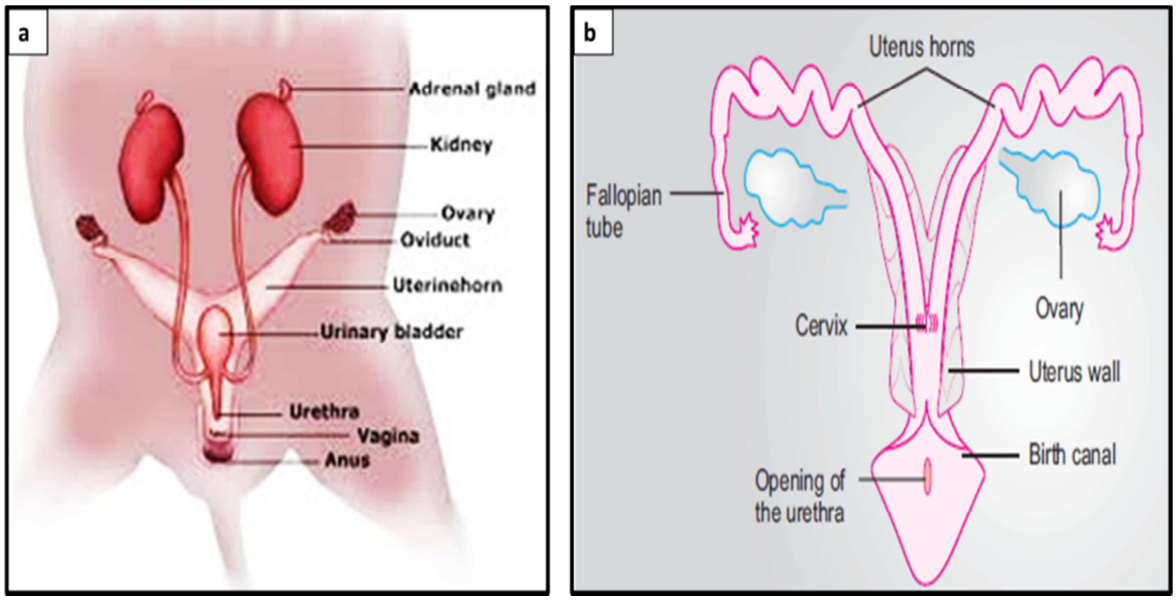



Figure 9.2. Ventral view of rabbit female reproductive system (a) with the detail of uterus horn (b)

### *9.3. Semen cryopreservation*

As previously illustrated, **semen** is generally composed of spermatozoa and seminal plasma. Rabbit semen is characterized by an opalescent white appearance. During the collection, semen contains a gel plug (deriving from accessory prostate glands) that has to be removed. The ejaculate volume usually ranges from 0.19 to 1.19 mL, but on average is about 0.6 mL while the concentration is 600 million sperm per ml.

**Rabbit spermatozoon** has a head that carries the genetic information and a tail that provides propulsion by its whip-like movements. The rabbit spermatozoan is about 50-60 μm long, it has a wide, flattened head and a long, thin and cylindrical segment composed by a neck, an intermediate part and a flagellum or protoplasmic tail (figure 9.3). The whole sperm cell is enveloped by the plasma membrane, whose function is to contain organelles and intracellular components. Thanks to its semi-permeability, it maintains the chemical gradient of ions and of the other soluble components. Moreover, specific membrane proteins facilitate the transport of fructose and glucose from the extracellular environment to the inside of the cell, thus providing the energetic substrates required for the cell metabolism.
The head includes two structures of fundamental importance: the acrosome and the nucleus. The acrosome is a structure derived from the Golgi apparatus and it is delimited by a trilaminar membrane: it covers two thirds of the front part of the sperm head. Above the acrosome, another structure covers the remaining front part of the head: it is the head cap, composed by sulphur- and fibrous proteins conferring great resistance. Its biological role, in effect, is to protect the acrosomal content: this includes enzymes with a high proteolytic and glycolytic power, which allow the spermatozoon to penetrate through the zona pellucida during the fecundation of the egg cell. The most important enzyme is acrosin, followed by hyaluronidase, collagenase, acid phosphatase, phospholipase A, arylsulfatase. Inside the head, in the central part, it is possible to find the nucleus, whose function is to transport and transmit the chromosomes, i.e. the genetic information. The nucleus of a ripe spermatozoon is very dense, as it is composed by tightly packed chromatin. The segment adjacent to the head is called neck: this region contains centrioles, which give the impulse for the meiotic cell division. This is also the starting point of the axoneme, a thigh bundle of axial contractile fibres, which continues in the following parts as well.
The segment after the neck, in rabbits, is about 8.8 μm long. It is called intermediate piece and it contains mitochondria, organized in a layer surrounding the axoneme. This part,

therefore, constitutes the main energy reservoir for the cell. The long tail segment (40 μm) following the intermediate piece is called main piece: it is not surrounded by any coat of mitochondria but it contains arginine, leucine and other essential amino acids. This segment is composed by bundles of parallel contractile fibres which end in thin fibrils constituting the final segment. These filaments are projected in the liquid medium like a helix, thus determining the helical movement of the spermatozoa. Moreover, they are able to change their descent energy into ascent energy, thereby advancing against the gravity force following the liquid currents of the female genital apparatus.

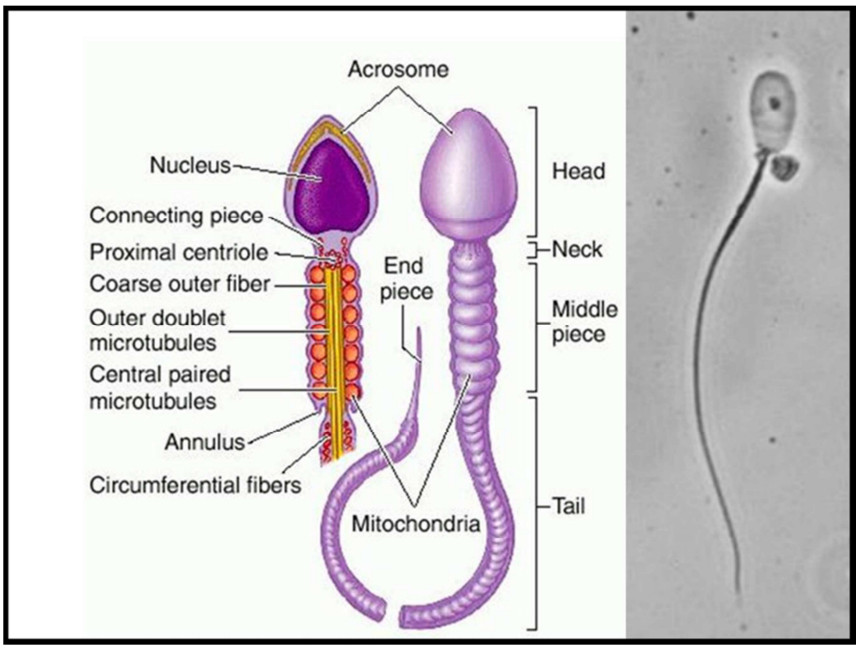



Figure 9.3. The structural components of rabbit spermatozoon and its microscopic view

The biochemical composition of rabbit **seminal plasma** is very complex and variable among species. Advances in reproductive technologies reveal seminal plasma as a nutritive-protective medium in which sperm cells are suspended. Seminal plasma is composed of some components such as proteins and enzymes, phospholipids, aminoacids, ions, which are very important for sperm metabolism, as well as sperm function, survival, and transport in the female reproductive tract.

### *9.4. Semen collection*

Semen collection from rabbit male occurs through specific artificial vagina (Morrell, 1995). The artificial vagina is composed of a rubber or latex tube, open on both sides, which is placed in a glass or plastic container. It must be filled with a warmed water at about 50°C in order to create the vaginal natural environment that it results be of 40°C. One side of the tube is needed for the ejaculation of the male, while the other one is connected to a graduated test tube for the collection of the semen. The test tube has to be maintained at about 38°C before its use, and changed after every collection. If the temperature of artificial vagina decreases below 40°C, the male refuses to ejaculate. During the collection, it is necessary with one hand to hold the artificial vagina, placed under the abdomen of the fake female rabbit, while the other hand, holds the fake rabbit female in front of the male. At the moment of the jump of the male, the opening of the artificial vagina is held between a thumb and an index finger to facilitate the insertion of the erect penis (figure 9.4a,b). The ejaculation is then helped with a light push backwards of the artificial vagina. Ejaculation usually takes place immediately following the appearance of the doe. During the collection, a good hygienic level of manipulation is essential to minimize the contamination of the semen sample.

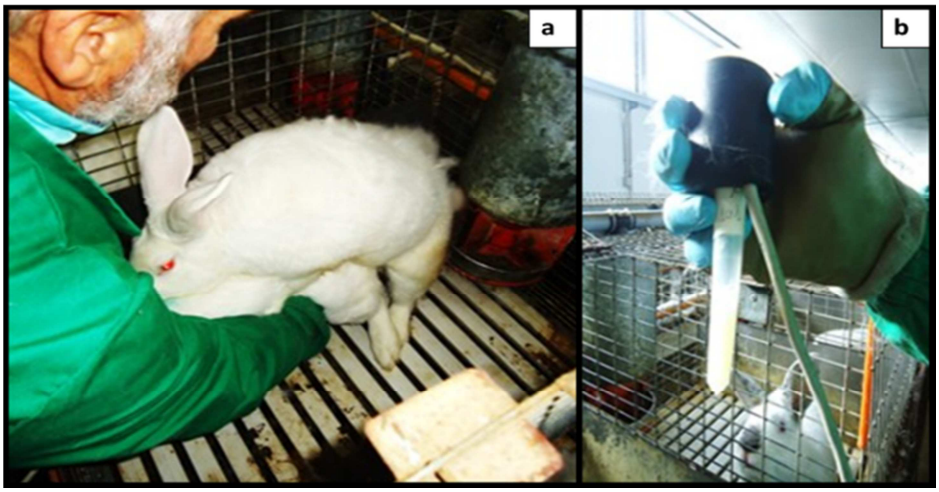



Figure 9.4. Semen collection from rabbit male performed by use of a specific artificial vaginaplaced under the abdomen of the fake female rabbit, while the other hand holds the fake rabbit female in front of the male (a); semen is collected into a plastic tube connected to artificial vagina (b).

### *9.5. Conservation methods for rabbit semen*

Since 60's a large number of protocols for the cryopreservation of rabbit semen have been developed by many researchers and their results have been reviewed (Mocé and Vicente, 2009). Each of these protocols has involved in the study of some aspects that can affect the success of the rabbit sperm cryopreservation, such as the composition of the freezing medium, nature of CPA and its concentration, freezing conditions and cooling and warming temperatures (Mocé and Vicente, 2009). The semen storage technologies available for rabbit semen are two: hypothermic-liquid storage and semen cryopreservation.

The **hypothermic-liquid storage** enables the storage of semen for up to 24 or 48 h at chilling temperatures around 5-15°C. The semen is placed in a tube containing the extender, previously incubated at 37-38°C. Subsequently, the diluted semen is transferred in a refrigerator set at specific temperature (see above), in order to induce its gradual decrease, and to avoid thermic shock which would affect the sperm survival.After this procedure, resulting semen (chilled semen) has to be applied for artificial insemination within 24-36 h. The key factors for the success of the semen storage in the liquid phase are the choice of extender, the dilution ratio and the storage temperature. To perform its function, the extender should supply the nutrients needed for the metabolic maintenance of the sperm cell, control the pH and osmotic pressure of the medium, and inhibit microbial growth (antibiotics) (Gadea, 2003).

The extenders should have an osmotic pressure similar to seminal plasma (approximately 300-320 mOsm/kg). Hypotonic or hypertonic solutions may cause transient or permanent loss of sperm motility, although it seems that hypertonic solutions are better tolerated in rabbit sperm than hypotonic ones (Costantini, 1989).

The diluent must also contain buffer substances that allow to maintain the pH neutral and invariable. The optimum pH value is 7, but considerable fluctuations are tolerated (Watson, 1990). Generally, motility and metabolic processes are suppressed at low pH and stimulated at higher values (Brun *et al.*, 2002). The buffer substances mainly used for the preparation of diluents are citrates, phosphates, sulfates, organic molecules such as the zwitterionic Tris-hydroxymethyl amino-methane (Tris) or Tes and electrolytic solutions of sodium or magnesium (Foote, 1972). In particular, the buffer-based organic Tris seems more appropriate for storage in the liquid phase (Maertens and Luzi, 1995; Castellini, 1996; Roca *et al.*, 2000).

To avoid bacterial contamination of the semen and the influence of pathogens that can affect fertility in the female, the addition of antibiotics in the semen is required. The most used are kanamycin, penicillin, streptomycin or gentamicin, alone or in each other combination. The sources of energy most commonly used in rabbit semen extenders are glucose or fructose (Watson, 1990; Roca *et al.*, 2000), although it has been observed that the viability of rabbit sperm is higher when glucose is used (Costantini, 1989; Roca *et al.*, 2000).

The dilution ratio for semen storage is related to the sperm concentration, viability and motility. Generally, it is preferred to use a dilution rate of 1:10, in order to obtain a number of sperm at least 20-30 million/dose (Alvariño *et al.*, 1996). Some authors have also shown that high dilution rates (greater than 1: 100) exert a detrimental effect on motility due to high dilution of seminal plasma, which appears to play a key role in preserving the quality of spermatozoa (Castellini *et al.*, 2000; Minelli *et al.*, 2001).

The storage temperature is another important factor that affects the semen quality, although the optimum temperature may depend on the extender. Studies conducted on extenders based on the use of Tris, showed a strong decline of sperm motility after 48 h of storage at 5°C (El-Gaafary, 1994), while Roca *et al.* (2000) at a temperature of 15°C, observed a motility which was still acceptable at the same time of conservation. According to the guidelines of the International Rabbit Reproduction Group (2005), 15-18°C is an optimal temperature for rabbit semen storage up to 48 h. Rosato and Iaffaldano (2011), reported that a temperature of 5°C proved to be more beneficial than 15°C in retaining the overall semen quality during long-term storage.

Different studies were performed to identify a suitable extender for long-term survival of rabbit spermatozoa (Castellini, 1996; Roca *et al.*, 2000; Carluccio *et al.*, 2004; El-Kelawy *et al.*, 2012).

However, there are still limited *in vivo* studies conducted to assess the levels of fertility achieved with chilled semen. The most encouraging results, were observed by Alvariño *et al.* (1996), who observed a fertility rate of 77% in multiparous rabbit does using semen stored for 24 h at 18°C with a commercial diluent (MA 24, Laboratorios Ovejero, Spain). While, Roca *et al.* (2000), reported 78% fertility in multiparous does inseminated with semen diluted in a TCG (Tris-citrate-glucose) and stored for 48 h at 15°C.

Recently, Di Iorio *et al.*, 2014 observed that Cortalap® with TCG preserved *in vitro* rabbit semen quality better than Merk III® and Lepus® during 72 h of storage.

Then the use of Cortalap® in AI trial evidenced that fertility and prolificacy obtained with chilled semen were significantly lower respect to those of fresh semen (20% and 2.2 vs. 93.3% and 10.1).Therefore, Cortalap® use is not recommended in AI programs.

**Semen cryopreservation**, allows long-term storage at -196°C (the temperature of liquid nitrogen). The method of slow freezing involves a brief pre-equilibration of cells in CPA solutions followed by slow, gradual, controlled cooling at rates optimized for the type of cells to be cryopreserved. The whole process, is carried out with the use of special programmable cell freezing equipment or exposure at different heights above liquid nitrogen level (between 2 and 10 cm) (Mocé and Vicente, 2009) and requires 3-6 h.

CPAs are used to protect the cells from damage due to intracellular ice crystal formation. The temperature of the cells is lowered to a super cooled state and ice crystal growth is initiated within the extra-cellular solution by a process called seeding. During ice crystals extension, water in the solution is converted from liquid state to solid state. This increases the concentration of solute in the extracellular medium which draws water out of the cell. As a result, the cell dehydrates with consequent increase in intracellular solute concentration, which further lowers the freezing point of the cell to approximately -35°C. The cell is almost devoid of any water at this point and therefore, ice crystal formation is negligible when the cell ultimately freezes at this temperature. Rate at which water leaves the cell, depends on the rate of cooling. When the cells are cooled at rapid rate, water present inside the cell, is not able to move out fast enough, leading to the formation of intracellular ice crystals which are lethal for the cell. If the cells are cooled too slowly, there is severe volume shrinkage leading to high intracellular solute concentration, which has deleterious effects on the lipid-protein complexes of cell membranes. Hence, the rate of cooling and CPA concentration employed in the protocol, should be optimized in order to avoid the intracellular ice crystallization and high solute concentration, the two main events involved in cellular injury during cryopreservation. The success of slow cooling depends on achieving this optimal balance between the rate at which water can leave the cell and the rate at which it is cooled before it is converted into ice.

Effective semen freezing protocols for rabbit are based on using slow freezing in liquid nitrogen vapour (Mocé and Vicente, 2009) whilst few reports regarding the use of ultrarapid freezing techniques (vitrification) (Hoagland and Pincus, 1942; Li *et al.*, 2010; Rosato and Iaffaldano, 2013).

Although cooled semen recorded high fertility and prolificacy rates, there is a need of reliable methods for rabbit sperm cryopreservation, to store rabbit semen for indefinite period of time. At present, cryopreserved sperm is not used for commercial purposes but it represents the main instrument for rabbit sperm resource banking, especially if it is considered that this species is a valuable laboratory animal (Mocè and Vincènte 2009). Several protocols developed for the cryopreservation of rabbit semen resulted unsatisfactory because of lower fertility and prolificacy rate than fresh semen (Mocè and Vincènte 2009). For this reason, many researchers turned their attention to optimize the cryopreservation protocol for rabbit semen to find the best freezing conditions without affecting its fertilization ability. Moreover, the combination of AI and cryopreservation of rabbit semen can be useful technique contributing to an enhancement of productivity and reduction of the number of rabbits for colony maintenance.

### *9.6. Cryopreservation procedure*

The **cooling step** is an important phase during which semen is subjectto a decrease of temperature before CPA addition. Tipically, rabbit semen cooling occurs at 5° C for 45 min in the fastest protocols. Although more recent research indicated that this step can be reduced to 10 min (Mocè e al., 2010), there are many protocols in which cooling step is longer (Mocè and Vicènte 2009; Iaffaldano *et al.*, 2011). As reported by Iaffaldano *et al.*(2011) for example, rabbit semen cooled at 5°C for 90 min recorded high fertility and prolificacy rates. During cooling time, spermatozoa are subject to cold shock inducing an irreversible loss of motility upon re-warming. Cooling alters the cellular membrane, changing it from a crystalline-liquid phase to a gel phase (lipid phase transition) and inducing lipid packaging faults, affecting plasma membrane fluidity.

Indeed, depending on different membrane constituents including cholesterol/phospholipid ratio, the effect of cooling differs among different species. Although mammalian spermatozoa are very sensitive to cooling from body temperature to near the freezing point (about 4°C), rabbit spermatozoa are not so susceptible. This is due to the composition of their plasma membrane which is characterized by higher cholesterol/phospholipid ratio that increases membrane fluidity. Due to this characteristic, reducing cooling time for rabbit sperm was possible. This permitted to simplify the cryopreservation process allowing preservation of more ejaculates in a given time. Many attempts were performed to reduce cooling time required for equilibrium phases in rabbit spermatozoa (Maeda *et al.*, 2012;

Mocè *et al.*, 2010; 2014;) and also in other species such as monkey (Dong *et al.*, 2008) wolf (Zindl, 2009) and stallion (Torres *et al.*, 2015). However, cooling step reduction has been made only for canine and buck spermatozoa (Du Bois, 2009; Ahmad *et al.*, 2015). Attempts to reduce time for cooling step involved in freezing rabbit sperm have not yet been performed.

After cooling, semen is diluted by use of specific **extenders**. In general, Tris-based extenders (Tris, citric acid and fructose or glucose) are the base of the extenders commonly used for rabbit sperm cryopreservation (Mocé and Vicente, 2009). Moreover, other extenders for rabbit sperm cryopreservation such as sodium citrate, Illinois Variable Temperature (IVT), MIII have been tested. None of them offered better results than the Tris-based extender (Rohloff and Laiblin, 1976; Cortell and Viudes de Castro, 2008).

**Cryoprotective agents** are essential for sperm defense against freezing damage, but nowadays there is no a universal CPA or an appropriate CPA concentration for cryopreservation of rabbit sperm.

In general, results of *in vitro* quality and fertility obtained with rabbit sperm frozen with glycerol, are lower than those obtained with sperm frozen with other CPAs (EG, DMSO or amides) (Mocé and Vicente, 2009). Therefore, glycerol is not the CPA of choice for rabbit sperm. Fertility problems associated with rabbit sperm frozen with glycerol are due to its effect on sperm after cryopreservation, and not to a contraceptive effect of this CPA on the female reproductive tract or on the fresh sperm (Smith and Polge, 1950; Griffin *et al.*, 1974). Unlike sperm from other species, rabbit sperm present a low water permeability coefficient and a high activation energy (Curry *et al.*, 1995). This low water permeability value is consistent with the need to use CPAs with lower molecular weight and higher permeability (such as DMSO or amides) than glycerol for rabbit sperm cryopreservation (Curry *et al.*, 1995).

Most of the freezing extenders used for rabbit sperm cryopreservation include one P-CPA in combination (or not) with N-CPAs (Mocé and Vicente, 2009). However, two P-CPAs (usually DMSO and glycerol) were used also for rabbit sperm cryopreservation (Rohloff and Laiblin, 1976; Weitze *et al.*, 1976; Götze and Paufler, 1976; Bamba and Adams, 1990).

The reduction in the concentration of CPAs has, in general, a beneficial effect on sperm quality after cryopreservation process.

As reported by Fox and Burdick (1963), they observed that sperm quality improved when the concentration of glycerol or EG in the extender was lowered to 4% (instead of 8%), which was also confirmed in later studies (Hsieh, 1996). Nevertheless high DMSO concentrations, can be used in some extenders (17.5% Sawada and Chang, 1964; 12.4% Vicente and Viudes de Castro, 1996; Mocé and Vicente, 2002; Mocé *et al.*, 2003a,b,c; 2005), avoiding the inclusion of egg yolk or skim milk. However, a detrimental effect of DMSO on sperm acrosomes (Hellemann *et al.*, 1979a; Martín-Bilbao, 1993) and *in vivo* fertility (Hellemann *et al.*, 1979b), has been confirmed in some studies as the concentration of this CPA increased above 4.5–5% in extenders containing egg yolk, although sperm motility increased as DMSO level increased.

Since 1980, amides have also been used for rabbit sperm cryopreservation, thanks to the studies performed by Hanada and Nagase (1980). Amides present lower molecular weight than glycerol, and cause less osmotic damage to the sperm cells. These authors reported that CPAs containing hydroxyl groups seemed to be less effective cryoprotective agents for rabbit sperm than those containing amide or methyl groups. From all P-CPAs tested (different amides, alcohols and DMSO), the ones that offered the best results were lactamide, acetamide or DMSO at a concentration 1 M in the extender. These results were confirmed in later studies conducted by Kashiwazaki *et al.* (2006), comparing in vitro 1 M glycerol, lactamide, acetamide and DMSO. Glycerol offered the worst results, lactamide and acetamide offered the best results and DMSO gave intermediate results. These results were also observed when lower concentrations of CPAs were used (2% acetamide vs. 2% glycerol; Okuda *et al.*, 2007). Other studies revealed that both sperm transport and fertility decreased when acetamide exceeded 0.83 M in the final mixture (Arriola and Foote, 2001).

Some studies were performed to compare DMSO and acetamide as P-CPAs, but none of them, were conclusive about the optimal CPA for rabbit sperm (Castellini *et al.*, 1992; Martín- Bilbao, 1993; Dalimata and Graham, 1997). Acetamide remains as one of the CPAs of choice for rabbit sperm cryopreservation (Parrish and Foote, 1986; Chen *et al.*, 1989a; Chen and Foote, 1994; Courtens, 1995; Fargeas, 1995; Dalimata and Graham, 1997). Unfortunately, neither extenders containing a mixture glycerol-DMSO nor extenders containing DMSO as the only CPA, provided repeatable results or results similar to those obtained with fresh sperm.

Although the combination of P-CPAs most commonly used is DMSO-glycerol, other combinations have been tested. Thus, Castellini *et al.* (1992) tested the combination 1 M acetamide with 0%, 2% or 5% glycerol. However, the inclusion of glycerol in the extenders did not improve the results after cryopreservation.

In light of what has been said before, it is evident that two types of P-CPAs seem to offer the best results for rabbit sperm cryopreservation. These are DMSO (in combination or not with glycerol) and acetamide. Unfortunately, none of these extenders provided repeatable results therefore, none of them has excelled as the P-CPA of choice for rabbit semen.

The most common N-CPAs used, for cryopreservation of rabbit semen are egg yolk, sugar (disaccharides) and skin milk.

Egg yolk is usually used in extenders for rabbit sperm freezing at concentration varying from 10% to 20% (Fox, 1961; Stranzinger *et al.*, 1971; Weitze *et al.*, 1976; Götze and Paufler, 1976; Theau-Clément *et al.*, 1996; Si *et al.*, 2006; Liu *et al.*, 2007). Skim milk (at a final concentration of 8-10%) has also been used in some extenders for rabbit sperm (Wales and O'Shea, 1968; O'Shea and Wales, 1969), although its use is less common than the use of egg yolk. Some authors even observed that skim milk or Laciphos (commercial skim-milk based extender) did not offer such good results as egg yolk for rabbit sperm (Rohloff and Laiblin, 1976).

The main disaccharides used as N-CPAs are lactose, sucrose, maltose, raffinose or trehalose (Hanada and Nagase, 1980; Liu, 1985; Vicente and Viudes de Castro, 1996; Dalimata and Graham, 1997). They have been used in combination with P-CPAs (DMSO, glycerol or acetamide) in rabbit cryopreservation extenders and their concentrations varying from 0.05 to 0.08 M. These disaccharides in general interact with the polar head groups of membrane phospholipids, and stabilize the membrane during cryopreservation process (Dalimata and Graham, 1997).

Moreover, some macromolecules (methyl-cellulose, gelatin, surfactants) have been tested, as N-CPAs for rabbit sperm cryopreservation. The inclusion of methyl-cellulose (0.5%) to the freezing extender (Dalimata and Graham, 1997) increased the percentage of motile sperm and live sperm after cryopreservation.

Gelatin provides protection to sperm during freezing by modifying or inhibiting ice crystal formation, due to its property to form gel with water. Nevertheless, addition of gelatin (1% final concentration, w/v) to the extender, did not improve rabbit sperm quality or sperm fertility (Olivares *et al.*, 2005; Cortell and Viudes de Castro, 2008).

Detergent inclusion (triethanolamine lauryl sulfate or sodium lauryl sulfate) has been tested in several works. Surfactants act as emulsifiers of egg yolk granules and favor the interaction between egg yolk and sperm (Arriola and Foote, 2001), offering acrosome protection (Hellemann *et al.*, 1979a; Hellemann and Gigoux, 1988).

After CPA addition, semen is **packaged** in specific devices. Although in recent years, the most widely used devices are the straws (0.25 or 0.5 mL capacity) (Mocé and Vicente, 2009), some other devices (such as glass ampoules) were used in the first studies (O'Shea and Wales, 1969). Other ways of sperm freezing have included pellets, plastic ampoules, or polyvinylchloride tubing (Stranzinger *et al.*, 1971). Recently a new device which allows freezing large volumes of sperm (2 mL) was used for rabbit sperm cryopreservation (Si *et al.*, 2006).

**Equilibration time** is the period during which cryoprotectants are able to penetrate sperm cells. The longest part of the cryopreservation process is cooling the sperm to 5 °C and equilibrating the sperm. These steps require 45 minutes in the fastest protocols for cryopreserving rabbit sperm, but for many protocols these steps may take 90 minutes or longer (Mocè and Vicente, 2009) and account for greater than 80% of the total time required to cryopreserve the sperm. Reducing the time required for cooling and equilibration, could considerably reduce the time required to cryopreserve rabbit sperm and permit more ejaculates to be preserved in a given time (Mocè *et al.*, 2014).

Finally, semen is **frozen in liquid nitrogen**. In 1942 Hoagland and Pincus (1942), observed that rabbit sperm could scarcely survive following immersion in liquid nitrogen after various plasmolytic pre-treatments, contrary to human sperm which remained highly active after freezing and thawing (Sawada and Chang, 1964; Mocè and Vicente, 2009). However, over the years many reaserchers turned their attention to develop effective freezing protocol finding a number of suitable extenders and cryoprotectants with the final goal of improvingthe survivability of rabbit spermatozoa during cryopreservation. In particular, to reduce the impact of sperm cells with liquid nitrogen (at -196°C), the best strategy was represented by exposure of semen on liquid nitrogen vapour prior to its plunging into liquid nitrogen. This procedure, was successfully used for rabbit semen using DMSO as cryoprotectant placing semen at 5cm from liquid nitrogen vapour compared to vitrification (Rosato and Iaffaldano, 2013).

The **thawing procedure** (temperature and time) is also crucial for the post-freezing quality of semen. When frozen samples are returned to room temperature, a reversal of the freezing process takes place. The rate of thawing depends on the freezing rate. During slow thawing (low temperature, long time), the small ice crystals formed during freezing start to melt, turning into large crystals (recrystallization) that are harmful to the spermatozoa (Watson, 1995). During fast thawing (high temperature, short time) the time for recrystallization to occur, is limited and this increases the survivability of spermatozoa.

Thawing, in general, is performed in water baths at 37–39°C or at 50°C for 10-12 s (Mocé and Vicente, 2009). Chen and Foote (1994) compared different thawing rates for 0.5mL straws (25°C, 1 min vs. 45°C, 30 s vs 65°C, 7 s) and observed that sperm quality improved when the slow thawing rate was used.

Mocé *et al.* (2003b) compared two thawing rates for 0.5 mL straws (50 or 70°C, 10–12 s) and observed similar kindling rates for both of the thawing protocols (67% and 68%), although sperm prolificacy was higher for sperm thawed at 50°C (7.1 live born) than sperm thawed at 70°C (5 live born).

### *9.7. Assessment of semen quality in vitro and in vivo*

*In vitro* assessment of fresh and frozen quality of rabbit semen, is performed taking in consideration specific macroscopic parameters including: volume, color, smell, density, presence or absence of gel plug, pH and microscopic parameters such as sperm motility, viability, osmotic resistance, acrosome integrity, DNA integrity that will be described in detail in chapter 10.

*In vivo* assessment of semen quality is performed by Artificial insemination (AI) procedure (figure 9.5). The latter, has become a routine practice in rabbit production (Alvariño, 2000). The technique offers significant benefits, including genetic selection, prolonged fertility even during unfavourable times of the year, cycle-based production, more efficient breeding programmes and last, but certainly not least, improved health monitoring (Bergonzoni *et al.*, 1994). AI in rabbits is generally performed with 0.5 ml of extended semen by use of a 25 cm cannula that is introduced into vagina (figure 9.5). Theoretically it is possible to obtain 30-40 doses per ejaculate, but in everyday practice it is preferable to have a dilution rate from 1:5 to 1:10, meaning approximately 10-15 doses/ejaculate, to ensure that there are at least 10 million viable, non damaged spermatozoa (Paufler, 1985; Facchin *et al.*, 1991; Facchin,

1995; Viudes de castro and Vicente, 1997; Castellini and Lattaioli, 1999). After extension, insemination must take place within 24-48 hours, since the sperm survival strongly decrease after 36 hours and its fertilizing capacity tends to diminish after about 16 hours of storage (Paufler, 1985; Facchin *et al.*, 1991).

However, independently of semen dilution, it should be kept in mind that the type of extender used will have an impact on the reproduction rate (Kiprianidis and Facchin, 1994).

Figure 9.5. Artificial insemination practice on rabbit female by use of 25 cm cannula contained extended semen

# Chapter 10

## Methods for evaluation of fresh and frozen semen quality in poultry and rabbit species

### *10.1. Evaluation of macroscopic and microscopic parameters in vitro*

The numerous effects that cryopreservation can induce in spermatozoa, range from lethal injuries to those which merely impair their subsequent function. In the last few years, the considerable increase in our understanding of both, the cell physiology of spermatozoa, and the stress of cryopreservation, have contributed to a renewed interest in improving the performance of cryopreserved semen. Despite the significant progress, the post-thaw viability and fertility of the cryopreserved sperm are still reduced, as a consequence of accumulated cellular injuries that arise throughout the cryopreservation process. Many laboratory tests have already carried out experiments to verify these detrimental effects and their origin (Partyka *et al.*, 2012). For evaluating fresh and frozen semen quality macroscopic and microscopic parameters can be taken into account.

**Macroscopic parameters** include volume, viscosity, visual appearence and pH of the ejaculate. These parameters depend on semen density that is determined by sperm concentration, composition of seminal plasma and its contents. Semen pH usually has a specific range. The visual appearance is based on the colour. In particular it should be milky, white evenly turbid but without clots and residues of urine, feces or blood residues that can affect its quality. The viscosity varies with decreasing sperm number from creamy, milky to watery and is regarded as an indication of reduced sperm concentration.

According to FAO guidelines, the evaluation of fresh and frozen semen quality can be performed based on three **microscopic parameters** e.g. sperm concentration, motility, and morphology (FAO, 2012). However, there are some other additional parameters that can improve the assessment of semen quality such as sperm viability, acrosome integrity, DNA integrity, osmotic resistance, apoptosis and ultrastructure as follows.

**Sperm concentration** is traditionally determined by means of cell-counting chamber known as Neubauer chamber or hemacytometer (figure 10.1). Hemacytometer was developed for counting blood cells, but can also be used to count spermatozoa. It consists of two chambers and each chamber has a microscopic grid etched on the glass surface. The chambers are overlaid with a glass coverslip that rests on pillars exactly 0.1 mm above the chamber floor. Thus, the volume of fluid above each square of the grid is known with precision. The semen must be killed to prevent movement and diluted before loading into the hemacytometer. This, can be done by diluting the semen into a buffer containing a small quantity of sodium chloride. The dilution factor must be recorded to allow calculating the concentration taking in consideration the number of sperm cell within five of the 16 smaller squares (thickness 1mm), that compose the central counting area of the hemacytometer. Currently, more feasible techniques, to determine sperm concentration are represented by spectrophotometer, electronic particle counter. However, because of the disadvantage of missing differentiation between sperm and other cells in the semen sample these procedures are not really preferred respect to traditional method. Additional possibility arises out of the integration of computer assisted sperm analysis (CASA) that measures sperm motility and concentration simultaneously.

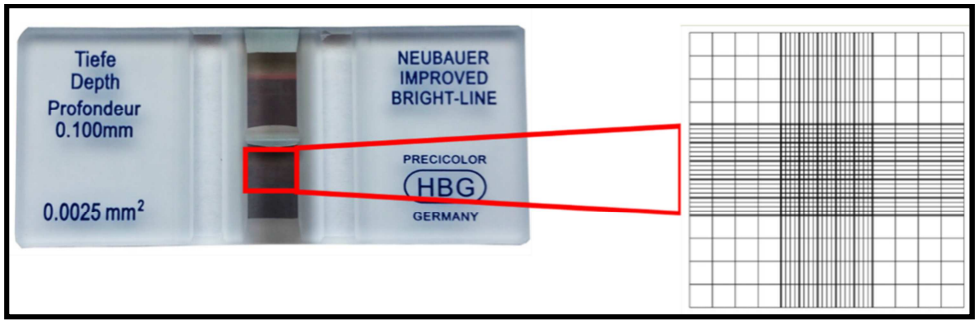



Figure 10.1. The Neubauer chamber

**Sperm motility** is an important semen parameter in predicting sperm fertility as it is indicative of a decrease in the functional competence of spermatozoa and is directly related to the quality of spermatozoa. Motility, can be considered as a physiologycal variable as it is indicative of the spermatogenesis process. Motility is classified according to a grading system. Any tipe of motility with an absence of progression is defined as total motility (TM) or non progressive motility (NP), while active linearly spermatozoa moving is known as progressive motility (PR). The percentage of progressive motility in the ejaculate is critical to ensure adequate sperm transport and fertilization (Nagy *et al.*, 2012)

Immotile sperm are the cells that are not able to move. Motility is assessed by visual extimation of a wet-mount slide (20 mm deep) under phase contrast microscope at × 200 or 400 magnification. In some cases, it is possible to use also the Leja counting chamber slide that is disposable created for sperm motility analysis. After the sample has stopped drifting, approximately 200 spermatozoa should be scored. The procedure can be performed at room temperature or at 37°C. For each sample analysed, is attributed a percentage score. Semen samples showing less than 70% motility are discarded, therefore they are not taken in consideration for *in vivo* trials. Traditional semen analysis by subjective analysis, is associated to the experience of the operator, the method of sample preparation and the number of cells evaluated. Variation in the results of conventional evaluation of the same samples by different observers and laboratories may achieve up to 30-60% (Coetze *et al.*, 1999). Alternatively, to subjective motility, the computer assisted sperm analysis (CASA) is currently becoming very popular for assessing sperm motility. CASA system is functionally able to project successive images of a sperm suspension onto a detector array and to detect objects based on intensity of pixels in a frame or light scatter (Amann and Waberski, 2014). The system is supplemented by special software to extract desired information and produce the desired output. Motion of each sperm is recorded as changes in centroid location in successive frames (Figure 10.2). The computations, provide output measures describing the motion through different specific parameters: curvilinear velocity (VCL) calculated as the time- average velocity of a sperm head along its actual curvilinear path as perceived in two dimensions in the microscope (µm/s), average path velocity (VAP) corresponding to the time-average velocity of a sperm head along its average path that is computed by smoothing the actual path (µm/s), straight line velocity (VSL) which is the measure of time-average velocity of a sperm head along the straight line between its first detected position and its last position (µm/s), amplitude of lateral head displacement (ALH) measuring the sperm head oscillation (µm), linearity of the curvilinear path (LIN) calculates the departure from linear progression as VSL/VCL × 100 (%), straightness of the average path (STR) measured as the departure of the cell path from a straight line and defined as VSL/VAP ×100 (µm/s), and beat-cross frequency (BCF) that is the frequency of sperm head crossing the sperm average path in either direction (Hz).

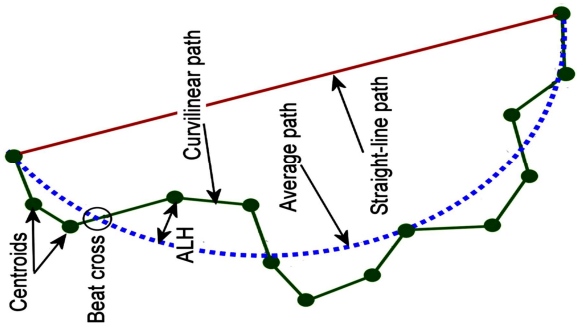



Figure 10.2. Illustration showing CASA terminology. Initial image processing provides a centroid for each spermatozoon in the first frame of a scene, and for each cell location of the most probable centroid in successive frames is deduced. Connecting the centroids for a spermatozoon provides its actual trajectory, termed curvilinear path. The time-averaged velocity along this trajectory is termed curvilinear velocity (VCL; mm/s). The average path is computed, and time-averaged velocity along this trajectory is termed average path velocity (VAP; mm/s). A straight-line path from the first to last position of a sperm head is plotted, and velocity along this trajectory is termed straight line velocity (VSL; mm/s). For each centroid location there is a deviation from the average path, and this is termed the amplitude of lateral head displacement (ALH; mm). Similarly, there are points where the curvilinear path intersects the average path, and the number of such intersections is termed beat-cross frequency (BCF; number per second) (Amann and Waberski, 2014).

S**perm morphology** provides vital information on the quality of the sperm sample. Morphology has been considered to be an essential parameter when establishing the fertility of the species of interest. During the maturation of spermatozoa the morphogenic process can result in imperfections and anomalies which can be seen in routine semen analysis. Mammalian spermatozoa abnormalities can be divided into primary and secondary abnormalities or in some classification systems into major and minor abnormalities. Primary sperm defects occur during spermatogenesis, and secondary defects are assumed to occur during maturation in the epididymis and the transit through the ductal system and specimen preparation (Partyka *et al.*, 2012). It must be considered that several factors can influence the spermatogenesis process such as chemical and environmental factors, which could result in anomalies in the morphology of spermatozoa. Many reports have shown the common classification system for the morphology of spermatozoa from different species. However, classification categories are different for the various species and the adoption of uniform system within each species is needed (Partyka *et al.*, 2012).

Sperm from different species vary in size and shape. Bull and human sperm, for example, have paddle-shaped heads, rodent sperm have hook-shaped heads, and the heads of chicken sperm are spindle-shaped and almost difficult to distinguish from the midpiece. Various types of sperm abnormalities, which result in a decrease in fertility and can lead to infertility, have been identified in mammals, including humans, and detailed descriptions of the defects including their ultrastructure have been documented (Plessis and Soley, 2011). The most common sperm abnormalities in mammalian sperm, are related to abnormal acrosomal regions/heads, detached head, proximal droplets, distal droplets, abnormal midpieces, bent/coiled tails. Acrosome defects include knobbed, roughed, and detached acrosomes. In ddition, there are a number of reports on the incidence of sperm abnormalities in birds, particularly in domestic poultry such as the fowl (Siudzinska and Lukascewivz, 2008), turkey (Alkan *et al.*, 2002), duck (Penfold *et al.*, 2000) and goose (Ferdinand, 1992). Spermatozoa within the fraction of live cells were classified as morphologically normal (spindle-shaped head with well-marked acrosome and visible tail) or with a swollen head, bent neck, defective midpiece, or other deformity (coiled tail, lack of tail, spermatides, etc.) (Siudzinska and Lukascewicz, 2008). Vital dye in combination with different stains, are commonly utilised to assess the spermatozoa morphology and the viability together. For this purpose, India ink, William's, Karras, Spermac, Diff-Quick, Papanicolaou, Fuelgen or combination: Trypan blue and Giemsa, Trypan blue, Bismarck Brown and Rose Bengal, and finally eosin-nigrosin have been used in birds and mammals including human to analyze sperm morphology (Partyka *et al.*, 2012).

**Sperm viability** is a key determinant of sperm quality and a prerequisite for successful fertilization. The principle of the test, is based on the exclusion of the dye by the sperm membrane. In living cells, the membrane remains intact and therefore excludes the dye from penetrating. However, in dead sperm the membrane's integrity is compromised and hence cellular staining by the dye occurs. The most common staining method used for viability assessment is the one-step eosin- nigrosin staining technique have been used in birds and mammals including humans (Siudzińska and Lukascewicz 2008; Halili *et al.*, 2014). When stained smears are viewed under the oil immersion objective of light microscope, the percentage of viable, live, properly formed spermatozoa, nonviable and also partially-damaged spermatozoa can be determined. In eosin-nigrosin stain under the microscope, live spermatozoa appear white, unstained against the purple background of nigrosin.

Dead and damaged spermatozoa having a permeable plasma membrane are pink. The evaluation of the percentage of live and dead spermatozoa and the percentage of morphology defects can be performed on the same nigrosin-eosin stained slides.

Membrane integrity of mammalian and avian spermatozoa may be assessed by using many fluorescent probe combinations including: carboxyfluorescein diacetate (CFDA) in combination with propidium iodide (PI), SYBR-14 with PI, carboxy-seminaphthorhodfluor (Carboxy-SNARF) with PI, calcein-AM with ethidium homodimer (EthD-1) and Hoechst 33258 (Partyka *et al.*, 2012).

SYBR-14 and CFDA, usually used as detectors of live cells, are membrane-permeant compounds, which are immediately deacylated and thus rapidly converted into high fluorescent compounds by intracellular esterases. These green fluorochromes, are maintained intracellular by intact membranes. As plasma membrane deteriorates at cell death, cells loose their ability to resist the influx of red fluorescent PI. The latter, replaces or quenches green fluorochromes (Garner and Johnson, 1995). Live, viable, intact spermatozoa show a green fluorescence (CFDA, SYBR-14, calcein-AM) while dead sperm showa red fluorescence (PI). Carboxy-SNARF is pH indicator which stains live spermatozoa orange, while bisbenzimide stain Hoechst 33258 labels dead spermatozoa bright blue (Hewitt and England, 1998).

Indeed SYBR-14/PI fluorochromes are more sensitive in comparison with conventional method of live-dead cell assessment. The advantage of the use of fluorochromes, is the possibility to assess the semen without the interference of fat particles and others material present in the extended semen (Rijsselaere *et al.*, 2005). Sperm viability can be assessed by numerous methods, some manual and other automated (Hossain *et al.*, 2011).

The most automated technology to assess sperm viability is represented by flow citometry (FC). Today, FC is a recognized methodology within animal spermatology, and has moved from being a research tool to become routine in the assessment of animal semen destined to breeding. An FC is an instrument that can measure physical, as well as multicolor fluorescence properties of particles of cells flowing in a flowing stream. It is basically composed of four main systems, fluidics, optics, electronics and software handling. Cells analyzed, are centrifuged sufficiently before so the supernatant fluid can be removed with little loss of cells. After centrifugation, the samples are marked using the fluorescent probes described above, and resuspended. When the cell suspension is introduced into this instrument for analysis, it flows through a tubular system and is exposed to laser (or mercury arc lamp in some older instruments) illumination at particular spots. The emission is

recorded from the cells as a result of this illumination and is digitized and computer-handled to provide understandable results. A fluid that is moving under a laminar flow specifies the movement and velocities that cells possess when passing across the detector. During this transport, cell properties like fluorescence, absorbance and light scattering can be detected, making use of one or several illumination sources, which might differently excite uploaded markers. In this way, FC detects labeling by multiple fluorochromes associated with individual spermatozoa, so that more than one sperm attribute, can be assessed simultaneously, increasing our capacity to correlate these attributes to, for instance, potential fertilizing capacity.

Data analysis consists of displaying the data from a list-mode file in a plot, then measuring the distribution of the events within the plots. It is possible to make a single-parameter histogram plot with histogram markers, a two parameters dot plot with a quadrant marker, a two-parameter dot plot with regions, and three-dimensional plots. A dot plot provides a two-parameter display of data. Each dot represents one or more events or particles present within cell suspension. Each dot plot is divided into four sections (quadrants) that allow to distinguish populations in negative (lower-right), single positive (upper-right), or double positive (upper-left). The lower-left quadrant displays events that are negative for both parameters and corresponds to debris (figure 10.3).

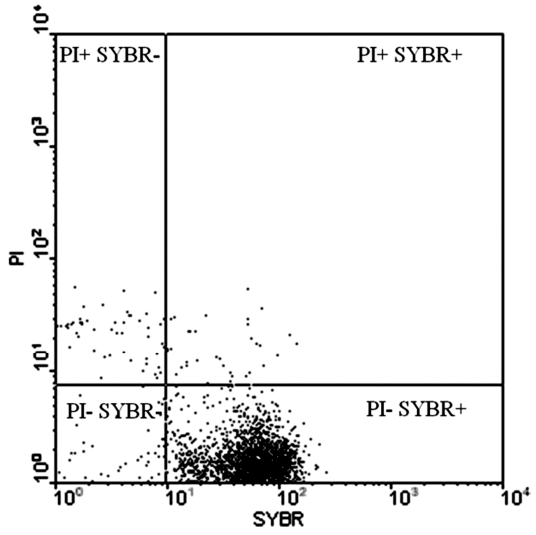



Figure 10.3. Flow cytometric dot plot of Canada goose (Branta canadensis) sperm analyzed for both SYBR-14 and propidium iodide (PI) fluorescence. PI- SYBR- quadrant contains debris; PI- SYBR+ quadrant contains live spermatozoa; PI+ SYBR- quadrant contains dead spermatozoa; and PI+ SYBR+ quadrant contains dying spermatozoa (Partyka *et al.*, 2011a).

**Sperm acrosome integrity** is a key factor for successful fertilization. The acrosome is located in front of the sperm head and it is formed by two membranes that contain inside glycoproteins, sugars and enzymes such as hyaluronidase and acrosin (Harrison, 1983). These enzymes help in the fertilization process by participating in the digestion of the zona pellucida (in mammals) and egg envelope (in birds) and allowing the entry of the genetic material of the sperm into the oocyte. Numerous probes can be used to evaluate acrosome status. Particularly acrosome integrity may be measured using conjugated lectins because of its glycoprotein environment.

Conjugated lectins bind to glucose, mannose, galactose, N-acetylglucosamine and other carbohydrate-specific glycoproteins that are exclusively located in the acrosome. The most commonly used lectins are PSA (*Pisum sativum agglutinin*) or PNA (peanut agglutinin from *Arachis hypogaea*), depending on the mammal species (Carretero *et al.*, 2015b). These agglutinins should be conjugated to fluoresceins, such as FITC (Fluorescein isothiocyanate) (Baker *et al.*, 2004) or RPE (R-phycoerythrin), for microscopy visualization (Cunha *et al.*, 2015). Lectins are impermeable to intact acrosomes, but reacted or damaged acrosomes allow the entry of these macromolecules. PNA binds to glycoproteins of the outer acrosome membrane, and PSA identifies binds to matrix enzymatic components of the acrosome lumen by binding specifically to the α-mannosidase sugar in the acrosome content. The presence of a green color region, indicates a reacted acrosome in non-fixed cells. A fluorescent acrosome, is considered intact in sperm fixed and permeabilized sperm. No difference, was found using PSA or PNA alternatively. Similar results, were obtained using different lectins (Chuna *et al.*, 2015).

**Sperm DNA** consists of a complex of macromolecules within nuclear compartment named chromatin. The evaluation of chromatin damage is an important aspect of sperm analysis because some sperm with chromatin abnormalities can fertilize the oocyte, but impairing the embryo development leading to a high rate of embryonic death (Kato and Nagao, 2015). Changes in chromatin may occur due to various factors, including, toxic and pathogenic agents, gene mutations, chromosomal abnormalities, high levels of oxidative stress and the loss of testicular thermoregulation (Karabinus *et al.*, 1997).

All of these factors can result in DNA damage, such as strand breaks or fragmented DNA. Sperm DNA fragmentation is assessed using the terminal deoxynucleotidyl transferase mediated dUDP Nick end labeling (TUNEL) test (Caglar *et al.*, 2007) and the single cell gel electrophoresis (COMET) test (Mynou *et al.*, 2012).

The *TUNEL* test consists of the incorporation of nucleotides labeled with FITC at the free region (3'OH) of all free breaks in the DNA strands. The level of labeling in each sperm is quantified using flow cytometry to indicate the degree of strand breakage within the sperm head. The incorporation of fluorochrome conjugated deoxyribonucleotide on the free breaks regions of DNA is amplified using a secondary enzymatic reaction. The labeling of sperm with green fluorescence indicates the presence of DNA fragmentation, and sperm that exhibit blue fluorescence are negative for fragmentation.

The *COMET* assay or single-cell gel electrophoresis, is a relatively simple and sensitive method for measuring strand breaks in DNA in individual sperm. is based on the principle that when sperm DNA breaks, the region where this occurs, becomes negatively charged. Therefore, exposure of the DNA fragment to an electric field results in the migration of the DNA fragment out of the nucleus and migration to the positive pole. During this procedure sperm cells are embedded in a thin layer of agarose on a microscope slide and lysed with detergent under high salt conditions. This process removes protamines and histones allowing the nucleus to form a nucleoid-like structure containing supercoiled loops of DNA.

Alkaline pH conditions result in unwinding of double-stranded DNA, and subsequent electrophoresis, results in the migration of broken strands towards the anode, forming a comet tail, when observed under fluorescence microscope. The amount of DNA in the head and tail, is reflected by its fluorescent intensity. The relative fluorescence in the tail compared with its head serves as a measure of the level of DNA damage evaluated based on the fluorescence intensity and the length of the tail extension (Farah *et al.*, 2013).

Another test to evaluate chromatin structure is *acridine orange* (AO) dye. This fluorophore assesses DNA stability to acid or heat denaturation. The fluorochrome intercalates the double-stranded DNA as a full monomer and fluoresces green. The fluorochrome binds to the single-stranded DNA in the form of aggregates and emits a red/orange fluorescence if the DNA is damaged. Therefore, sperm with normal DNA emit green fluorescence, and sperm with abnormal DNA, emit a color ranging from yellow to red color (Farah *et al.*, 2013).

The hypo osmotic swelling test (HOST) is a simple laboratory test is used for the assessment of **osmotic resistance** associated to the functional sperm membrane integrity. This test, is based on the principal of semi-permeability of integral cell membrane of spermatozoa. This ability helps the sperm to swell under the hypo osmotic situations. Under the exposure of hypo osmotic conditions, swelling of the sperm tail indicates the movement of water across the membranes to get the equilibrium. When the sperm are put in hypo osmotic solution, water moves within cell membrane leading to its swelling. If the membrane is already damaged,the swelling of membrane does not occur. The percentage of swollen spermatozoa is the measure of intact membrane. Living sperm can maintain an osmotic gradient under hypo-osmotic conditions whereas a dead cell cannot tolerate this stress.

In a normal sample more than 60% of spermatozoa react to hypo-osmotic challenge. The evaluation of membrane integrity and motility, predict the good fertility rate of an individual. Due to high importance of plasma membrane in the process of fertilization, the evaluation of plasma membrane integrity received the significant attention in the semen evaluation of male animal. The process of fertilization, which comprises the certain physiological events (union of sperm to egg, reaction of acrosome and capacitation) require the active plasma membrane of sperm and it is not possible for the occurrence of fertilization with inactive plasma membrane (Zubair *et al.*, 2013). Sometimes, HOST is used as vital marker in combination with fluorescent dyes. As reported in Iaffaldano *et al.* (2011, 2016a) in fact, the combination SYBR-PI has been used for turkey sperm cell under hypo-osmotic conditions. As result, viable thawed spermatozoa with intact membranes developed a green fluorescence conferred by SYBR and excluded PI. Conversely, damaged membranes permitted the passage of PI, staining spermatozoa that have lost their functional integrity red.

**Apoptosis** is the programmed cell death that occurs because of the DNA fragmentation. Some of the earliest detectable apoptotic events involve sperm plasma membrane, including changes in membrane asymmetry and permeability. The first step is represented by the disruption of sperm membrane phospholipid through the destabilization of Phosphatidylserine (PS), which is a component of the cell membrane. Early apoptosis and late apoptosis can be detected using two different fluorochromes: Annexin-V and YO-PRO-1 respectively. During early apoptosis, sperm membrane is subject to its early destabilization induced by the translocation of PS detectable by use of Annexin-V.

The latter, is able to produce highly fluorescent conjugates with PS which is translocated from the inner to the outer leaflet of the membrane and asymmetrically exposed to the external cellular environment in early apoptotic cells. Spermatozoa with disordered PS asymmetry exhibit green fluorescence whilst intact spermatozoa remain unstained.
The evaluation of late apoptotic sperm instead is performed using fluorescein YO-PRO-1 which is unpermeable nuclear dye crossing selectively plasma membrane of apoptotic cells and labeling them with moderate green fluorescence.
In contrast with apoptosis, the necrosis is a form of cell injury which results in the premature death of cells in living tissue by autolysis. Necrotic sperm cells can be detected by use of Propidium iodide (PI) which is a popular red-fluorescent nuclear and chromosome counterstain. Since it is not permeant to live cells, it is also commonly used to detect dead cells in a population. PI binds to DNA by intercalating between the bases with little or no sequence preference and stains the nuclei of membrane-damaged cells red. All samples analyzed for apoptosis have to be centrifuged and then stained after the removal of surnatant. Annexin-V and YO-PRO-1 and PI are often used in combination with DAPI (4',6-diamidino-2-phenylindole) that is a fluorescent stain which strongly binds to A-T rich regions in DNA. Resulting cells are observed by use of fluorescence microscope (magnification 400 x) using 488nm, 560nm and 420nm filters for annexin-V/YO-PRO-1, PI and DAPI, respectively.

The study of **ultrastructure** allows to analyse the level of damage caused by freezing and thawing. It is possible to evaluate sperm structure in detail, verifying the integrity of their components including mitochondria, mid-piece, nucleus, and perforatorium. The procedure known as “ultrastructure analysis” is able to predict sperm fertilization ability through the study of sperm abnormalities. Therefore, it represents a feasible method to establish the complete integrity of sperm cell. Indeed, for deeply observing spermatozoa by microscope, staining methods should be used. However, this technique is unable to detect interior organelles of sperm cell with high definition. The limits of light microscopy can be overcome by the use of transmission electron microscopy (TEM) which permits the exploration of the ultrastructural organelles rigorously, which are characterizing sperm abnormalities. The ultrastructural organization of sperm organelles plays a significant role for cell function and, therefore, for the reproductive process.

### *10.2. Assessment of fresh/frozen semen quality in vivo*

The most valid assessment of semen quality *in vivo* is to obtain viable pregnancies and normal offspring following **artificial insemination (AI)**. Over the past twenty years, the assisted reproduction techniques reached a rapid advance in domestic species of economic interest. By the time, the male gamete has provided to be the most successful tool used to improve animal breeding programs. Freezing and stock of semen is a safe procedure to preserve reproductive potential of animals with superior genetic heritage. The fertilizing capacity of frozen semen is influenced by several factors including: semen characteristics, freezing technique, insemination dose, as well as status and management of the females.

AI programs have developed in response to a variety of needs in avian propagation (Smyth, 1968; Martin, 1975; Cooper 1977; Gee and Temple 1978). The most obvious need was to reduce or eliminate infertility (Szumowski *et al.* 1976, Lake, 1978; Sexton, 1979). In some mated pairs, natural copulation can be difficult because of differences in body size, injury, or deformity, and in some, natural copulation maybe inhibited by behavioral difficulties.

In other situations, some females may be maintained in separate pens because of incompatibility or the lack of a mate. Occasionally a productive female may be in a distant location separate from the male, where transfer of semen is the only alternative to infertility. In addition, poor fertility in a mated pair, can be improved through insemination with semen from another male. AI is useful in carrying out the goals of special breeding programs.

# PART II
# RESEARCH STUDIES

# PREFACE

The alarming decline in biological diversity, particularly in local poultry and rabbit breeds, due to the introduction of modern intensive production methods associated to increasing income, human population and urbanization, prompted conservation biologists to develop strategies to conserve and maintain genetic diversity of these breeds. Local poultry and rabbit breeds consist of locally adapted stocks that have been evolved to specific environments for thousands of years. Moreover they have been considered particularly precious because of their disease resistance capacity and their major robustness against pathogen threats. Thanks to these characteristics, local breeds can result specifically adequate for agroecological production systems, therefore they need to be preserved.

Many countries as illustrated above, have been involved in specific conservation programs for poultry and rabbit breeds. However, the majority of these programs, were accomplished by only *in situ* conservation strategy that is going on a relatively slow pace because of the complexity of maintaining live animals. Therefore, although the Convention on Biological Diversity considered *ex situ* conservation to be an essential complementary activity of *in situ* conservation strategy (FAO, 2012), the development and operation of a gene bank for cryoconservation of animal genetic resources including poultry and rabbit breeds, occurred only in few countries in the World. Currently also in Italy, there is an urgent need of national actions aiming at specific conservation programs for Italian rabbit and poultry breeds because of the limited number of their individuals becoming in risk of extinction. Conservation and valorisation projects of Italian poultry and rabbit breeds have been developing thanks to the financial support of regional and local public institutions.

However, Italian projects for poultry and rabbit breeds are only based on *in situ* conservation strategy, whilst nothing has been done about the *ex situ in vitro* strategy based on endless storage of genetic material in the form of haploid (semen and oocytes) or diploid cells (embryos, somatic cells) by cryoconservation.

Regarding the *ex situ in vitro* management of poultry breeds including turkey and chicken, and rabbit breeds, the semen cryopreservation represents a valuable tool to safeguard the genetic animal resources by cryobanks.

This technology becomes particularly precious in poultry species including turkey and chicken, since it is the only reproductive technology which is currently available for these species because of the inability to freeze embryos and oocytes (Gee, 1995; Hammerstedt,

1995; Blesbois *et al.*, 2008; Mosca *et al.*, 2016). In many countries, successful semen cryopreservation has enabled the creation of semen banks for several wild and some poultry species (Saint Jalme *et al.*, 2003; Blackburn, 2006; Woelders *et al.*, 2006; Blesbois, 2007; Blanco *et al.*, 2009; Santiago-Moreno *et al.*, 2011; Kowalczyk *et al.*, 2012). However, the conservation of poultry genetic resources in Italy was accomplished only by *in situ* strategies.

In light of this, the principal aim of the present thesis is to find a reference procedure for freezing turkey (study 1) and chicken (study 2) semen by implementation of the first semen cryobank of Italian poultry breeds.

Regarding the *ex situ in vitro* management of local rabbit breeds, semen cryopreservation has been more studied and the results are more satisfactory respect to those of poultry species (Iaffaldano *et al.*, 2014; Safaa *et al.*, 2014;Mocè *et al.*, 2014; Zhu *et al.*, 2015). Moreover, the semen cryopreservation results easier and cheaper than embryos cryoconservation (Mocè and Vicente, 2009). However, definition of optimal freezing protocols is still needed for rabbit semen and to improve the sperm management of rabbit breeds (Rosati *et al.*, 2007). Currently in Italy there are 43 local rabbit breeds which have been enclosed in a specific register (*Registro Anagrafico della specie cunicola, ANCI*) and three of them (Bianca Italiana, Macchiata Italiana and Argentata Italiana), have been already involved in genetic improvement programs.

The present doctoral thesis is part of a more extended project realized by some Italian universities including the University of Molise, aiming to preserve and valorize Italian poultry and rabbit breeds by a lot of actions including even the construction of a national semen cryobank.

My research activity during my PhD period includes three studies whose aims and results are reported in short below :

The first study was designed to identify a suitable protocol for freezing turkey semen in straws exposed to nitrogen vapour by examining the effects of dimethylacetamide (DMA) or dimethylsulfoxide (DMSO) as cryoprotectant (CPA), CPA concentration, freezing rate and thawing rate on *in vitro* post-thaw semen quality. Cryosurvival of turkey sperm was affected by DMSO concentration. Freezing rate affected the motility of sperm cryopreserved using both CPAs, while thawing rates showed an effect on the motility of sperm cryopreserved using DMA and on the viability of sperm cryopreserved using DMSO.

Significant interactions between freezing rate × thawing rate on sperm viability in the DMA protocol were found.
The most effective freezing protocol was the use of 18% DMA or 10% DMSO with freezing 10 cm above the $LN_2$ surface and a thawing temperature of 50°C. An efficient protocol for turkey semen would improve prospects for sperm cryobanks and the commercial use of frozen turkey semen.

The second study aimed to compare effect of four different permeating cryoprotectants and two thawing temperatures (37 vs. 5°C) on sperm post-thaw motility and to analyse combined effect of the best permeating cryoprotectant (P-CPA) with one of four non-permeating cryoprotectants (N-CPA) on post-thaw quality of rooster semen evaluated *in vitro*. Our results indicate that the combination of EG and the thawing at 5°C improves sperm post-thaw motility. Moreover, ficoll addition to EG-based freezing extender, provided additional beneficial effect on progressive movement and apoptosis incidence. Further work should evaluate different N-CPA concentrations to improve freezing protocol. In addition, fertility evaluation and testing on different chicken lines, are needed, in order to contribute to animal genetic resources bank.

The third study intends to investigate the effect of two cooling times at 5°C (45 min vs 90 min) in order to improve the post-thaw quality of rabbit semen *in vitro* and *in vivo.* Our results indicate that the 90 min cooling incubation prior to dilution with cryoprotectants improves the post-thaw sperm viability, motility and fertility when compared to 45 min.
In fact, reproductive performance obtained with semen cooled for 90 min before cryopreservation and with fresh semen were similar. Hence, the present research provides an effective freezing protocol for rabbit semen that will allow for the introduction of a sperm cryobank for the conservation of Italian rabbit genetic resources, as well as for the use of frozen semen doses in commercial farms.

# Chapter 11

## STUDY1

**Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality**

http://dx.doi.org/10.1080/00071668.2016.1148261

### 11.1. AIM

The cryopreservation and storage of germplasm has long been valued for the indefinite preservation of genetic material, especially in cases of high-risk populations. An immediate need for this practice was identified for research using unique poultry lines (Long and Kulkarni, 2004). Today, however, semen cryopreservation seems to be the only effective method of storing reproductive cells for the ex situ management of genetic diversity in birds (Blesbois, 2011; Kowalczyk and Łukaszewicz, 2015). Successful semen cryopreservation has enabled the creation of semen banks for several wild and some domestic chicken species and breeds (Saint Jalme *et al.*, 2003; Blackburn, 2006; Woelders *et al.*, 2006; Blesbois, 2007; Blanco *et al.*, 2009; Kowalczyk *et al.*, 2012). However, research has not yet advanced sufficiently to facilitate a turkey semen cryobank. The possibility of using turkey semen in frozen form for artificial insemination (AI), besides maintaining and ensuring the long-term conservation of this bird's genetic diversity, would have practical benefits for turkey production. Turkeys are the only commercial livestock species that depend entirely upon AI for fertile egg production. Hence, the turkey industry would greatly benefit if semen could be cryopreserved soon after its collection and used for subsequent AI (Rosato *et al.*, 2012). Protocols for cryopreserving turkey semen are unsatisfactory, leading to poor post-thaw sperm quality with obvious consequences on fertility (Blesbois, 2007; Iaffaldano *et al.*, 2011). Due to their different biophysical and biological characteristics, turkey spermatozoa are much more sensitive to damage caused by cooling, freezing and thawing than chicken semen (Blanco *et al.*, 2000, 2008; Blesbois, 2007; Iaffaldano *et al.*, 2011). Thus the freezing and thawing procedures developed for chickens or other birds are inefficient for turkey spermatozoa.

Researchers have turned their attention to developing freezing protocols for the improved cryopreservation of turkey semen by reducing the cell damage caused by freezing and thawing. Among the procedures tested, the pellet method has shown some promise. Recently, we optimised the pellet procedure by examining the effects of different combinations of critical steps (Iaffaldano *et al.*, 2011). However, unlike straws, as a packaging system, pellets do not ensure sperm traceability or the safe transport of semen for breeding and the identification of each sample, which is required in cryobanks. Each cryopreservation procedure has its own particular variables influencing sperm cryosurvival. Numerous factors may affect the success of turkey semen cryopreservation, although a decisive role is played by combinations of factors such as the cryoprotectant (CPA) used and its concentration, the speed of freezing and the packaging system (Tselutin *et al.*, 1995; Blanco *et al.*, 2011, 2012; Iaffaldano *et al.*, 2011; Long *et al.*, 2014). Optimal freezing and thawing rates minimise the damage caused by intracellular ice formation, cell shrinkage and exposure to multiple osmotic gradients; these factors are critical for developing successful semen cryopreservation protocols. The effects of freezing rates on the quality of cryopreserved chicken sperm have been established (Blanco *et al.*, 2000; Woelders *et al.*, 2006) though some of these data are still lacking for turkey sperm (Blanco *et al.*, 2012). The most important factors for an effective freezing protocol are the choice of CPA and its concentration. The CPAs mainly involved in freezing protocols for turkey semen are glycerol, dimethylsulfoxide (DMSO), ethylene glycol, and dimethylacetamide (DMA) (Blesbois, 2007; Iaffaldano, 2015). DMA and DMSO have been used as alternative CPA to glycerol because of its contraceptive effect (Hammerstedt and Graham, 1992; Blanco *et al.*, 2000). DMA was largely adopted as CPA for turkey semen cryopreservation using rapid or low freezing-thawing procedures and pellets or packaging in straws (Blanco *et al.*, 2011, 2012; Iaffaldano *et al.*, 2011; Long *et al.*, 2014), whereas little is known about the use of DMSO.There is a clear need to standardise the complete freezing and thawing process to improve the post-thaw quality of turkey semen and minimise variability in results. This study aimed to identify a suitable protocol for the in-straw freezing in nitrogen vapour of turkey semen using DMA or DMSO as CPA without any special freezing equipment. We tested the effects of two concentrations of DMA or DMSO and different freezing and thawing rates on in vitro post-thaw semen quality.

## 11.2. MATERIALS AND METHODS

### *11.2.1. Experimental design*

The model used for the cryopreservation of the turkey semen for both CPAs (DMA or DMSO) was a 2 × 3 × 2 design: CPA concentration (8% and 18% DMA, 4% and 10% DMSO), freezing rate (three different heights, 1, 5 and 10 cm, above the liquid nitrogen level) and thawing rate (4°C for 5 min and 50°C for 10 s). Samples of pooled turkey semen were processed for freezing using the full combinations of these factors.

### *11.2.2. Chemicals*

The LIVE/DEAD Sperm Viability Kit was purchased from Molecular Probes, Inc. (Eugene, OR, USA). DMSO, DMA and all the other chemicals used in this study were purchased from Sigma Chemical Co. (Milan, Italy).

### *11.2.3. Birds*

A total of 50 turkey males of the Hybrid Large White line were supplied by Agricola Santo Stefano (Amadori Group, TE, Italy). Turkeys were reared in a poultry house in a controlled environment with artificial lighting (14 h light- 10 h dark cycle) and given free access to a standard commercial feed and water. The 7-week trial began when the birds were 45 weeks of age.

## 11.3. SEMEN PROCESSING

Semen was collected once a week by abdominal massage, yellow and abnormal semen samples were discarded. Ejaculates were pooled (1 ejaculate/ male; 4–6 ejaculates/pool) to avoid the effects of individual differences among males. Seven pools were used, each containing at least 4 ml of semen and an average concentration of $10.12 \pm 0.32 \times 10^9$ spermatozoa (spz)/ml.

The quality of the fresh semen was assessed in an aliquot taken from each pool as described below and the remaining undiluted semen pool was cooled at 4°C for 25 min before freezing.After cooling, the pools were diluted 1:1 (v:v) with a pre-cooled freezing extender composed of Tselutin diluent (Tselutin *et al.*, 1995) containing DMA or DMSO (as permeable CPAs) to give final concentrations of 8% and 18% DMA, and 4% and 10% DMSO. The extended semen was packaged in 0.25 ml plastic straws sealed with polyvinyl chloride powder. The straws were grouped by treatment and equilibrated at 4°C for 20 min (equilibration time). Semen was frozen by exposure to liquid nitrogen vapour at different heights above the liquid nitrogen surface (1, 5 and 10 cm) for 10 min to give three different freezing rates. During these 10 min, the temperature of straws at 1 cm fell from +4°C to −140°C, at 5 cm from +4°C to −125°C and at 10 cm from +4°C to −90°C, indicating a slower freezing rate as the distance from the liquid nitrogen increases. Temperatures were monitored by a temperature sensor (Ascon M1). Subsequently, the straws were plunged into liquid nitrogen for storage at −196°C. Sperm samples were thawed by immersion of the straws in a water bath: (1) at 4°C for 5 min; or (2) at 50°C for 10 s.

## 11.4. SPERMATOZOA QUALITY

In both the fresh and thawed semen samples, spermatozoa motility, viability and osmotic tolerance were determined in duplicate. Spermatozoa motility was subjectively evaluated by visual estimation. A 5 µl-drop was diluted in 45 µl of Tselutin extender, and 5 µl of extended semen was deposited on a clean glass slide prewarmed to 38°C and covered with a coverslip. The mounted slides were observed on a warm-plate at ×400 using a phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany). Percentage motility was estimated in 5 microscopy fields. Spermatozoa viability was determined as described previously by Rosato *et al.* (2012) using the fluorescent stains SYBR-14 and propidium iodide (PI). This procedure was performed on 5 µl of semen, which was added to 80 µl of extender containing 2 µl SYBR-14 (diluted 1:100 in DMSO). The extended semen was incubated at 38°C for 10 min, and 5 µl PI (diluted 1:100 in PBS) added followed by incubation at 38°C for a further 5 min.

Next, 10 μl of the suspension were placed on microscope slides, covered with a coverslip and viable/nonviable spermatozoa were determined by fluorescence microscopy (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany; blue excitation filter λ = 488 nm; ×100 oil immersion objective; total magnification ×1000). SYBR- 14 is a membrane-permeable DNA stain for live spermatozoa producing bright green fluorescence of nuclei. PI stains the nuclei of membrane damaged cells red, so that spermatozoa showing green fluorescence are recorded as live and those fluorescing red as dead. After counting at least 200 spermatozoa, percentages of viable spermatozoa were calculated as a ratio: green cells/(green cells + red cells) × 100. To determine the osmotic tolerance of the sperm membrane, a hypo-osmotic swelling test (HOST) was used (Iaffaldano *et al.*, 2011). Aliquots of 5 μl of diluted semen were added to 80 μl of distilled H2O and stained with SYBR and PI and counted as described for sperm viability. This test is effective for assessing the percentage of viable spermatozoa that are capable of withstanding hypo-osmotic stress in vitro. Under hypo-osmotic conditions, viable thawed spermatozoa with intact membranes will fluoresce green (SYBR) and exclude PI. Conversely, damaged membranes permit the passage of PI, staining spermatozoa that have lost their functional integrity red.

## 11.5. STATISTICAL ANALYSIS

To compare the different treatments, we used a randomised block design in a 2 × 3 × 2 factorial arrangement (2 CPA concentrations × 3 freezing rates × 2 thawing rates), with 7 replicates per treatment. Sperm variables (motility, viability and osmotic tolerance) were compared among by ANOVA followed by Duncan's test. A generalised linear model procedure was used to determine the fixed effects of CPA concentration, freezing rate, thawing rate and their interactions on the sperm quality variables. Significance was set at $P \leq 0.05$. All tests were performed using SPSS software (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA).

## 11.6. RESULTS

Spermatozoa motility in fresh semen was 77.2 ± 2.0%, sperm viability and sperm osmotic tolerance were 78.8 ± 1.3% and 58.9 ± 2.2%, respectively. Semen quality variables assessed after thawing were motility, viability and osmotic tolerance (Tables 11.1 and 11.2).

The fixed effects of CPA concentration, freezing rate, thawing rate and their interactions on spermatozoa quality variables for the DMA freezing protocol are shown in Table 11.1. Effects of freezing and thawing rates ($P \leq 0.05$) were detected on spermatozoa motility, while there was a significant interaction between freezing rate × thawing rate on spermatozoa viability. Regarding the remaining treatments, the greatest motile spermatozoa percentages ($P < 0.05$) were recorded for semen frozen 10 cm above the liquid nitrogen in the presence of 18% DMA and thawed at 50°C (18% DMA/10 cm/50°C) with the exception of the treatment combination 8% DMA/10 cm/50°C. Lowest motile spermatozoa percentages were observed for the treatments 8% DMA/5 cm/4°C and 8% DMA/5 cm/50°C. Higher spermatozoa viability percentageswere observed for the combinations 18% DMA/10 cm/50°C or 4°C with no significant differencebetween the two or with respect to the other treatments except 8% DMA/5 cm/50°C. The fixed effects of CPA concentration, freezing rate, thawing rate and their interactions observed on the spermatozoa quality variables for the DMSO freezing protocols are in Table 11. 2. An effect of CPAconcentration was observed on all sperm quality variables examined. In addition, freezing rate affected spermatozoa motility, while thawing rateaffected viability. Better post-thaw spermatozoa motility was recorded for semen frozen using the treatment combination 10% DMSO/10 cm/50°Cwith respect to all the other treatment combinations ($P < 0.05$). The best treatment combination in terms of effects on viability was also 10% DMSO/10 cm/50°C and showed significance with respect to all other treatment combinations with the exceptions 10% DMSO/10 cm/4°C and 10% DMSO/5 cm/50°C. Higher rates of spermatozoa osmotic tolerance were also observed for 10% DMSO/10 cm/50°C versus 4% DMSO/1 cm/4 and 50°C or 4% DMSO/5 cm/4 and 50°C.

Table 11.1. Sperm quality variable (mean ± SE) recorded for semen frozen using DMA as cryoprotectant according to CPA concentration, freezing rate and thawing rate (N = 7).

| Semen treatment | | | Sperm variable (%) | | |
|---|---|---|---|---|---|
| DMA concentration (%) | Freezing rate (cm above $LN_2$) | Thawing rate (°C × min or s) | Motility | Viability | Osmotic resistance |
| 8 | 1 | 4 | $10.71 \pm 1.57^{bc}$ | $22.28 \pm 1.51^{a}$ | $12.22 \pm 1.22^{a}$ |
| 8 | 1 | 50 | $15.07 \pm 2.46^{bc}$ | $24.65 \pm 1.70^{a}$ | $10.01 \pm 1.64^{a}$ |
| 8 | 5 | 4 | $10.21 \pm 1.55^{c}$ | $22.51 \pm 1.91^{a}$ | $12.31 \pm 1.90^{a}$ |
| 8 | 5 | 50 | $10.28 \pm 1.49^{c}$ | $17.08 \pm 1.49^{b}$ | $10.06 \pm 0.96^{a}$ |
| 8 | 10 | 4 | $13.14 \pm 1.57^{bc}$ | $22.58 \pm 1.00^{a}$ | $14.76 \pm 2.08^{a}$ |
| 8 | 10 | 50 | $16.93 \pm 1.59^{ab}$ | $20.97 \pm 0.82^{ab}$ | $13.24 \pm 1.35^{a}$ |
| 18 | 1 | 4 | $11.71 \pm 1.13^{bc}$ | $20.72 \pm 1.03^{ab}$ | $13.32 \pm 2.19^{a}$ |
| 18 | 1 | 50 | $13.57 \pm 2.20^{bc}$ | $24.09 \pm 1.59^{a}$ | $15.12 \pm 2.21^{a}$ |
| 18 | 5 | 4 | $10.50 \pm 1.14^{bc}$ | $22.22 \pm 1.60^{a}$ | $12.61 \pm 1.34^{a}$ |
| 18 | 5 | 50 | $12.92 \pm 3.14^{bc}$ | $22.26 \pm 1.68^{a}$ | $12.19 \pm 1.53^{a}$ |
| 18 | 10 | 4 | $14.78 \pm 2.70^{bc}$ | $25.48 \pm 2.91^{a}$ | $13.84 \pm 1.11^{a}$ |
| 18 | 10 | 50 | $21.28 \pm 2.05^{a}$ | $24.12 \pm 1.17^{a}$ | $15.40 \pm 1.71^{a}$ |
| Concentration effect | | | $P \leq 0.223$ | $P \leq 0.122$ | $P \leq 0.089$ |
| Freezing rate effect | | | $P \leq 0.001$ | $P \leq 0.111$ | $P \leq 0.099$ |
| Thawing rate effect | | | $P \leq 0.007$ | $P \leq 0.642$ | $P \leq 0.595$ |
| Concentration × freezing rate effect | | | $P \leq 0.512$ | $P \leq 0.165$ | $P \leq 0.544$ |
| Concentration × thawing rate effect | | | $P \leq 0.709$ | $P \leq 0.236$ | $P \leq 0.124$ |
| Freezing rate × thawing rate effect | | | $P \leq 0.385$ | $P \leq 0.045$ | $P \leq 0.826$ |
| Concentration × freezing rate × thawing rate effect | | | $P \leq 0.584$ | $P \leq 0.474$ | $P \leq 0.896$ |

[a-c]Different superscript letter within the same column indicates a significant difference ($P < 0.05$).

CPA: cryoprotectant; DMA: dimethylacetamide; $LN_2$: liquid nitrogen.

Table 11.2. Sperm quality variable (mean ± SE) recorded for semen frozen using DMSO as cryoprotectant according to CPA concentration, freezing rate and thawing rate (N = 7).

| Semen treatment | | | Sperm variable (%) | | |
|---|---|---|---|---|---|
| DMSO concentration (%) | Freezing rate (cm above $LN_2$) | Thawing rate (°C × min or s) | Motility | Viability | Osmotic resistance |
| 4 | 1 | 4 | $14.36 \pm 1.63^{e}$ | $25.16 \pm 2.70^{d}$ | $14.68 \pm 1.97^{b}$ |
| 4 | 1 | 50 | $17.93 \pm 1.50^{de}$ | $27.52 \pm 2.41^{cd}$ | $15.81 \pm 2.41^{b}$ |
| 4 | 5 | 4 | $20.14 \pm 1.34^{cde}$ | $25.85 \pm 2.55^{cd}$ | $16.34 \pm 1.38^{b}$ |
| 4 | 5 | 50 | $17.71 \pm 2.25^{de}$ | $30.90 \pm 2.99^{bcd}$ | $17.40 \pm 2.78^{b}$ |
| 4 | 10 | 4 | $22.00 \pm 1.98^{bcd}$ | $25.75 \pm 3.01^{cd}$ | $18.78 \pm 1.45^{ab}$ |
| 4 | 10 | 50 | $23.57 \pm 3.60^{bcd}$ | $31.09 \pm 4.95^{bcd}$ | $19.36 \pm 2.61^{ab}$ |
| 10 | 1 | 4 | $21.35 \pm 2.38^{bcde}$ | $30.52 \pm 2.04^{bcd}$ | $17.92 \pm 2.11^{ab}$ |
| 10 | 1 | 50 | $24.78 \pm 2.83^{bcd}$ | $33.31 \pm 2.57^{bcd}$ | $19.50 \pm 1.66^{ab}$ |
| 10 | 5 | 4 | $26.57 \pm 3.12^{bc}$ | $31.61 \pm 2.16^{bcd}$ | $20.31 \pm 2.29^{ab}$ |
| 10 | 5 | 50 | $28.50 \pm 3.20^{b}$ | $36.83 \pm 3.40^{ab}$ | $21.93 \pm 2.66^{ab}$ |
| 10 | 10 | 4 | $28.57 \pm 2.58^{b}$ | $34.91 \pm 2.03^{abc}$ | $20.21 \pm 2.20^{ab}$ |
| 10 | 10 | 50 | $36.57 \pm 1.78^{a}$ | $42.10 \pm 1.50^{a}$ | $25.15 \pm 2.66^{a}$ |
| Concentration effect | | | $P \leq 0.000$ | $P \leq 0.000$ | $P \leq 0.004$ |
| Freezing rate effect | | | $P \leq 0.000$ | $P \leq 0.102$ | $P \leq 0.053$ |
| Thawing rate effect | | | $P \leq 0.056$ | $P \leq 0.006$ | $P \leq 0.162$ |
| Concentration × freezing rate effect | | | $P \leq 0.699$ | $P \leq 0.452$ | $P \leq 0.965$ |
| Concentration × thawing rate effect | | | $P \leq 0.203$ | $P \leq 0.804$ | $P \leq 0.488$ |
| Freezing rate × thawing rate effect | | | $P \leq 0.308$ | $P \leq 0.640$ | $P \leq 0.875$ |
| Concentration × freezing rate × thawing rate effect | | | $P \leq 0.613$ | $P \leq 0.975$ | $P \leq 0.780$ |

[a-e]Different superscript letter within the same column indicates a significant difference ($P < 0.05$).

CPA: cryoprotectant; DMSO: dimethylsulfoxide; $LN_2$: liquid nitrogen.

## 11.7. DISCUSSION

This study sought to identify effective freezing protocols for the cryopreservation of turkey semen using straws and nitrogen vapour, and DMA or DMSO as the CPA. The treatment combinations that were most effective for the DMA protocol were: a CPA concentration of 18% DMA, sample freezing 10 cm above the liquid nitrogen (LN2) surface and a thawing temperature of 50°C. This combination (18% DMA/ 10 cm/50°C) returned recovery rates (value in frozen semen/value in fresh semen × 100) of about 30.5% for spermatozoa viability, 27.5% for motility and 26% for osmotic tolerance. For DMSO, the best treatment combination was 10% DMSO/10 cm/50°C which yielded recovery rates of 47% viability, 53% motility and 42% osmotic tolerance. As previously reported, many factors affect the success of semen cryopreservation including freezing medium, CPA and its concentration, along with freezing and thawing conditions (Iaffaldano, 2015), all of which affect the structure and function of spermatozoa (Garner *et al.*, 1999; Bailey *et al.*, 2003). In particular, the combination of these factors plays an important role (Tselutin *et al.*, 1995; Blanco *et al.*, 2011, 2012;
Iaffaldano *et al.*, 2011; Long *et al.*, 2014). The choice of CPA is among the most important factors for an effective turkey semen freezing protocol. In this study, DMA and DMSO were used as an alternative to glycerol because this compound has to be removed from the semen before insemination due to its contraceptive effect (Hammerstedt and Graham, 1992). Both DMSO and DMA are penetrating CPAs. Such CPAs are membrane-permeable solutes that act intra- and extracellularly, dehydrating spermatozoa by an osmotically driven flow of water, which varies according to CPA composition (Purdy, 2006). Penetrating CPAs also cause membrane lipid and protein reorganisation. This improves membrane fluidity causing greater dehydration at lower temperatures, and thus an increased ability to survive cryopreservation (Holt, 2000). Permeable CPAs may paradoxically have a toxic effect on sperm, causing membrane destabilisation and protein and enzyme denaturation. This toxicity is directly related to the CPA concentration used and the time of cell exposure (Swain and Smith, 2010; Iaffaldano *et al.*, 2014). In the present study, an effect of CPA concentration on post-thaw semen quality was observed although this was significant only for DMSO. Thus, concentrations of 18% DMA and 10% DMSO better protected the spermatozoa from cryodamage. It is assumed that these CPA concentrations were, on one hand, able to increase osmolarity to suitably dehydrate the cells avoiding ice crystal formation during cryopreservation and, on the other, produced no toxic effects. Blanco *et al.* (2011) using

cryovials as the packaging system also reported 18% DMA out of different concentrations tested (6%, 10%, 18%, 24%, 26%) as the most effective in protecting in vitro post-thaw semen quality. Compared with the present results, Long *et al.* (2014) recorded higher intact sperm-membrane and similar motility rates using 6% DMA, although work conditions differed. Little is known about the use of DMSO as a CPA for freezing turkey semen. Published results only exist for studies performed around the 1980s and the semen processing conditions and in vitro sperm quality were not always specified (Bakst and Sexton, 1979; Sexton, 1981). The best DMSO freezing protocol in the present study gave rise to a better quality of semen than the DMA freezing protocol previously identified as best. Although both DMA and DMSO are permeable CPAs and share many physical-chemical properties, their different molecular structures confer different permeabilities in a given phospholipid bilayer. This could account for variation in these CPAs in relative permeabilities through the turkey sperm membrane and thus explain their relative cryoprotection efficiencies as reported for the cryopreservation of rabbit semen (Iaffaldano *et al.*, 2012). Although it was observed that DMSO performs better than DMA (data not shown), there is still a need to further improve post-thaw semen quality by including non-permeable CPAs in semen freezing protocols and to test both DMSO and DMA *in vivo*.

Another step that emerged as critical for semen cryopreservation was freezing rate. The present results revealed an effect of freezing rate only on sperm motility for both CPAs tested. Loaded straws frozen 10 cm above the liquid nitrogen surface returned better post-thaw sperm motility results compared to other heights. It is hypothesised that the slower freezing rate (10 cm) led to reduction of ice crystal formation owing to better cellular dehydration and adequate cell shrinkage. This finding is consistent with previous reports that the cooling rate is crucial and that inaccurate cooling rates can negatively affect sperm survival, motility, plasma membrane integrity and mitochondrial function (Henry *et al.*, 1993). A sufficiently slow cooling rate means there is sufficient time for intracellular water efflux and balanced dehydration. However, if cooling is too slow, damage may occur due to exposure of cells to high concentrations of intracellular solutes. Extreme cellular dehydration leads to shrinkage of cells below the minimum cell volume necessary to maintain their cytoskeletons, genomic structures and ultimately cell viability (Mazur, 1984). Conversely, if cooling rates are too fast, external ice can induce intracellular ice formation and potential rupture of the plasma membrane, thus damaging intracellular organelles. In addition, mechanical damage to cells is possible due to extracellular ice compression and close proximity of frozen cells can lead to cellular deformation and membrane damage

(Fujikawa and Miura, 1986). Blanco *et al.* (2012) using cryovials and a programmable freezer observed better semen quality when turkey semen was frozen via a moderate (5°C/min from +4°C to −70°C) or slow (1°C/min from +4°C to −20°C) cooling rate compared to rapid cooling (plunging directly into liquid nitrogen). Conversely, the best results using 10 cm above liquid nitrogen (slower freezing rate) were poorer than those of Long *et al.* (2014) using a height above liquid nitrogen of 1.25 cm (faster freezing rate) though their experimental conditions differed from those of the present study.

A further factor that emerged as critical was warming temperature; a higher warming temperature (50°C) over a shorter period (10 sec) was better than longer exposure to a cooler temperature (4°C for 5 min). During thawing, sperm cells suffer additional damage due to recrystallization.Recrystallization refers to the growth of large ice crystals from small crystals. This process exerts additional tension on entrapped proteins and causes further cell damage (Cao *et al.*, 2003). Rapid thawing improves survival (Farrant, 1980) by avoiding recrystallization, while slow thawing is more damaging because of a longer total exposure time to sub-zero ice temperatures (Mazur, 2004).

Many factors may affect the success of turkey semen cryopreservation, while a given combination of factors is important, such as the CPA used and its concentration, the freezing and thawing rate and freezing vehicle (Tselutin *et al.*, 1995; Iaffaldano *et al.*, 2011). In the present study, a significant interaction effect on sperm viability was only observed for freezing rate × thawing rate when DMA was present as the CPA.

## 11.8. REFERENCES

BAILEY, J.L., MORRIE, A. & CORMIER, N. (2003) Semen cryopreservation: successes and persistent problems in farm species. Canadian Journal of Animal Science, 83: 393–401. doi:10.4141/A03-024.

BAKST, M.R. & SEXTON, T.J. (1979) Fertilizing capacity and ultrastructure of fowl and turkey spermatozoa before and after freezing. Journal of Reproduction and Fertility, 55: 1–7. doi:10.1530/jrf.0.0550001.

BLACKBURN, H.D. (2006) The National Animal Germplasm Program: challenges and opportunities for poultry genetic resources. Poultry Science, 85: 210–215. doi:10.1093/ps/85.2.210.

BLANCO, J.M., GEE, G., WILDT, D.E.&. & DONOGHUE, L. (2000) Species variation in osmotic cryoprotectant, and cooling

rate tolerance in poultry, eagle and peregrine falcon spermatozoa. Biology of Reproduction, 63: 1164–1171. doi:10.1095/biolreprod63.4.1164.

BLANCO, J.M., LONG, J.A., GEE, G., DONOGHUE, A.M. & WILDT, D.E. (2008) Osmotic tolerance of avian spermatozoa: influence of time, temperature, cryoprotectant and membrane ion pump function on sperm viability. Cryobiology, 56: 8–14. doi:10.1016/j.cryobiol.2007.09.004.

BLANCO, J.M., LONG, J.A., GEE, G., WILDT, D.E. & DONOGHUE, A. M. (2011) Comparative cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. Animal

Reproduction Science, 123: 242–248. doi:10.1016/j. anireprosci.2010.12.005.

BLANCO, J.M., LONG, J.A., GEE, G., WILDT, D.E. & DONOGHUE, A. M. (2012) Comparative cryopreservation of avian spermatozoa: effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. Animal Reproduction Science, 131: 1–8. doi:10.1016/j.anireprosci.2012.02.001.

BLANCO, J.M., WILDT, D.E., HOFLE, U., VOELKER, W. & DONOGHUE, A.M. (2009) Implementing artificial insemination as an effective tool for ex situ conservation of endangered avian species. Theriogenology, 71: 200–213. doi:10.1016/j.theriogenology.2008.09.019.

BLESBOIS, E. (2007) Current status in avian semen cryopreservation. World’s Poultry Science Journal, 63: 213–222. doi:10.1017/S0043933907001419.

BLESBOIS, E. (2011) Freezing avian semen, in: PIKE, T., SANDERCOCK, D. & WASCHER, C. (Eds) Avian Biology Research, Vol. 4, pp. 52–58 (UK, Science Reviews 2000).

CAO, E., CHEN, Y. & CUI, Z. (2003) Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnology and Bioengineering, 82: 684–690. doi:10.1002/bit.10612.

FARRANT, J. (1980) General observations on cell preservation, in: ASHWOOD-SMITH, M.J. & FARRANT, J. (Eds) Low Temperature Preservation in Biology and Medicine, pp.1–18 (Tunbridge Wells, UK, Pitman Medical).

FUJIKAWA, S. & MIURA, K. (1986) Plasma membrane ultrastructural changes caused by mechanical stress in the formation of extracellular ice as a primary cause of slow freezing injury in fruit-bodies of basidiomycetes (Lyophyllum ulmariuam). Cryobiology, 23: 371–382. doi:10.1016/0011-2240(86)90042-8.

GARNER, D.L., THOMAS, C.A. & GRAVANCE, C.G. (1999) The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. Reproduction in Domestic Animals, 34: 399–404. doi:10.1111/rda.1999.34.issue-5.

HAMMERSTEDT, R. & GRAHAM, J.K. (1992) Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology, 29: 26– 38. doi:10.1016/0011-2240(92)90004-L.

HENRY, M.A., NOILES, E.E., GAO, D., MAZUR, P. & CRITSER, J.K. (1993) Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. Fertility and Sterility, 60: 911–918.

HOLT, W.V. (2000) Basic aspects of frozen storage of semen. Animal Reproduction Science, 62: 3–22. doi:10.1016/S0378-4320(00)00152-4.

IAFFALDANO, N. (2015) Storage of turkey semen with special focus on cryopreservation. Proceedings of the 9th Turkey Science and Production Conference, Chester, UK, pp. 34–40.

IAFFALDANO, N., DI IORIO, M. & ROSATO, M.P. (2012) The cryoprotectant used, its concentration, and the equilibration time are critical for the successful cryopreservation of rabbit sperm: dimethylacetamide versus dimethylsulfoxide. Theriogenology, 78: 1381–1389. doi:10.1016/j. theriogenology.2012.06.009.

IAFFALDANO, N., DI IORIO, M., ROSATO, M.P. & MANCHISI, A. (2014) Cryopreservation of rabbit semen using non-permeable cryoprotectants: effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or

sucrose. Animal Reproduction Science, 151: 220–228. doi:10.1016/j.anireprosci.2014.10.020.

IAFFALDANO, N., ROMAGNOLI, L., MANCHISI, A. & ROSATO, M.P. (2011) Cryopreservation of turkey semen by the pellet method: effects of variables such as the extender, cryoprotectant concentration, cooling time and warming temperature on sperm quality determined through principal components analysis. Theriogenology, 76: 794–801. doi:10.1016/j.theriogenology.2011.04.012.

KOWALCZYK, A. & LUKASZEWICZ, E. (2015) Simple and effective methods of freezing capercaillie (Tetrao urogallus L.) semen. PLoS ONE, 10: 1–11. doi:10.1371/journal.pone.0116797.

KOWALCZYK,, A., ŁUKASZEWICZ, E. & RZOŃCA, Z. (2012) Successful preservation of capercaillie (Tetrao urogallus L.) semen in liquid and frozen states. Theriogenology, 77: 899–907. doi:10.1016/j.theriogenology.2011.09.015.

LONG, J.A. & KULKARNI, G. (2004) An effective method for improving the fertility of glycerol-exposed poultry semen. Poultry Science, 83: 1594–1601. doi:10.1093/ps/83.9.1594.

LONG, J.A., PURDY, P.H., ZUIDBERG, K., SIPKE-JOOST, H., VELLEMAN, S.G. & WOELDERS, H. (2014) Cryopreservation of turkey semen: effect of breeding line and freezing method on post-thaw sperm quality, fertilization, and hatching. Cryobiology, 68: 371–378. doi:10.1016/j.cryobiol.2014.04.003.

MAZUR, P. (1984) Freezing of living cells: mechanisms and implications. American Journal of Physiology, 247: C125–142.

MAZUR, P. (2004) Principles of cryobiology, in: BENSON, E., FULLER, B. & LANE, N. (Eds) Life in the Frozen State, pp.214– 315 (London, CRC Press).

PURDY, P.H. (2006) A review on goat sperm cryopreservation. Small Ruminant Research, 63: 215–225. doi:10.1016/j.smallrumres.2005.02.015.

ROSATO,, M.P., CENTODUCATI, G., SANTACROCE, M.P. & IAFFALDANO, N. (2012) Effects of lycopene on in vitro sperm quality and lipid peroxidation in refrigerated and cryopreserved turkey spermatozoa. British Poultry Science, 53: 545–552. doi:10.1080/00071668.2012.716508.

SAINT JALME, M., LECOQ, R., SEIGNEURIN, F., BLESBOIS, E. & PLOUZEAU, E. (2003) Cryopreservation of semen from endangered pheasants: the first step towards a cryobank for endangered avian species. Theriogenology, 59: 875–888. doi:10.1016/S0093-691X(02)01153-6.

SEXTON,, T.J. (1981) Development of a commercial method for freezing turkey semen: I. Effect of prefreeze techniques on the fertility of processed unfrozen and frozen-thawed semen. Poultry Science, 60: 1567–1573. doi:10.3382/ps.0601567.

SWAIN, J.E. & SMITH, G.D. (2010) Cryoprotectants, in: CHIAN, R. C. & QUINN, P. (Eds) Fertility Cryopreservation, Vol. 4, pp. 24– 38 (New York, Cambridge University Press).

TSELUTIN,, K., NARUBINA, L., MAVRODINA, T. & TUR, B. (1995) Cryopreservation of poultry semen. British Poultry Science, 36: 805–811. doi:10.1080/00071669508417825.

WOELDERS, H., ZUIDBERG, C.A. & HIEMSTRA, S.J. (2006) Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poultry Science, 85: 216–222. doi:10.1093/ps/85.2.216.

# Chapter 12

## STUDY 2

## Effect of cryoprotectants and thawing temperatures on chicken sperm quality

https://doi.org/10.1111/rda.13070

### 12.1 AIM

To date, semen cryopreservation is the only effective method of storing reproductive cells for the *ex situ* management of genetic diversity in birds ensuring the creation of semen cryobanks (Iaffaldano, Di Iorio, Miranda, *et al.*, 2016; Kowalczyk & Łukaszewicz, 2015).
In this regard, following the international agreements on animal biodiversity, the need to improve and standardize germplasm cryopreservation technology has been emphasized by the demand of *ex situ in vitro* conservation programs. In birds, semen cryopreservation is the only technology currently available to develop *ex situ* conservation programs; cryopreservation of oocytes cannot be used due to the characteristic of the megalecithal egg (Blesbois, 2007). Despite a number of reports are dealing with chicken semen cryopreservation (Mosca *et al.*, 2016; Mphaphathi, Luseba, Sutherland, & Nedambale, 2012; Mphaphathi, Seshoka, Luseba, Sutherland, & Nedambale, 2016; Zaniboni, Cassinelli, Mangiagalli, Gliozzi, & Cerolini, 2014), this procedure cannot still be successfully used due to cryopreservation-induced damages. Consequently, fertility rate with frozen-thawed semen is highly variable and not reliable enough for use in commercial productionor preservation of genetic stocks (Kowalczyk & Łukaszewicz, 2015; Long, 2006; Mphaphathi *et al.*, 2016). In this regard, literature surveys in chicken show that the average fertility after artificial insemination of frozen-thawed semen is ranging from 0% to 90% (Abouelezz *et al.*, 2015; Iaffaldano, Di Iorio, Cerolini, & Manchisi, 2016). Therefore, analysis of different processing conditions on the post-thaw sperm quality is still actual. Semen cryopreservation involves several steps affecting sperm structure and function such as extension, cooling, cryoprotectant addition, packaging system (pellets or straws), freezing and thawing (Bailey, Morrier, & Cormier, 2003).

Moreover, their interaction can also influence the effectiveness of sperm cryopreservation (Abouelezz *et al.*, 2015; Iaffaldano, Di Iorio, Miranda, *et al.*, 2017; Iaffaldano, Di Iorio, Cerolini, *et al.*, 2016; Tselutin, Seigneurin, & Blesbois, 1999). To date, dimethylsulphoxide (DMSO), dimethylacetamide (DMA), dimethylformamide (DMF), ethylene glycol (EG) and methylacetamide (MA; Hanzawa, Niinomi, Miyata, Tsutsui, & Tajima, 2010; Mosca *et al.*, 2016; Mphaphathi *et al.*, 2012, 2016; Sasaki *et al.*, 2010) were tested for rooster semen cryopreservation as alternatives to glycerol that acts as contraceptive (Hammerstedt & Graham, 1992; Long & Kulkarni, 2004). The use of DMSO and EG resulted in higher sperm motility rates than the propanediol-treated group (Mphaphathi *et al.*, 2016). In an *in vivo* study, Sasaki *et al.* (2010) recorded higher fertility rates (81.7%) using MA compared to DMA (40.7%; Santiago-Moreno *et al.*, 2011). Moreover, Hanzawa *et al.* (2010) reported higher fertility results of MA (60.8%) in contrast to DMF, DMA and DMSO (47.6%, 32.9%, 41.3% respectively). Moreover, the addition of non-permeating substances such as proteins (mainly albumin), amino acid (glycine) or carbohydrates (trehalose, sucrose etc.) has been reported to improve the cryosurvival of mammalian, fish and avian spermatozoa (Blanco, Long, Gee, Wildt, & Donoghue, 2012; He & Woods, 2003; Mosca *et al.*, 2016). As described by Blanco, Long, Gee, Wildt, and Donoghue (2011), the inclusion of sucrose and trehalose in the cryodiluent-containing DMA as permeating cryoprotectant improved post-thaw motility of turkey sperm. In a recent study by Mosca *et al.* (2016), it was shown that trehalose at concentration of 0.1 mol/L, but not sucrose at the same concentration, plays a positive protective action during the cryopreservation of chicken sperm. Glycine is known to be beneficial for the cryosurvival of sperm. In particular in rooster sperm, the inclusion of glycine into the diluent may improve sperm integrity by direct interaction with plasma membrane phospholipids (Cerolini, Zaniboni, Mangiagalli, & Gliozzi, 2007; Gliozzi, Zaniboni, & Cerolini, 2011). In addition, there is little information available in literature regarding use of Ficoll (polysaccharide polymer) as non-permeating cryoprotectant for avian species. Nevertheless, addition of Ficoll 70 in the freezing extender improved the rabbit sperm post-thaw quality (Kulíková *et al.*, 2015).

Apart from the type of cryoprotectant (CPA), also thawing procedure is crucial for avian post-thaw semen quality (Iaffaldano, Di Iorio, Cerolini, *et al.*, 2016). Lower semen quality values were observed using a thawing rate of 37°C for 30 s (Santiago-Moreno *et al.*, 2011; Choi *et al.*, 2013; Madeddu *et al.*, 2016) when compared to 5°C for 2 min (Hanzawa *et al.*, 2010; Mphaphathi *et al.*, 2016; Sasaki *et al.*, 2010).

Therefore, due to need for minimizing variability in results and standardization of freezing–thawing protocol, our study was aimed to (i) compare effect of DMA, DMF, MA and EG as permeating cryoprotectant(P-CPA) and two thawing temperatures (37 vs. 5°C) on sperm post-thaw motility; (ii) analyse combined effect of the best P-CPA with sucrose, trehalose, glycine or ficoll as non-permeating cryoprotectant (N-CPA) on post-thaw quality of rooster semen.

## 12.2. MATERIALS AND METHODS

### *12.2.1. Animals*

Twelve sexually mature males of Ross PM3 heavy line (12–18 months) kept at a private breeding facility (Liaharensky podnik Nitra Ltd., Močenok, Slovakia) were used in our study. All chickens were housed in individual cages, maintained under an artificial photoperiod (14 hr of light at 10 lux and 10 hr of dark) and were fed a commercial standard diet (TEKRO Nitra, s.r.o., Slovakia) with water given *ad libitum*. The experiments have been carried out in accordance with The Code of Ethics of the EU Directive 2010/63/EU for animal experiments.

### *12.2.2. Chemicals*

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Germany).

## 12.3. Experiment 1: Effect of different permeating cryoprotectants and thawing rates on frozen-thawed rooster sperm motility

Semen was collected from 12 roosters three times a week by dorso-abdominal massage and pooled to avoid intermale differences. Altogether, six replicates of pooled samples were used. An aliquot taken from each pool (3–4 ml) was immediately used in the experiments on fresh semen, as described below, whereas the rest of the pool was divided into five aliquots. Pooled semen was pre-extended (1:1; v/v) with Kobidil$^{+}$ extender (Landata Cobiporc, France). Although Kobidil$^{+}$ extender is widely used for diluting boar semen, we decided to

test this extender for freezing rooster semen according to positive results provided by Mphaphathi *et al.* (2016).

These authors used Kobidil extender for freezing Venda chicken sperm. Furthermore, in our preliminary work, we have found that Kobidil$^{+}$ showed better motility results when compared to Beltsville poultry semen extender II (BPSE; Continental Plastic Corp., USA; data not shown). Once pre-extended at room temperature, semen was cooled at 5°C for 30 min.

Thereafter, the pre-extended semen was diluted (1:1; v/v) with the freezing extender Kobidil$^{+}$ supplemented with 12% of DMA, 15% DMF, 16% EG or 18% MA to reach the final concentration of 6%, 7.5%, 8% or 9%, respectively. Permeating cryoprotectant concentrations used in our study were selected according to available literature (Hanzawa *et al.*, 2010; Mphaphathi *et al.*, 2016; Santiago-Moreno *et al.*, 2011; Sasaki *et al.*, 2010). After the second dilution, semen was loaded into 0.25-mL plastic straws and equilibrated at 5°C for 45 min.

Then, the straws were frozen by exposure to liquid nitrogen vapours (5 cm above) for 15 min and plunged into liquid nitrogen for storage. Sperm samples were thawed by immersing the straws in a water bath at 5 or 37°C for 2 min or 30 s, respectively.

### 12.4. Experiment 2: Effect of different non-permeating cryoprotectants on quality of frozen/ thawed rooster sperm

Semen was collected from 12 roosters three times a week by dorso-abdominal massage and pooled to avoid intermale differences. Each semen pool (six replicates) was diluted (1:1 v/v) with *Kobidil*$^{+}$ extender at room temperature and then cooled at 5°C for 30 min. Afterwards, semen was diluted to 1:1 (v/v) with the freezing extender composed of *Kobidil*$^{+}$ extender containing the best P-CPA identified in experiment 1 (16% of EG) in the combination with one of the following N-CPAs: 1.5 mol/L ficoll (KEG-F), 0.4 mol/L sucrose (KEG-S), 0.4 mol/L trehalose (KEG-T) or 0.1 mol/L glycine (KEG-G) to reach the final concentration of 0.75 mol/L ficoll, 0.2 mol/L sucrose, 0.2 mol/L trehalose and 0.05 mol/L glycine. The control without N-CPA was KEG containing only *Kobidil*$^{+}$ extender supplemented with 16% of EG. Non-permeating cryoprotectants concentrations used in our study were selected according to the available literature (Blanco *et al.*, 2011; Gliozzi *et al.*, 2011). Ficoll concentration was chosen according to post-thaw motility results in our previous experiment

(data not shown). After dilution and equilibration (45 min at 5°C), samples were frozen as stated above.

Three days later, frozen samples were thawed. According to results obtained in experiment 1, only thawing at 5°C was used as it showed higher post-thaw motility when compared to thawing at 37°C. Sperm motility (as above) and viability was determined in duplicate in both fresh and frozen-thawed semen samples.

## 12.5. MOTILITY ANALYSIS

For CASA analysis, fresh and frozen-thawed semen was diluted in a saline 1:100 (v/v) and 1:25 (v/v), respectively, to give a final concentration of $25 \times 10^6$/ml and loaded into Leja Standard Counting Analysis Chamber (depth of 20 μm; MiniTübe, Tiefenbach, Germany). Each sample was evaluated under a Zeiss Axio Scope A1 microscope using the CASA system (Sperm Vision™; MiniTübe, Tiefenbach, Germany) for total motility (motility > 5 μm/s), progressive movement (motility > 20 μm/s), VCL (curvilinear velocity; μm/s) and VSL (straight-line velocity; μm/s) considering seven microscopic fields and two replicates for each sample. For each sample chamber, 800 sperm tracks were evaluated. Image acquisition was obtained at 60 Hz (corresponding to 60 frames/s).

## 12.6. VIABILITY ANALYSIS

### *12.6.1. Fluorescence analysis*

Plasma membrane destabilization, apoptosis and necrosis of fresh and frozen-thawed sperm were evaluated using Annexin V-Fluos, YO-PRO-1 and propidium iodide (PI), respectively. Semen samples ($3 \times 10^6$ sperm) were washed in a binding buffer (Annexin V; supplied with a kit) or in phosphate-buffered saline (PBS; YO-PRO- 1/ PI staining) and centrifuged at 500 *g* for 6 min. The semen pellet was resuspended in: (i) 50 μl of working solution of Annexin V-Fluos (4 μl of Annexin V in a 200 μl of binding buffer), (ii) 50 μl of YO-PRO- 1 staining solution (1 μl of YO-PRO- 1 in 399 μl of PBS) or in (iii) 50 μl of PI staining solution (4 μl of PI in 200 μl of PBS). Following 15 min of incubation in a dark, sperm was washed in a binding buffer (Annexin V) or in PBS (YO-PRO- 1/ PI) and centrifuged (500 *g* × 6 min).

Subsequently, 4 μl of the stained sample was placed on a microslide into 4 μl of the Vectashield antifade medium containing DAPI fluorescent dye (total sperm number).
All the sperm samples were observed under a Leica fluorescence microscope or laser scanning microscope (LSM 700; ZEISS) equipped with an Axio Imager Z2 scanning unit, at magnification 400× using 488, 560 and 420 nm filters for Annexin V, YO-PRO- 1, PI and DAPI, respectively. Spermatozoa with disordered phosphatidylserine (PS) asymmetry (Annexin V) and apoptotic spermatozoa (YO-PRO- 1) exhibited green fluorescence, whilst necrotic spermatozoa (PI) exhibited red fluorescence.

### *12.6.2. Flow cytometry*

Sperm viability of fresh and frozen sperm was determined using fluorescein SYBR-14 and PI. Approximately $3 \times 10^6$ sperm were added to 40 μl of PBS containing 2 μl of SYBR-14 (diluted first in DMSO (1: 100; v/v) and then in PBS (1:1,000; v/v) and incubated at 37°C for 10 min in dark. Afterwards, 5 μl of PI were added and incubated at 37°C for 5 min. Each sample was then diluted with 200 μl of WBFB buffer (special buffer for flow cytometry: 1.5 g of BSA, 0.5 g of $NaN_3$, 50 ml of 10× PBS and 450 ml of distilled water) and analysed using BD FACSCalibur flow cytometry analyser. At least, 10,000 events were analysed for each sample. The emitted green fluorescence of SYBR-14 positive cells and red fluorescence of PI positive cells were recorded in the FL-1 and FL-3 channels, respectively. The different labelling patterns in bivariate SYBR/PI analysis identified three different sperm populations (Figure 12.1). Spermatozoa were classified as viable (SYBR+/PI-), dying (SYBR+/PI+) or dead (SYBR-/ PI+).

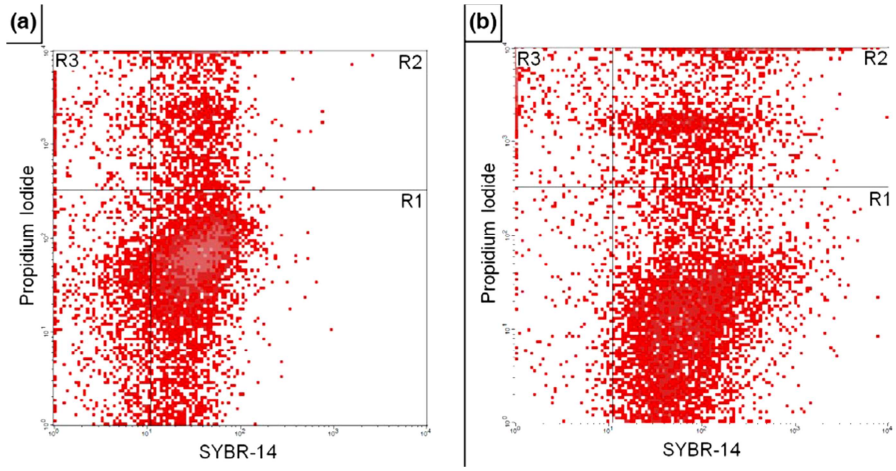



**Figure 12.1.** Representative flow cytometry dot plots for fresh semen (a) and KEG-F (KEG and 0.75 mol/L ficoll) (b). Regions R1, R2 and R3 represent viable, dying and dead spermatozoa, respectively.

## 12.7. MORPHOLOGICAL ANALYSIS

To detect the proportion of normal and abnormal sperm cells in fresh and frozen-thawed semen, slides which were prepared for fluorescence analysis (YO-PRO-1/PI staining) were used for evaluation of morphology observed by a contrast-phase microscope at 630× magnification. Spermatozoa with spindle-shaped head and visible tail were regarded as normal whilst spermatozoa with structural defects such as swollen head, bent neck, coiled tail, lacking tail and others were considered abnormal.

## 12.8. ULTRASTRUCTURE ANALYSIS

For a more comprehensive overview of changes caused by freezing, transmission electron microscopy was used to compare fresh and frozen-thawed sperm ultrastructure. Sperm was fixed in Karnovsky fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/L sodium cacodylate buffer, pH 7.1–7.3) during 1 hr at 4°C. Subsequently, the sperm was washed three times in cacodylate buffer for 15 min. Sperm pellets were embedded into 2% agar and post-fixed in 1% osmium tetroxide in cacodylate buffer during 1 hr. Samples were then dehydrated by passing them through an acetone series and embedded into Durcupan ACM (Fluka). Semithin sections (1 μm) were stained with methylene blue. Ultrathin sections (70 nm) were placed on nickel grids, contrasted with uranyl acetate and lead citrate and examined on a transmission electron microscope (JEM100CXII, Jeol, Japan) operating at 80 kV. For each group, electronograms were made at the magnification × 10,000.

## 12.9. STATISTICAL ANALYSIS

Sperm variables with a skewed distribution (as determined by the Shapiro–Wilk's test: $p < .05$) were arcsine-transformed before statistical analysis. In experiment 1, to compare the different treatments, we used a randomized block design in a four × two factorial arrangement (four P-CPAs × two thawing rates) with six replicates per treatment.

Sperm variables (total motility and progressive movement) were compared among the treatments by Analysis of variance (ANOVA) followed by Duncan comparison test.

A generalized linear model procedure (GLM) was used to determine the fixed effects of type of CPA, thawing rate and their interactions on the sperm quality variables. In experiment 2, four different N-CPAs were tested in combination with EG. The Duncan post hoc test was applied when significant differences within sperm quality parameters were observed by ANOVA. All statistical tests were performed using the SPSS software package (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA). Significance was set at $p \leq .05$.

## 12.10. RESULTS

### *12.10.1. Experiment 1: Effect of different P-CPAs and thawing rates on frozen/thawed rooster sperm motility*

The total motility and progressive movement in fresh semen were 60.7% and 45.3%, respectively. The fixed effect of the kind of CPA, thawing rate and their interaction on sperm quality are shown in Table 12.1. Significant effect of thawing rate was detected on total and progressive movement, VCL and VSL, whilst no significant effect of the CPA excluding VSL, was observed. A significant interaction between CPA and the thawing temperature was observed for all sperm parameters except VSL. Significantly higher values of total motility, progressive movement, VCL and VSL were obtained in semen frozen in the presence of 8% EG and thawed at 5°C for 2 min when compared to all other treatments except for DMF/5°C treatment.

**Table12.1.** Effect of four P-CPAs and two thawing rates on post-thaw motility of rooster semen.

| **Semen treatment** | | **Semen quality** | | | |
|---|---|---|---|---|---|
| **Cryoprotectant** | **Thawing rate (37°C × 30 s or 5°C × 2 min)** | **Total Motility** | **Progressive Movement** | **VCL** | **VSL** |
| DMA 6 % | 37 | $32.3 \pm 4.1^{b}$ | $16.9 \pm 2.9^{bc}$ | $73.6 \pm 1.8^{c}$ | $25.8 \pm 1.5^{c}$ |
| DMF 7.5 % | 37 | $28.6 \pm 3.1^{b}$ | $13.6 \pm 2.3^{c}$ | $72.1 \pm 2.4^{c}$ | $26.9 \pm 1.2^{c}$ |
| MA 9 % | 37 | $30.7 \pm 1.0^{b}$ | $17.2 \pm 1.0^{bc}$ | $67.8 \pm 3.6^{c}$ | $25.9 \pm 1.9^{c}$ |
| EG 8 % | 37 | $27.9 \pm 2.1^{b}$ | $12.1 \pm 0.9^{c}$ | $70.6 \pm 2.8^{c}$ | $33.3 \pm 0.7^{b}$ |
| DMA 6 % | 5 | $31.1 \pm 3.1^{b}$ | $14.9 \pm 1.9^{bc}$ | $81.6 \pm 2.6^{b}$ | $28.1 \pm 0.7^{c}$ |
| DMF 7.5 % | 5 | $39.8 \pm 1.6^{a}$ | $19.7 \pm 0.6^{b}$ | $81.6 \pm 2.1^{b}$ | $32.9 \pm 1.2^{b}$ |
| MA 9 % | 5 | $31.3 \pm 1.9^{b}$ | $14.6 \pm 1.2^{bc}$ | $84.8 \pm 2.7^{ab}$ | $28.5 \pm 1.5^{c}$ |
| EG 8 % | 5 | $46.6 \pm 1.8^{a}$ | $25.9 \pm 1.5^{a}$ | $91.9 \pm 1.6^{a}$ | $39.8 \pm 1.5^{a}$ |
| CPA effect | | P≤0.070 | P≤0.229 | P≤0.218 | P≤0.000 |
| Thawing rate effect | | P≤0.000 | P≤0.003 | P≤0.000 | P≤0.000 |
| CPA × Thawing rate effect | | P≤0.001 | P≤0.000 | P≤0.037 | P≤0.285 |

CPA, cryoprotectants; DMA, dimethylacetamide; DMF, dimethylformamide; MA, methylacetamide; EG, ethylene glycol.

Different superscripts within the column indicate significant difference ($p \leq .05$).

### *12.10.2. Experiment 2: Effect of different N-CPAs on the quality of frozen/thawed rooster sperm*

The results obtained showed that cryopreservation process impaired the motility of sperm when compared to fresh semen. However, the motility of frozen semen was affected by the N-CPAs used (Table 12.2). In fact, significantly higher value of progressive movement was found in KEG-F, whilst higher total motility was recorded in the KEG treatment and in KEG-F treatment. Fluorescence analysis showed a decrease ($p \leq .05$) in the percentage of apoptotic sperm (YO-PRO-1) in KEG-F and KEG-G when compared to other treatments. No significant difference was observed in the percentage of necrotic sperm among the treatments (Figure 12.2). According to fluorescence results, flow cytometric analysis showed no significant differences in numbers of viable, dying and dead sperm among the treatments (Table 12.3).

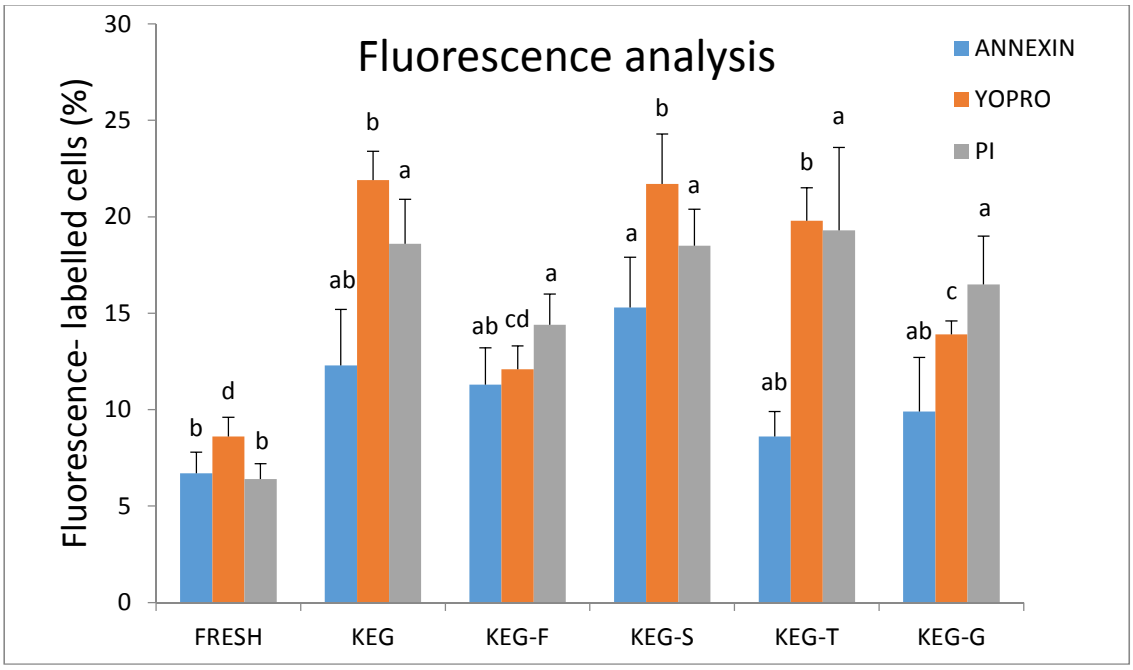
Morphological analysis showed significant decrease in the percentage of normal sperm with spindle-shaped head and visible tail together with significant increase in the percentage of abnormal cells showing structural defects (swollen head, bent neck, coiled tail, lacking tail and others) in frozen semen, when compared to its fresh counterparts (Figure 3). No difference was observed among treatments of cryopreserved semen. A further overview of the comparison between fresh and frozen semen was performed by ultrastructure analysis (Figure 12.4). Fresh sperm (Figure 12.4a) showed a slightly waved plasma membrane (PM) on the sperm head surface followed by intact acrosome membrane (A). Perforatorium (P) and nucleus (N) are well visible. In contrast, frozen sample represents markedly swollen plasma membrane (PM) mitochondria (M), proximal (PC) and distal (DC) centriole in fresh semen (Figure 12.4c), whilst evidenced the disruption of plasma (PM) and nuclear (NM) membrane accompanied by damaged mitochondria within midpiece of a frozen sperm (Figure 12.4d).

**Table 12.2.** Effect of various combinations of KEG and four N-CPAs on post-thaw motility of rooster sperm

| Sperm treatment | Sperm quality (mean ± SE) | | | |
|---|---|---|---|---|
| **P-CPA+N-CPA** | **Total Motility** | **Progressive Movement** | **VCL** | **VSL** |
| Fresh | 62.5 ± 3.9$^{a}$ | 50.1 ± 4.2$^{a}$ | 94.1 ± 2.4$^{a}$ | 38.6 ± 1.9$^{a}$ |
| KEG | 44.4 ± 2.1$^{b}$ | 23.3 ± 1.8$^{c}$ | 84.4 ± 3.4$^{a}$ | 25.5 ± 0.9$^{b}$ |
| KEG-F 0.75 mol/L | 48.8 ± 1.6$^{b}$ | 30.1 ± 1.3$^{b}$ | 91.6 ± 1.7$^{a}$ | 30.8 ± 0.8$^{b}$ |
| KEG-S 0.2 mol/L | 23.6 ± 2.7$^{d}$ | 9.8 ± 1.4$^{d}$ | 52.5 ± 3.1$^{c}$ | 27.4 ± 1.6$^{b}$ |
| KEG-T 0.2 mol/L | 30.7 ± 1.9$^{cd}$ | 16.8 ± 1.8$^{c}$ | 67.0 ± 3.6$^{b}$ | 29.5 ± 1.9$^{b}$ |
| KEG-G 0.05 mol/L | 31.2 ± 2.1$^{c}$ | 17.5 ± 1.6$^{c}$ | 60.1 ± 4.6$^{bc}$ | 26.6 ± 2.2$^{b}$ |

CPA, cryoprotectant; KEG, Kobidil + EG; KEG-F, Kobidil +EG +Ficoll; KEG-S, Kobidil + EG+ Sucrose; KEG-T, Kobidil +EG + Trehalose; KEG-G, Kobidil + EG+ Glycine. Different superscripts within the column indicate significant difference ($p \leq .05$).

**Figure 12.2.** Effect of various combinations of EG and four different N-CPAs on sperm viability.




Different superscripts indicate a significant difference among groups ($p \leq .05$)

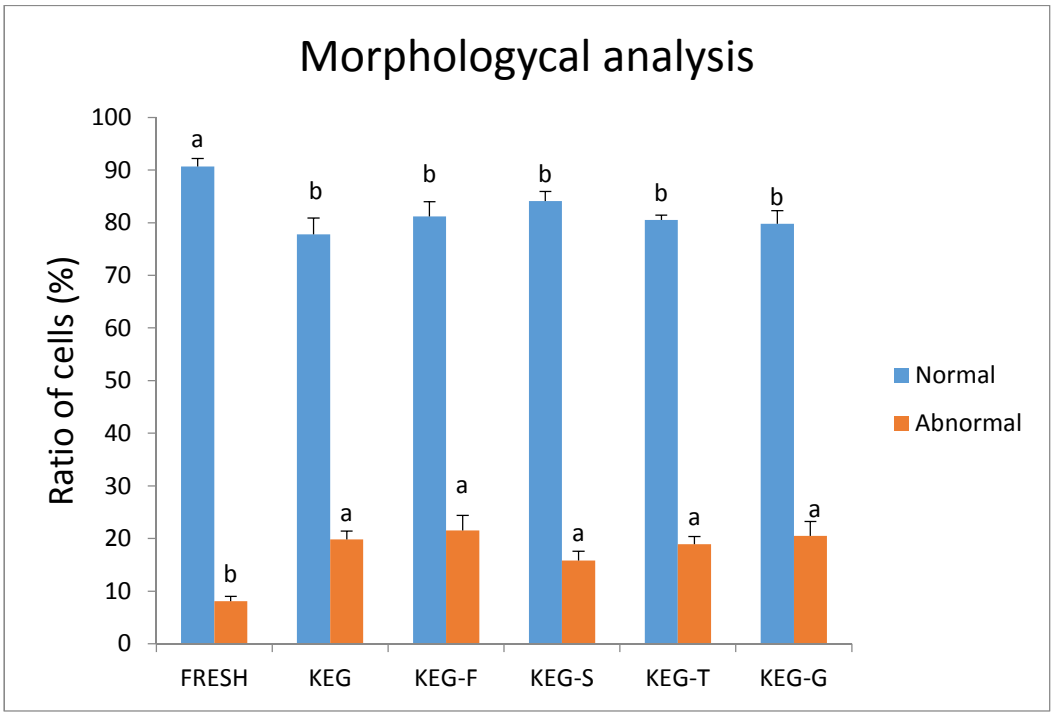
**Table 12.3.** Percentage of viable, dying and dead sperm of fresh and frozen semen, determined by flow cytometry

| | | Sperm quality (mean ± SE) | | |
|---|---|---|---|---|
| Sperm | Sperm treatment | SYBR+/PI- | SYBR+/PI+ | SYBR-/PI- |
| | P-CPA+ N-CPA | Live | Dying | Dead |
| Fresh | - | 67.9 ±1.9[a] | 13.9 ± 0.7[b] | 3.0 ± 0.7[b] |
| Frozen | KEG | 47.5 ± 1.5[b] | 34.1 ± 5.1[a] | 9.1 ± 2.3[a] |
| | KEG-F 0.75 M | 45.1 ± 1.5[b] | 33.6 ± 5.7[a] | 9.4 ± 2.1[a] |
| | KEG-S 0.2 M | 41.9 ± 3.7[b] | 38.8 ± 4.9[a] | 12.1 ± 2.7[a] |
| | KEG-T 0.2 M | 47.1 ± 3.8[b] | 37.5 ± 4.3[a] | 10.5 ± 2.0[a] |
| | KEG-G 0.05 M | 48.2 ± 2.7[b] | 33.3 ± 4.5[a] | 10.4 ± 1.7[a] |

CPA, cryoprotectant; KEG, Kobidil + EG.

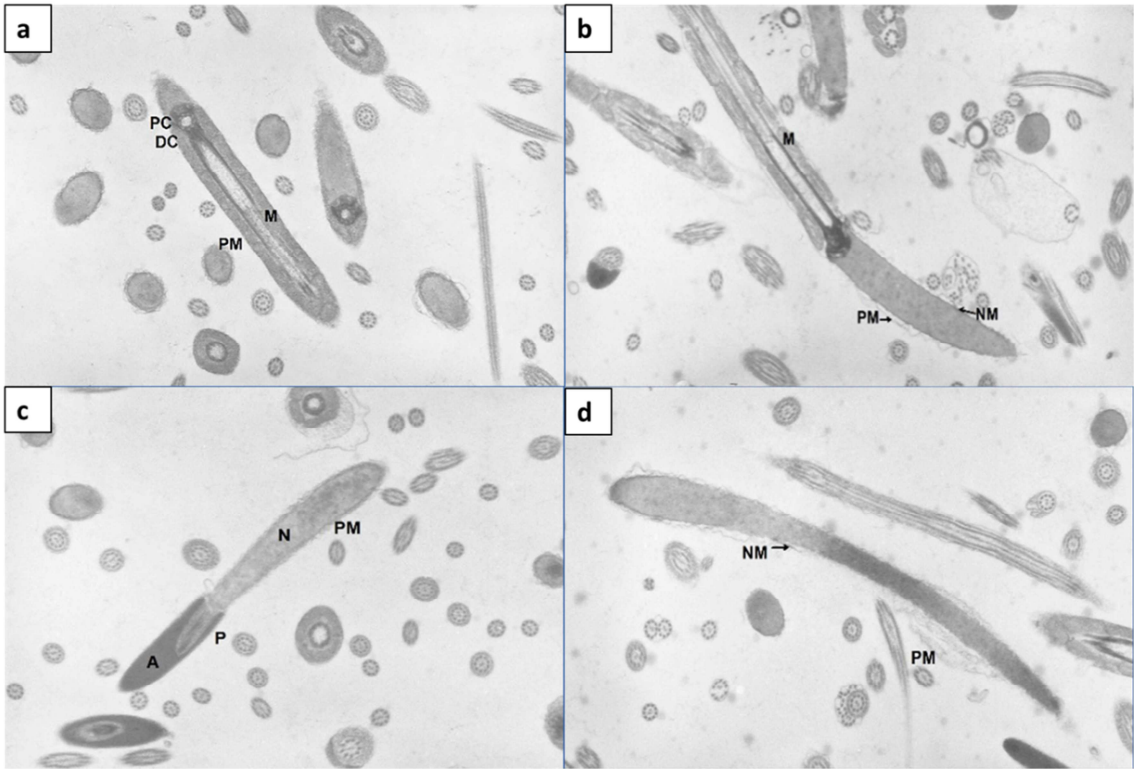
Different superscripts within the column indicate significant difference ($p \leq .05$).

**Figure 12.3.** Morphological analysis of fresh and frozen-thawed semen by contrast-phase microscope.




Different superscripts indicate a significant difference among groups ($p \leq .05$)

**Figure 12.4.** Longitudinal and diagonal section of fresh (a–c) and frozen sperm cell (b–d) obtained by ultrastructural analysis. PM, plasma membrane; M, mitochondria; PC and DC, proximal and distal centrioles; NM, nuclear membrane; P, perforatorium; A, acrosome; N, nucleus. Magnification × 10,000.




## 12.11. DISCUSSION

A variety of permeating cryoprotectants, such as DMSO, DMA, DMF, MA and EG (Hanzawa *et al.*, 2010; Mosca *et al.*, 2016; Mphaphathi *et al.*, 2012, 2016; Sasaki *et al.*, 2010), were tested for rooster sperm cryopreservation. Nevertheless, due to differences among conditions used, results reported in a literature are very variable. Here, the standardized *in vitro* comparison of four permeable cryoprotectants and two thawing temperatures showed that according to post-thaw motility, EG as P-CPA and thawing at 5°C demonstrated as the best option for rooster sperm. Moreover, significant interaction between the P-CPA and thawing temperature for all sperm parameters except VSL was found. Surprisingly, the type of P-CPA itself did not significantly affect the rooster semen quality except VSL.

As was reported in various papers, the success of sperm cryopreservation procedure may also depend on the interaction among the type of cryoprotectant, the semen freezing, thawing conditions and packaging system used, each one affecting sperm structure and function (Iaffaldano, Di Iorio, Cerolini, *et al.*, 2016; Long *et al.*, 2014). In this regard, an interesting point emerging from our study was that the thawing temperature at 5°C affected significantly the cryoprotective effect of EG and DMF, when compared to 37°C. However, these findings are in disagreement with those of Iaffaldano, Di Iorio, Cerolini, *et al.*, 2016; that thawing conditions of 50°C/10 s were more efficient than 4°C/5 min for turkey sperm. On the other hand, Mphaphathi *et al.*, 2016; showed that EG as well as DMSO are more suitable CPAs than propanediol for freezing of an indigenous Venda cock sperm using a thawing temperature of 5°C. According to our data, we hypothesize that EG because of the affinity of its chemical groups towards the sperm membrane, its low molecular weight (62.07 g/mol) and greater membrane permeability acts better when a lower thawing temperature is used. Moreover, lower temperature might have better preserved avian sperm membrane due to its high sensitivity to lipoperoxidation, compared to mammalian species (Fujihara & Howarth,1978). Addition of N-CPAs to freezing medium serves to offset the cryodamage caused by P-CPAs. At similar concentrations, these substances are less toxic than P-CPAs and they have multiple protective roles, such as inhibiting ice crystal growth and helping the sperm to stabilize internal solute concentrations under osmotic stress (Iaffaldano, Di Iorio, Cerolini, *et al.*, 2016; Mosca *et al.*, 2016). In chicken, carbohydrates (sucrose and/or trehalose) and glycine with DMA or DMSO (Mosca *et al.*, 2016) were already tested; however, no information on the use of polymers (such as ficoll) for avian species is available. In our study, addition of ficoll resulted in significantly higher post-thaw motility compared to trehalose, sucrose or glycine. Moreover, even though significantly higher percentage of dead sperm was detected in frozen semen when compared to its fresh counterparts, numbers of apoptotic sperm in KEG-F were similar to fresh sperm in contrast to other treatments. This finding is consistent with our previous report in rabbit sperm freezing (Kulíková *et al.*, 2015). Effectiveness of Ficoll was attributed mainly to its capability of affecting solution viscosity, ensuring a greater stability of the sperm membrane, reducing mechanical stress and ice crystals formation, therefore, increasing the ability to survive cryopreservation. In addition, ficoll was successfully used also in vitrification solution for embryos of different animal species (Lagares *et al.*, 2009; Makarevich *et al.*, 2008). Another interesting point emerging from our study is that the addition of trehalose, sucrose and glycine decreased post-thaw motility compared to EG

alone. This could be due to increased osmolarity of the extender resulting in deleterious effects to sperm. Beneficial effect of sugar supplementation varies among species and depends on their concentration (El-Sheshtawy, Sisy, & El-Nattat, 2015). In literature, 0.2 mol/L trehalose was successfully used for turkey semen cryopreservation(Blanco *et al.*, 2011); however, it was deleterious for Thai native chicken semen (Thananurak, Vongpralup, Sittikasamkit, & Sakwiwatkul, 2016). Nevertheless, lower trehalose concentration (0.1 mol/L) might play a positive protective action during chicken sperm cryopreservation (Mosca *et al.*, 2016). Even though other studies are needed to specify an optimal concentration of N-CPA in a freezing extender, the present study showed only 20%–30% decrease in motility and 20% decrease in viability of frozen-thawed semen when compared to its fresh counterpart. With regard to relatively low motility of fresh semen in this study (60.7%), which might be attributed to interbreed differences (Kuželová, Vašíček, & Chrenek, 2015), such decrease is not dramatic. To better understand changes caused by freezing–thawing, sperm morphology and ultrastructure were analysed. Our study revealed increased incidence of abnormal sperm in frozen thawed semen. Accordingly, sperm ultrastructure was markedly altered by cryopreservation. The plasmalemma was the primary site of damage. These changes were attributed to adverse osmotic conditions. It is already known that cryopreservation causes numerous negative effects including damage to cell membranes (plasma and mitochondrial) and, in some cases, to the nucleus with devastating consequences for sperm survival (Blesbois, 2007).

In conclusion, our findings indicate that the combination of EG and the thawing at 5°C improves sperm post-thaw motility. Moreover, addition of ficoll to EG-based freezing extender provided additional beneficial effect on progressive movement and apoptosis incidence.

These results contribute to the finding of an effective chicken semen cryopreservation procedure that might be implemented in national chicken breed *ex situ* conservation programs. Further work should seek optimal concentration of N-CPA to obtain an effective freezing protocol. In addition, fertility evaluation and testing on different chicken lines are needed in order to contribute to a bank of animal genetic resources.

## 12.12. REFERENCES


Abouelezz, F. M. K., Castaño, C., Toledano – Díaz, A., Esteso, 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. *Reproductionin Domestic Animals, 50*, 135–141.

Bailey, J. L., Morrier, A., & Cormier, N. (2003). Semen cryopreservation: Success and persistent problems in farm species. *Canadian Journal ofAnimal Science, 83*, 393–401.

Blanco, J. M., Long, J. A., Gee, G., Wildt, D. E., & Donoghue, A. M. (2011). Comparative cryopreservation of avian spermatozoa: Benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. *Animal Reproduction Science, 123*(3–4), 242–248.

Blanco, J. M., Long, J. A., Gee, G., Wildt, D. E., & Donoghue, A. M. (2012). Comparative cryopreservation of avian spermatozoa: Effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. *Animal Reproduction Science, 131*, 1–8.

Blesbois, E. (2007). Current status in avian semen cryopreservation. *World Poultry Science Journal, 63*, 213–222.

Cerolini, S., Zaniboni, L., Mangiagalli, M. G., & Gliozzi, T. M. (2007). Effect of glycine on cryopreservation of chicken spermatozoa. *Avian and PoultryBiology Reviews, 18*(2), 65.

Choi, J. S., Shin, D. B., Ko, Y. G., Do, Y. J., Byun, M., Park, S. B., … Kim, S. W. (2013). Effects of kinds of cryoprotectants on the characteristics of frozen fowl semen. *Korean Journal of Poultry Science, 3*, 171–178.

El-Sheshtawy, R. I., Sisy, G. A., & El-Nattat, W. S. (2015). Effects of different concentrations of sucrose or trehalose on the post thawing quality of cattle bull semen. *Asian Pacific Journal of Reproduction, 4*(1), 26–31.

Fujihara, N., & Howarth, B. (1978). Lipid peroxidation in fowl spermatozoa. *Poultry Science, 57*, 1766–1768.

Gliozzi, T. M., Zaniboni, L., & Cerolini, S. (2011). DNA fragmentation in chicken spermatozoa during cryopreservation. *Theriogenology, 75*, 1613–1622.

Hammerstedt, R. H., & Graham, J. K. (1992). Cryopreservation of poultry sperm: The enigma of glycerol. *Cryobiology, 29*, 26–38. Hanzawa, S., Niinomi, T., Miyata, T., Tsutsui, M., & Tajima, A. (2010). Cryopreservation of chicken semen using N-methylacetamide as cryoprotective agent. *Japanese Poultry Science, 47*, J27–J32.

He, S., & Woods, L. C. (2003). III The effects of osmolality, cryoprotectant and equilibration time on striped bass (*Morone saxatilis*) sperm motility. *Journal of the World Aquaculture Society*, *34*, 255–265.

Iaffaldano, N., Di Iorio, M., Cerolini, S., & Manchisi, A. (2016). Overview of turkey semen storage: Focus on cryopreservation. *Annals of AnimalScience*, *16*(4), 961–974.

Iaffaldano, N., Di Iorio, M., Miranda, M., Zaniboni, L., Manchisi, A., & Cerolini, S. (2016). Cryopreserving turkey semen in straws and nitrogen vapor using DMSO or DMA: Effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. *British Poultry Science*, *57*, 264–270.

Kowalczyk, A., & Łukaszewicz, E. (2015). Simple and effective methods of freezing capercaillie (*Tetrao urogallus* L.) Semen. *PLoS ONE*, *10*(1), 1–11 e0116797.

Kulíková, B., Di Iorio, M., Kubovicova, E., Kuželová, L., Iaffaldano, N., & Chrenek, P. (2015). The cryoprotective effect of Ficoll on the rabbit spermatozoa quality. *Zygote*, *23*(5), 785–794.

Kuželová, L., Vašíček, J., & Chrenek, P. (2015). Influence of macrophages on the rooster sperm quality. *Reproduction in Domestic Animals*, *50*, 580–586.

Lagares, M. A., Castanheira, P. N., Amaral, D. C. G., Vasconcelos, A. B., Veado, J. C. C., Arantes, R. M. E., & Stahlberg, R. (2009). Addition of Ficoll and disaccharides to vitrification solutions improve *in vitro* viability of vitrified equine embryos. *CryoLetters*, *30*(6), 408–413.

Long, J. A. (2006). Avian semen cryopreservation: What are the biological challenges? *Poultry Science*, *85*, 232–236.

Long, J. A., & Kulkarni, G. (2004). An effective method for improving the fertility of glycerol-exposed poultry semen. *Poultry Science*, *83*, 1594–1601.

Long, J. A., Purdy, P. H., Zuidberg, K., Hiemstra, S. J., Velleman, S. G., & Woelders, H. (2014). Cryopreservation of turkey semen: Effect of breeding line and freezing method on post-thaw sperm quality, fertilization, and hatching. *Cryobiology*, *68*, 371–378.

Madeddu, M., Mosca, F., Sayed, A. A., Zaniboni, L., Mangiagalli, M. G., Colombo, E., & Cerolini, S. (2016). Effect of cooling rate on the survival of cryopreserved rooster sperm: Comparison of different distances in the vapor above the surface of the liquid nitrogen. *Animal ReproductionScience*, *171*, 58–64.

Makarevich, A.V., Chrenek, P., Olexiková, L., Popelkova, M., Turanova, Z., Ostro, A., & Pivko, J. (2008). Post-thaw survival, cell death and actin cytoskeleton in gene-microinjected rabbit embryos after vitrification. *Theriogenology*, *70*, 675–681.

Mosca, F., Madeddu, M., Sayed, A. A., Zaniboni, L., Iaffaldano, N., & Cerolini, S. (2016). Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm. *Cryobiology*, *73*, 343–347.

Mphaphathi, M. L., Luseba, D., Sutherland, B., & Nedambale, T. L. (2012). Comparison of slow freezing and vitrification methods for Venda cockerel's spermatozoa. *Open Journal of Animal Science*, *2*(3), 204–210.

Mphaphathi, M. L., Seshoka, M. M., Luseba, D., Sutherland, B., & Nedambale, T. L. (2016). The characterisation and cryopreservation of Venda chicken semen. *Asian Pacific Journal of Reproduction*, *5*(2), 132–139.

Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Coloma, M. A., López- Sebastián, A., Prieto, M. T., & Campo, J. L. (2011). Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: Optimization of freezing rate and equilibration time. *Poultry Science*, *90*, 2047–2053. Sasaki, K., Tatsumi, T., Niinomi, T., Imai, T., Naito, M., Tajima, A., & Nishi, Y. (2010). A method for cryopreserving semen from Yakido roosters using N-Methylacetamide as a cryoprotective agent. *The Journal of Poultry Science*, *47*, 297–301.

Thananurak, P., Vongpralup, T., Sittikasamkit, C., & Sakwiwatkul, K. (2016). Optimization of trehalose concentration in semen freezing extender in Thai native chicken semen. *Thai Journal of Veterinary Medicine*, *46*(2), 287–294.

Tselutin, K., Seigneurin, F., & Blesbois, E. (1999). Comparison of cryoprotectants and method of cryopreservation of fowl spermatozoa. *PoultryScience*, *78*, 586–590.

Zaniboni, L., Cassinelli, C., Mangiagalli, M. G., Gliozzi, T. M., & Cerolini, S. (2014). Pellet cryopreservation for chicken semen: Effects of sperm working concentration, cryoprotectant concentration, and equilibration time during *in vitro* processing. *Theriogenology*, *82*, 251–258.

# Chapter 13

## STUDY 3

## The initial cooling time before freezing affects the post-thaw quality and reproductive performance of rabbit semen

### 13.1. AIM

The aim of this study was to investigate the effect of different cooling times, 45 and 90 min, at 5°C on the post-thaw quality and reproductive performance of rabbit semen. The low survival of rabbit sperm after cryopreservation is a major drawback for the widespread use of frozen semen in artificial insemination (AI) programs and also for the safeguard of genetic resources through the creation of sperm cryobank. Therefore, researchers have turned their attention to develop protocols for the improvement of cryopreservation in rabbit semen (Mocé & Vicente, 2009; Iaffaldano *et al.*, 2012, 2014; Rosato & Iaffaldano, 2013). Many factors affecting the success of cryopreservation have been considered in different studies: the composition of the freezing medium, the cryoprotectant (CPA) and its concentration, the freezing conditions, the cooling and warming temperatures, and the individual animal variation also (Mocé & Vicente, 2009). So far, most of the studies have focused on the identification of CPAs and their optimal concentrations (Mocé & Vicente, 2009; Rosato & Iaffaldano, 2013; Viudes-de-Castro *et al.*, 2014; Kulikova *et al.*, 2015). In particular, we developed an effective freezing protocol for rabbit semen based on the use of the permeant CPA DMSO in combination with the non permeant CPA sucrose (Iaffaldano *et al.*, 2012; 2014). This protocol includes a first cooling time of 90 min at 5°C before the addition of the CPAs, followed by an equilibration time, needed for the CPA to permeate the sperm cells, of 45 min at 5°C.The 90 min cooling time represents the majority of the time required to process semen samples for freezing, thus the time reduction of this step could considerably reduce the whole time required for semen processing and allow more ejaculates to be stored in a given time. In this regard, also Mocé et al (2014) performed a study on the equilibration time in order to shorten the semen cryopreservation process for rabbit semen.

Up to now, to the best of our knowledge no papers have reported results about the effect of the initial cooling time on the quality and fertility of cryopreserved rabbit semen.

## 13.2. MATERIALS AND METHODS

### *13.2.1.Chemical agents*

All chemicals used were of the highest commercially available purity. Unless stated otherwise, all the chemicals were purchased from Sigma, Chemical Co. (Milan, Italy).

### *13.2.2. Animals*

Forty adult bucks (8 months old) and one hundred and fifty does (8-9 months old) of the Bianca Italiana breed were used. Animals were kept at the ANCI-AIA Breeding Center (Volturara Appula, FG, Italy), housed in individual flat-deck cages, provided with a 16 h light/8 h dark photoperiod and fed a commercial standard breeder diet. Water was given ad libitum. The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments.

### *13.2.3. Semen collection*

Semen samples were collected using a pre-heated artificial vagina. Ejaculates were pooled (4 ejaculates/pool) to avoid the effects of individual differences among males. Ejaculates with undesired color (not plain white) and containing urine and/or cell debris were discarded, whereas gel plugs were removed. In total, 6 pooled semen samples were used.

## 13.3. Experiment 1. Effect of different cooling time on post-thaw semen quality

### *13.3.1. Semen processing*

Semen samples were processed and frozen by the straw freezing method described by Iaffaldano *et al.* (2012). Semen samples were transferred from the farm to the laboratory after 40 min in a water bath at 30°C in order to maintain semen at a temperature that was closer to that of male reproductive system. Once in the laboratory, an aliquot of semen was immediately taken from each pool for the evaluation of fresh semen quality, as described below.

Each pool was divided into two subsamples and cooled at 5°C for 45 or 90 min before freezing to minimize cold-shock damage. The temperature of semen was measured at the end of each cooling time by a temperature sensor (Ascon M1). After the cooling period, semen samples were diluted to a ratio 1:1 (v:v) with a freezing extender composed of Tris-citrate-glucose (TCG; 250 mmol/L Tris-hydroxymethylaminomethane, 88 mmol/L citric acid, and 47 mmol/L glucose) containing 16% DMSO, as permeable CPA, and 0.1 M sucrose, as non-permeable CPA. The diluted semen was aspirated into 0.25 mL plastic straws, equilibrated at 5°C for 45 min (equilibration time) and frozen by exposure to liquid nitrogen vapour 5 cm above the liquid nitrogen surface (temperature was approximately -125/-130°C) for 10 min. Finally, the straws were plunged into liquid nitrogen for storage at -196°C. Semen samples were thawed by immersion of the straws in a water bath at 50°C for 10 seconds.

### *13.3.2. Analysis of semen parameters*

Semen quality was assessed in both fresh and thawed samples and the following sperm quality parameters were measured: total motility (TM) and forward progressive motility (FPM), viability, osmotic resistance, acrosome and DNA integrity.

Rabbit sperm motility was subjectively evaluated by visual estimation. Briefly, a10 µL-drop of semen was transferred on a clean glass slide. The mounted slides were then observed on a warm-plate at ×400 magnification using a phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany). Percentage of TM (spermatozoa showing any type of sperm head movement) and FPM (spermatozoa showing linear movement) were measured in five microscopy fields (Di Iorio *et al.* 2014; Iaffaldano *et al.* 2014).

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit containing two fluorescent stains SYBR-14 and propidium iodide (PI). A drop of 5 µL of semen was added to 39 µL of TCG containing 1 µL SYBR-14 (diluted 1:100 in DMSO; final concentration 0.01 mM). The extended semen was then incubated at 37°C for 10 min, next 5 µL PI (diluted 1:100 in the TCG; final concentration 0.024 mM) were added and incubated for further 5 min at 37°C. Subsequently, 10 µL of this suspension were placed on microscope slides, covered with coverslips and examined at a magnification ×1000 using a ×100 oil immersion objective under epifluorescence illumination. For each sample, approximately 200 spermatozoa were examined in duplicate aliquots. SYBR-14, is a membrane permeant DNA stain that binds DNA of all the sperm and labels live sperm with green fluorescence, only stains live spermatozoa producing green fluorescence of the nuclei (live spermatozoa). PI stains the nuclei of membrane-damaged cells red (dead spermatozoa). The percentage of viable spermatozoa was determined as the number of green cells × 100 divided by the total number of sperm counted.

To assess sperm osmotic resistance, a hypoosmotic swelling test (HOS) was used. An aliquot of 10 µL semen was mixed with 80 µL of distilled water, incubating for 5 min at 37°C, then 10 µL of this mixture were deposited on a clean glass slide and observed under a phase-contrast microscope (magnification ×800). The typical sperm osmotic “coiled tail” reaction was detected and the number of HOS test-positive cells was recorded by counting spermatozoa showing this feature among 200 cells in at least five fields.

To determine acrosome integrity, duplicate smears were prepared using a drop of semen from each sample and air-dried. After fixation in methanol for 30 min, the slides were washed with water and air-dried and incubated with the Pisum sativum agglutinin FITC conjugate (FITC-PSA) for 30 min at room temperature. The slides were then mounted with 50% glycerol (vol/vol) and cover slipped. In each sample, assessment was made of 200 sperm at a magnification ×1000 using an oil immersion objective under epifluorescence illumination. This stain intensely labels the acrosomal region of acrosome-intact sperm, which emit a uniform apple-green fluorescence, while acrosome-damaged spermatozoa show scarce or no green fluorescence in the anterior part of the head. The percentage of acrosome-intact spermatozoa was calculated as a fraction of the total.

Sperm DNA integrity was evaluated using acridine orange (AO) following the method described by Gandini *et al.* (2006).

AO is a cationic fluorescent cytochemical stain that stains cell nuclei, specifically, DNA. Acridine orange fluoresces green when incorporated into native DNA (double-stranded and normal) as a monomer, and orange-red when it binds to denatured (single-stranded DNA) as an aggregate. From each sample, 10 µL of semen were smeared onto a microscope slide, air-dried and fixed overnight in a 3:1 methanol:glacial acetic acid solution and air-dried once more. Slides were washed with distilled water. Smears were then stained with an AO solution (0.2 mg/mL in water) in the dark at room temperature for 5 min. Next, each smear was washed with distilled water and mounted with a coverslip followed by examination using a fluorescence microscope with a 490 nm excitation light and 530 nm barrier filter. Nuclei in at least 200 spermatozoa per slide were examined and scored as green or yellow-orange-red fluorescing (intact DNA or damaged DNA, respectively) and the percentage of normal and abnormal chromatin condensation calculated.

### 13.4. Experiment 2. In vivo reproductive performance of rabbit does inseminated with semen cryopreserved using the different cooling times

An AI trial was performed to assess in vivo the efficacy of semen frozen after 45 and 90 min cooling times. One hundred and fifty multiparous (31 days postpartum) receptive rabbit does were randomly assigned to three treatment groups: (1) 50 does inseminated with fresh semen (control group); (2) 50 does inseminated with semen frozen after 45 min cooling time; (3) 50 does inseminated with semen frozen after 90 min cooling time.

All does were subjected to the following biostimulation protocol to synchronize the oestrus: flushing by increasing feed amount/doe from 180 g/day to *ad libitum* (3 days before insemination), changing cage (3 days before insemination), and increasing the photoperiod from 16 to 24 hours of light (2 days before insemination). Does in the control group were inseminated with a 0.5 mL dose of fresh semen diluted 1:10 (approximately 35 million sperm) in a commercial extender. Does in group 2 and 3 were inseminated with a 0.25 mL dose of thawed semen within 10 min after thawing. At the time of insemination, each female received an intramuscular injection of buserelin acetate to induce ovulation (1 µg/doe).

Fertility rate (number of pregnant does/number of inseminations), kindling rate (number of does giving births/number of inseminations), prolificacy (total born/kindling) and number of kids born alive (total live-born/kindling) were the reproductive performances considered.

Fertility rate (number of pregnant does/number of inseminations) was determined by abdominal palpation performed in each doe 17 days after AI. At parturition, the factors: kindling rate (number of does giving birth/number of inseminations), prolificacy (total born/fertility), and the number of kids born alive (total live-born/fertility) the above factors were determined.

## 13.5. STATISTICAL ANALYSIS

The effect of the cooling time on sperm quality parameters (motility, viability, osmotic resistance, acrosome and DNA integrity) was tested by independent-samples t-test. Reproductive performance (fertility rate, kindling, prolificacy and number of kits born alive) was compared among treatments by ANOVA, followed by Duncan's comparison test. All statistical tests were performed using the software package SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA). Significance was set at $P<0.05$.

## 13.6. RESULTS

### *13.6.1. Effect of two cooling times on post-thaw semen quality*

Over 80% of the fresh sperm population was viable (83.87 ± 1.85), motile (85.41 ± 1.36), and 73.75% (SEM=1.90) showed FPM. Acrosome and DNA integrity were observed in the 87.25% (SEM=0.85) and 99.01% (SEM=0.12) of sperm respectively and osmotic resistance in the 74.84% (SEM=1.12).

The temperatures of semen measured at the end of each cooling time by a temperature sensor (Ascon M1), after cooling time of 45 or 90 min resulted as 9°C after 45 min and 5°C after 90 min, respectively. Sperm quality parameters recorded in frozen-thawed semen samples are reported in Table 13.1. According to the results of the independent t-test, the initial cooling equilibration time at 5°C significantly affected sperm TM, FPM and viability, whereas no significant effect was found on acrosome and DNA integrity and sperm membrane osmotic resistance. As expected, cryopreservation caused sperm damages and a consequent great reduction in sperm quality. Sperm cooling for 90 min at 5°C before cryoprotectant addition improved their ability to survive cryopreservation when compared to

45 min incubation. Significantly higher proportion of viability, TM and FPM was found in sperm cooled for 90 min (Table 13.1).

In contrast, the longer cooling time did not affect the percentage of sperm with intact acrosome, DNA and plasma membrane (osmotic resistance).

Table 13.1. Probability of t-test and means (± SEM) of sperm quality parameters recorded in thawed rabbit semen samples (n=6) frozen after 45 and 90 min cooling time at 5 °C (n = 6)

| Sperm parameters (%) | 45 min | 90 min | *P* value |
|---|---|---|---|
| Viability | $36.77 \pm 1.79^{b}$ | $42.49 \pm 1.01^{a}$ | 0.024 |
| Total motility | $30.50 \pm 1.02^{b}$ | $37.34 \pm 1.13^{a}$ | 0.001 |
| Forward progressive motility | $22.42 \pm 0.72^{b}$ | $27.83 \pm 1.08^{a}$ | 0.003 |
| Osmotic resistance | $31.68 \pm 0.67^{a}$ | $34.80 \pm 1.29^{a}$ | 0.067 |
| Acrosome integrity | $35.64 \pm 1.14^{a}$ | $37.31 \pm 0.91^{a}$ | 0.280 |
| DNA integrity | $97.44 \pm 0.19^{a}$ | $98.83 \pm 0.12^{a}$ | 0.117 |

### *13.6.2. Artificial insemination*

The reproductive performances recorded after AI of does with fresh semen and semen frozen after the two different cooling treatments is reported in Table 13.2. Similar fertility and kindling rate were recorded after AI with fresh or frozen-thawed semen cooled for 90 min at 5°C before freezing. In contrast, the AI with frozen-thawed semen cooled for 45 min before freezing, caused a significant decrease in both fertility and kindling (Table 13.2). As a consequence, a similar number of young rabbits , born, were obtained from the does inseminated with treatment 1 (fresh semen) and 3 (90 min) (296 and 291, respectively), and in contrast only 220 young rabbits born from the does inseminated with treatment 2 (45 min) semen. No significant differences were found between treatments for prolificacy and the number of kits born alive (Table 13.2).

**Table 13.2.** Reproductive performance outcomes recorded in rabbit does after artificial insemination with fresh semen and frozen-thawed semen cooled for 45 and 90 min at 5°C before freezing.

| Reproductive performance | Semen treatment | | |
|---|---|---|---|
| | Fresh semen | 45 min | 90 min |
| % Fertility rate (n) | $74^{a}$ (37) | $64^{b}$ (32) | $76^{a}$ (38) |
| % Kindling rate (n) | $68^{a}$ (34) | $54^{b}$ (27) | $70^{a}$ (35) |
| Prolificacy (mean ± SEM) | $8.7 \pm 0.6^{a}$ | $8.2 \pm 0.6^{a}$ | $8.3 \pm 0.6^{a}$ |
| Live born (mean ± SEM) | $8 \pm 0.7^{a}$ | $7.8 \pm 0.6^{a}$ | $7.8 \pm 0.6^{a}$ |

Values with different superscript letters on the same row are significantly different ($P<0.05$).

## 13.7. DISCUSSION

The goal of our study was to find out if reduced cooling time from 90 to 45 min affects rabbit sperm quality or not. The present results clearly show that semen cooled at 5°C for 90 min before freezing showed better post-thaw quality and fertility compared to semen cooled for 45 min. Thus, we hypothesize that 90 min of cooling time prepares much better the sperm to the addition of the CPAs (DMSO and sucrose). Although the cooling step before the addition of the CPAs is not used (when an extender with high DMSO level is used), in our previous experiment (data not published), we observed that DMSO added at room temperature (20°C) decreased the post-thaw quality of rabbit semen. Therefore we cooled spermatozoa before CPA addition. Moreover, we found in literature that different works about rabbit sperm freezing without cooling step, achieved lower results than ours (Mocé *et al.* 2010, 2014; Viudes-de-Castro *et al.* 2014).

Fuller (2004) reported that, when the CPA is added to the sperm held at room temperature, it permeates quickly because of a higher permeability of the sperm membrane with the consequence of a greater toxic effect of the CPA. Instead, the sperm membrane at 5°C shows a lower permeability and the CPA permeates gradually inside the sperm, thus reducing its toxic effect.

It is generally accepted that the toxicity of permeant CPAs depends on their concentration, and temperature and time exposure (Holt 2000; Iaffaldano *et al.* 2012).

Therefore, in accordance with Fuller (2004), we can suppose that longer cooling time at 5°C for 90 min allows the sperm plasma membrane to cool down 5°C and to reduce its permeability, therefore DMSO permeates gradually through the sperm decreasing its toxic effect. In contrast, the shorter cooling time at 5°C for 45 min allows the sperm plasma membrane to cool down 9°C and then to maintain higher permeability, therefore DMSO could play a greater toxic effect.

The reproductive performances obtained by AI of semen processed for cryopreservation after a longer cooling phase confirms our previous data collected in similar experimental protocols (Iaffaldano *et al.* 2012, 2014; Rosato & Iaffaldano 2013). The cryopreservation protocol studied for rabbit semen and including 90 min initial equilibration time at 5°C is very efficient and able to preserve the full fertilizing ability of fresh sperm. In fact, very similar fertility and kindling rates were recorded with both fresh and 90 min cooled/cryopreserved semen, whereas a great significant decrease in kindling rate was recorded for the 45 cooled/cryopreserved semen. Moreover, the prolificacy and the number

of kits born alive were similar in all treatments irrespective of semen storage and processing. This result suggests that sperm resilient to the freezing/thawing process preserve their functional integrity and are able to give the same number of viable embryos as fresh semen.

Moreover, our in vitro (motility and viability) and *in vivo* results were higher than those obtained by authors (Mocé *et al.* 2010, 2014; Viudes-de-Castro *et al.* 2014) who diluted the semen with freezing extender (containing CPA) at room temperature i.e. without a cooling phase, while results similar to ours were obtained by Mocé *et al.* (2003) and Maeda *et al.* (2012).

In conclusion, our findings aimed to obtain an effective semen freezing protocol whilst they also tried to reduce the cryopreservation semen processing. The results clearly show that the cryosurvival of rabbit sperm is affected by the initial cooling time of 5°C. In fact, fresh semen and semen frozen/thawed after 90 min cooling time performed similar reproductive performances, whereas semen frozen/thawed after 45 min cooling showed a 20% reduction in fertility. Therefore, reducing cooling time to 45 min had a negative effect on rabbit sperm quality in vitro and in vivo. Thus the best semen freezing protocol (90 min cooling time) will allow the introduction of a sperm cryobank for the conservation of Italian rabbit genetic resources. However, further optimization work should seek to address the sperm concentration/inseminating dose as well as confirming the efficacy of the semen cryopreservation protocol in intensive rabbit rearing systems. By the optimization of this protocol the intensive rabbit farm may take advantages of the opportunities offered by extensive use of frozen semen doses.

## 13.8. REFERENCES


Di Iorio, M., Manchisi A., Rocco, M., Chrenek, P., & Iaffaldano, N. (2014).Comparison of different extenders on the preservability of rabbit semen stored at 5°C for 72 hours. *Italian Journal of Animal Science*, **13**, 710–714.

Fuller, B., Paynter, S.J., & Watson. P. (2004). Cryopreservation of human gametes and embryos, in: Fuller, B.J., Lane, N., Benson, E.E. (Eds), Life in the Frozen State. CRC Press Inc., New York, pp. 505–539.

Gandini, L., Lombardo, F., Lenzi, A., Spano, M., Dondero, F., (2006). Cryopreservation and sperm DNA integrity. *Cell Tissue Bank*, **7** (2), 91–98.

Holt, W.V.(2000). Basic aspects of frozen storage of semen. *Animal Reproduction Science*, **62**, 3–22.

Iaffaldano, N., Di Iorio, M. & Rosato, M.P.(2012). The cryoprotectant used, its concentration, and the equilibration time are critical for the successful cryopreservation of rabbit sperm: Dimethylacetamide versus dimethylsulfoxide. *Theriogenology*, **78**, 1381–1389.

Iaffaldano, N., Di Iorio, M., Rosato, M.P. & Manchisi, A. (2014). Cryopreservation of rabbit semen using non-permeable cryoprotectants: Effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or sucrose. *Animal Reproduction Science*, **151**, 220–228.

Kuliková, B., Di Iorio, M., Kubovicova, E., Kuželová, L., Iaffaldano, N. & Chrenek, P. (2015). The cryoprotective effect of Ficoll on the rabbit spermatozoa quality. *Zygote*, **23**, 785–794.

Maeda, T., Liu, E., Nishijima, K., Tanaka M., Yamaguchi S., Morimoto, M., Watanabe, T., Fan, J., Kitajima, S. (2012). Effect of the primary cooling rate on the motility and fertility of frozen-thawed rabbit spermatozoa. *World Rabbit Science*, **20**, 65–70.

Mocé E, Lavara R, Vicente JS. (2003). Effect of an asynchrony between ovulation and insemination on the results obtained after insemination with fresh or frozen sperm in rabbits. *Animal Reproduction Science*, **75**, 107–118.

Mocé, E. & Vicente, J.S.(2009). Rabbit sperm cryopreservation: A review. *Animal Reproduction Science*, **110**, 1–24.

Mocé, E., Lavara, R., Vicente, JS. (2010). Effect of cooling rate to 5 °C, straw size and farm on fertilizing ability of cryopreserved rabbit sperm. *Reproduction in Domestic Animals*, **45**, 1–7.

Mocé, E., Blanch, E., Talaván, A. & Viudes de Castro, M.P.(2014). Reducing the time rabbit sperm are held at 5 °C negatively affects their fertilizing ability after cryopreservation. *Theriogenology*, **82**, 1049–1053.

Rosato, M.P. & Iaffaldano, N.(2013). Cryopreservation of rabbit semen: Comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. *Theriogenology*, **79**, 508–516.

Viudes-de-Castro, M.P., Lavara R., Safaa, H.M., Marco-Jiménez, F., Mehaisen, G.M.K. & Vicente, J.S. (2014). Effect of freezing extender composition and male line on semen traits and reproductive performance in rabbits. *Animal*, **8**, 765–770.

# Chapter 14

## CONCLUSION

The research activities performed during the doctoral period have provided an important contribution in the optimization of the reference procedures of poultry (turkey and chicken) and rabbit semen cryopreservation. This will allow the take off of the first national semen cryobank for local poultry (turkey and chicken) and rabbit breeds.

The *ex situ in vitro* conservation strategy by semen cryobank,will provide an important tool for managing poultry and rabbit genetic resources supporting breeders associations involved in the management of small populations of these breeds enclosed into the register of autochtonous Italian breeds. Moreover, the construction of a national semen cryobank for local poultry and rabbit breeds, will provide also a backup of populations stored *in situ* in case of genetic problems (inbreeding, genetic drift) and health epidemics.

As it has been previously said, the national semen cryobank is a part of a more extended project realized by the some Italian universities including the University of Molise, aiming to preserve and valorize Italian poultry and rabbit breeds and also in maintaining of their genetic variability. The safeguard of the poultry and rabbit breeds represents also an important tool for preserving historical breeds of high genetic value and supporting the rural economy in some marginal agricultural areas and could also have a relevant role in developing new high quality products for niche markets.

## REFERENCES


Abe, Y., Hara, K., Matsumoto, M., Kobayashi, J., Sasada, H., Ekwall, H., Rodriguez-Martinez, M., Sato, E. 2005. Feasibility of nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. *Biol Reprod*, 72: 1416–1420.

Ahmad, M., Nasrullah, R., Ahmad, N. 2015. Effect of cooling rate and equilibration time on pre-freeze and post-thaw survival of buck sperm. *Cryobiology*, 70(3): 233–238.

Akhter, S., Ansari, M.S., Rakha, B.A., Andrabi, S.M.H., Khalid, M., Ullah, N. 2011. Effect of low density lipoproteins in extender on freezability and fertility of buffalo (Bubalus bubalis) bull semen. *Theriogenology,* 76: 759–764.

Alkan, S., Baran, A., Zdap B., Evecen, M. 2002. Morphological Defects in Turkey Semen. *Turk J Vet Anim Sci* 26, 1087–1092.

Allendorf, F.W., Hohenlohe, P.A., Luikart, G. 2010. Genomics and the future of conservation genetics. *Nat Rev Genet*, 11: 697–709.

Aller, J.F., Rebuffi, G.E., Cancino, A.K. 2002. Successful transfer of vitrified llama (Lama glama) embryos. *Anim Reprod Sci*, 73: 121–127.

Alvariño J.R.M. 2000. Reproductive performance of male rabbits. In Proceedings of 7th World Rabbit Congress. Valencia, Spain. ACAF, pp.13-35.

Amirat-Briand, L., Bencharif, D., Vera-Munoz, O., Pineau, S., Thorin, C., Destrumelle, S., Desherces, S., Anton, M., Jouan, M., Shmit, E., Tainturier, D., 2010. In vivo fertility of bull semen following cryopres-ervation with an LDL (low density lipoprotein) extender: preliminary results of artificial inseminations. *Anim Reprod Sci*, 122: 282–287.

Anton, M., Martinet, V., Dalgalarrondo, M., Beaumal, V., David-Briand, E., Rabesona, H., 2003. Chemical and structural characterization oflow-density lipoproteins purified from hen egg yolk. *Food Chem*, 83: 175–183.

Arav, A., Yavin, S., Zeron, Y., Natan, D., Dekel, I., Gacitua, H. 2002. New trends in gamete's cryopreservation. *Mol Cell Endocrinol*, 187: 77–81.

Armstrong, D.T. 1993. Recent advances in superovulation of cattle. Theriogenology, 39: 7–24.

Arriola, J., Foote, R.H. 2001. Accessory sperm as an indication of fertilizing ability of rabbit spermatozoa frozen in egg yolk acetamide with detergent. *J Androl*, 22: 458–463.

Athurupana, R., Takahashi, D., Ioki, S., Funahashi, S. 2015. Trehalose in glycerol-free freezing extender enhances post-thaw survival of boar spermatozoa. *J Reprod Develop*, 61(3): 205–210.

Bailey, J.L., Buhr, M.M. 1994. Cryopreservation alters the $Ca^{2+}$ flux of bovine spermatozoa. *Can J Anim Sci*, 74: 53-58.

Bailey, J.L., Morrier, A., Cormier, N. 2003. Semen cryopreservation: success and persistent problems in farm species. *Can J Anim Sci*, 83: 393−401.

Bakst, M.R., Sexton, T.J. 1979. Fertilizing capacity and ultrastructure of fowl and turkey spermatozoa before and after freezing. *J Reprod Fert*, 55: 1−7.

Bakst, M.R., Dymond, J.S. 2013. Artificial Insemination in Poultry. Chapter 10. In: Success in Artificial Insemination - Quality of Semen and Diagnostics Employed. edited by Alemayehu Lemma. InTech. T. pp. 175-195.

Baldassarre, H., Karatzas, C.N. 2004. Advanced assisted reproduction technologies (ART) in goats. *Anim Reprod Sci*, 82-83: 255–266.

Ballard, C.B., Looney, C.R., Lindsey, B.R., Pryor, J.H., Lynn, J.W., Bondioli, K.R., Godke, R.A. 2007. Using buoyant density gradient and nile red staining to evaluate lipid content of Bos taurus and Bos indicus oocytes. *Reprod Fert Develop*, 19: 170–171.

Bamba, K., Adams, C.E. 1990. Freezing rabbit semen by the use of BF5 diluent. *Lab Anim*, 24: 172–174.

Bank, H. 1973. Visualization of freezing damage. II. Structural alterations during warming. *Cryobiology*, 10: 157–170.

Bansal, A.K., Bilaspuri, G.S. 2011. Impacts of Oxidative Stress and Antioxidants on Semen Functions. *Vet Med International*, article, ID 686137, 7 pages.

Baruselli, P.S., Sa Filho, M., Martins, C.M., Nasser, L.F., Nogueira, M.F.G., Barros, C.M., Bo, G.A. 2006. Superovulation and embryo transfer in Bos indicus cattle. *Theriogenology*, 65: 77–88.

Baruselli, P.S., Sales, J.N.S., Crepald, G.A., Ferreira, R.M., Jacomini, J.O., Henryli de Sonza, A., Bo, G. 2008. Fixed-time insemination and embryo transfer in Bos indicus cattle. Proceedings of the World Brahman Congress, Fort Worth, USA.

Bednarczyk, M. 2014. Avian primordial germ cells and their application. The 2nd International Scientific Conference- Animal Biotechnology. *Slovak J Anim Sci*, 47, (4): 185–187.

Bellagamba F., Cerolini S., Cavalchini L.G. 1993. Cryopreservation for poultry semen: a review. *World's Poultry Sci J*, 49: 158–166.

Bellow, M.S., Didion, B.A., Davis, D.L., Murphy, C., Ferguson, C.E., Godke, R.A. & Day, B.N. 2001. A procedure for nonsurgical transvaginal ultrasound-guided oocyte aspiration in the sow. *Theriogenology*, 55: 528 (abstr.).

Bencharif, D., Amirat, L., Anton, M., Schmitt, E., Desherces, S., Delhomme, G., Langlois, M.L., Barriere, P., Larrat, M., Tainturier, D. 2008. The advantages of LDL (Low Density Lipoproteins) in the cryopreservation of canine semen. *Theriogenology*, 70: 1478–1488.

Benková, J. 2002: Biodiverzita génovej rezervy sliepok [Biodiversity of the chicken genetic reserve]. Research Institute of Animal Production. Nitra, Slovakia (in Slovak).

Berg, D.K., Berg, M.C., Hull, D.A., Bertram, M.F. 2000. Development of transvaginal oocyte pick-up for farmed red deer (Cervus elaphus). *Theriogenology*, 53: 326 (abstr.).

Bergeron, A., Crete, M.H., Brindle, Y., Manjunath, P. 2004. Low-density lipoprotein fraction from hen's egg yolk decreases the binding of themajor proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. *Biol Reprod* 70: 708–717.

Bergonzoni, M.L., Zambelli, D., Samoggia, G. 1994. Influenza del diluitore, della temperatura e del periodo di conservazione del seme nella F.A. cunicola. *Coniglicoltura Ricerca*, 4: 37–40.

Berri, C., Wacrenier, N., Millet, N., Le Bihan-Duval, E. 2001. Effects of selection for improved body composition on muscle and meat characteristics of broilers from experimental and commercial lines. *Poult Sci,* 80: 833–838.

Best, B.P. 2015. Cryoprotectant Toxicity: Facts, Issues, and Questions. *Rejuvenation Res*, 18: 422–436.

Betteridge, K.J. 1977. Embryo transfer in farm animals: A review of technique and applications. Agriculture Canada Monograph N°.16. Ottawa, Department of Agriculture.

Bezerra, F.S.B., Castelo, T.S., Alves, H.M., Oliveira, I.R.S., Lima, G.L., Peixoto, G.C.X., Bezerra, A.C.S.D., Silva, A.R. 2011. Objective assessment of the cryoprotective effects of dimethylformamide for freezing goat semen. *Cryobiology*, 63: 263–266.

Bittante G. 2011. L'attività del National Focal Point Italiano nella conservazione della biodiversità degli animali domestici. In: La salvaguardia della biodiversità animale. Iniziative generali ed azioni intraprese in Italia a tutela delle razze minacciate. Edizione a cura della Fondazione Iniziative Zooprofilattiche e Zootecniche, Brescia, vol.84, pp. 33-45.

Blackburn, H. 2004. Development of national animal genetic resource programs. *Reprod Fert Develop,* 16: 27–32.

Blackburn, H. D. 2006. The National Animal Germplasm Program: Challenges and Opportunities for Poultry Genetic Resources 1. *Poult Sci*, 85: 210–215.

Blackburn, H.D. 2009. Genebank development for the conservation of livestock genetic resources in the United States of America. *Livest Prod Sci*, 120: 196–203.

Blackburn, H.D., Silversides, F., Purdy, P.H. 2009. Inseminating fresh or cryopreserved semen for maximum efficiency: Implications for gene banks and industry. *Poult Sci,* 88: 2192–2198.

Blanco, J.M., Wildt, D.E., Hofle, U., Voelker, W., Donoghue, A.M. 2009. Implementing artificial insemination as an effective tool for ex situ conservation of endangered avian species. *Theriogenology,* 71: 200–213.

Blanco, J.M., Long, J.A., Gee, G., Wildt, D.E., Donoghue, A.M. 2011. Comparative cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. *Anim Reprod Sci*, 123: 242−248.

Blesbois, E. 2007. Current status in avian semen cryopreservation. *World Poultry Sci J*, 63: 213−222.

Blesbois, E., Seigneurin, F., Grasseau, I., Limouzin, C., Besnard, J., Gourichon, D., Coquerelle, G., Rault, P., Tixier-Boichard, M. 2007a. Semen cryopreservation for ex situ management of genetic diversity in chicken: creation of the French avian cryobank. *Poult Sci*, 87: 555−564.

Blesbois, E., Seigneurin, F., Grasseau, I., Limouzin, C., Besnard, J., Gourichon, D., Coquerelle, G., Rault, P., Tixier-Boichard, M. 2007b. Semen Cryopreservation for Ex Situ Management of Genetic Diversity in Chicken: Creation of the French Avian Cryobank. *Poult Sci,* 86: 555–564.

Blesbois, E., Grasseau, I., Seigneurin, F., Mignon – Grasteau, S., Saint Jalme, M., Mialon – Richard, M.M. 2008. Predictors of semen cryopreservation in chickens. *Theriogenology*, 69: 252–261.

Blesbois, E. 2011. Freezing avian semen. *Avian Biol Res*, 4: 52.

Boettcher, P.J., Stella, A., Pizzi, F., Gandini, G. 2005. The combined use of embryos and semen for cryogenic conservation of mammalian livestock genetic resources. *Genet Sel Evol*, 37: 657–675.

Bordas, A., Minvielle, F. 1999. Patterns of growth and feed intake in divergent lines of laying domestic fowl selected for residual feed consumption. *Poult Sci*, 78:317–323.

Bowden, R.M., Ewert, M.A., Lipar, J.L., Nelson, C.E. 2001. Concentrations of steroid hormones in layers and biopsies of chelonian egg yolks. *Gen Comp Endocrinol*, 121: 95–103.

Brinster, R.L., Zimmermann, J.W. 1994. Spermatogenesis following male germ-cell transplantation. *PNAS*, 91: 11298–11302.

Brun, J.M., Theau-Clément, M., Bolet, G., 2002. The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. *Anim Reprod Sci*, 70: 139–149.

Buhr, M.M., Canvin, A.T., Bailey, J.L. 1989. Effects of semen preservation on boar spermatozoa membranes. *Gamete Res*, 23: 441-449.

Burrows, W.H., Quinn, J.P. 1937. Artificial insemination of chicken and turkey. US Dept Agric. 525. *Poult Sci*, 14: 251–254.

Campo, J.L., Orozco, F. 1986. The genetic basis of the Melanotic Prat phenotype. *Br Poult Sci*. 27: 361–367.

Campo, J.L. 1998. Conservation and genetic study of Spanish chicken breeds (28). Pages 155–158 in Proc. 6th World Congr. Genet. Appl. Livestock Prod., Armidale, Australia. Univ. New England, Armindale, New South Wales, Australia.

Canvin, A.T., Buhr, M.M. 1989. Effect of temperature on the fluidity of boar spermatozoa membranes. *J Reprod Fertil*, 85: 533–540.

Carluccio, A., Robbe, D., De Amicis, I., Contri, A., Russo, F., Paoletti, M. 2004. Artificial insemination in rabbits: laboratory and field trial with three different semen extenders. *World Rabbit Sci*, 12: 65–79.

Carneiro, M., Afonso, S., Geraldes, A., Garreau, H., Bolet, G., Boucher, S., Tircazes, A., Queney, G., Nachman, M.W., Ferrand, N. 2011.The Genetic Structure of Domestic Rabbits. Mol. Biol. Evol. 28(6):1801–1816.

Carretero, M.I., Fumuso, F.G., Neilda, D.M., Giuliano, S.M., Ceticac, P., Miragay, M.H. 2015a. Evaluation of the acrosomal status in Lama glama sperm incubated with acrosome reaction inducers. *Anim Reprod Sci*, 160: 1–11.

Carretero, M. I., Neild, D. M., Ferrante, A., Caldevilla, M., Arraztoa, C.C., Fumuso, F. G., Giuliano, S. M. 2015b. Effect of cryoprotectant and equilibration temperature on cryopreservation of Lama glama spermatozoa. *Andrologia*, 47(6): 603–723.

Castellini, C., Battaglini, M., Lattaioli, P. 1992. Effects of cryoprotectants and freezing on rabbit semen quality. *J Appl Rabbit Res*, 15: 431–438.

Castellini, C. 1996. Recent advances in rabbit artificial insemination. In: Recent Advances in rabbit science. Lebas, F. Ed., INRA, Proc. 6th World Rabbit Congress. 2, 13–28.

Castellini, C., Lattaioli, P. 1999. Effect of number of motile sperms inseminated on reproductive performance of rabbit does. *Anim Reprod Sci*, 57: 111–120.

Castellini, C., Lattaioli, P., Minelli A., Moroni M. 2000. Effect of seminal plasma on the characteristics and fertility of rabbit spermatozoa. *Anim Reprod Sci*, 63: 275–282.

Castellini, C., Dal Bosco, A., Mugnai, C., Pedrazzoli, M. 2006. Comparison of two chicken genotypes organically reared: oxidative stability and other qualitative traits. *It J Anim Sci*, 5: 29–42.

Ceccobelli, S., Di Lorenzo, P., Lancioni, H., Castellini, C., Monteagudo Ibáñez, L.V., Sabbioni, A., Sarti, F.M. Weigend, S., Lasagna, E. 2013. Phylogeny, Genetic Relationships and Population Structure of Five Italian Local Chicken Breeds. *It J Anim Sci*, 12: 3, e66.

Cerolini, S., Surai, P., Maldjian, A., Gliozzi, T., Noble, R. 1997. Lipid composition of semen in different fowl breeders. *Poult Avian Biol Rev*, 8: 141–148.

Cerolini, S., Marzoni Fecia di Cossato, M., Romboli, I., Schiavone, A., Zaniboni, L. 2008. Avicoltura e Coniglicoltura. Cap: Apparato riproduttore pp. 412–439. Le Point Vétérinaire Italie. Milano Italy.

Cerolini, S. 2015. Attività di ricerca relative alla conservazione della biodiversità avicola in Italia. Atti del 51° Convegno Nazionale dell' Associazione Scientifica di Avicoltura – WPSA – Forlì.

Chen, Y., Foote, R.H. 1994. Survival of rabbit spermatozoa frozen and thawed at different rates with and without seeding. *Anim Reprod Sci*, 35: 131–143.

Choi, J.S., Shin, D.B., Ko, Y.G., Do, Y.J., Byun, M., Park, S.B., Kim, S.W. 2013. Effects of kinds of cryoprotectants on the characteristics of frozen fowl semen. *Korean J Poult Sci*, 3: 171–178.

Chrenek P, Kubovičova, E., Makarevich, A. 2017. Current Situation in Gene Bank of Animal Genetic Resources in Slovakia. The 5th International Scientific Conference "Animal Biotechnology". *Slovak J Anim Sci*, 50(4): 135–138.

Ciereszko, A., Dietrich, G.J., Nynca, J., Dobosz, S., Zalewski, T., 2014. Cryopreservation of rainbow trout semen using a glucose–methanol extender. *Aquaculture*, 420–421: 275–281.

Coles, B.H. 2007. BVSc, Dip. ECAMS, Hon. FRCVS. Third edition. Essentials of Avian Medicine and Surgery. Blackwell science.

Colli L., Negrini R., Ajmone Marsan P., Globaldiv Consortium. 2011. Marcatori molecolari, genoma e risorse genetiche animali. In: La salvaguardia della biodiversità animale. Iniziative generali ed azioni intraprese in Italia a tutela delle razze minacciate. Edizione a cura della Fondazione Iniziative Zooprofilattiche e Zootecniche, Brescia, vol.84, pp. 83-99.

Comizzoli P., Wildt D.E., Pukazhenthi B.S. 2003 Overcoming poor in vitro nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season. *Reproduction,* 126: 809–816.

Cooper, D.M. 1977. Artificial insemination. In R. F. Gordon (ed.), Poultry Disease. Bailliere Tindall, London. Pages 302–307.

Cortell C., Viudes de Castro M.P. 2008. Effect of gelatin addition to freezing extender on rabbit semen parameters and reproductive performance. In: Xicatto G, Trocino A, Lukefahr SD editors. Proceedings of the 9th World Rabbit Congress, Verona, Italy. Fondazione Iniziative Zooprofilattiche e Zootecniche-Brescia; pp. 327–332.

Costantini, F. 1989. Fecondazione Artificiale nel coniglio, sistemi di conservazione dello sperma. *Riv di coniglicoltura,* 4: 14–18.

Courtens, J.L. 1995. La congélation de la semence de lapin. *Cuniculture* 123: 101–102.

Critser, J.K., Agca, Y., Gunasena, K.T. 1997. The cryobiology of mammalian oocytes. In A. Karow & J.K. Critser, eds. Reproductive tissue banking: scientific principles, pp. 329–357, San Diego, CA, USA, Academic Press.

Cunha, A.T.M., Carvalho, J.O., Dode, M.A.N. 2015. Techniques for sperm evaluation using fluorescent probes. Semina: Ciências Agrárias, Londrina, v. 36, n. 6, suplemento 2, p. 4365–4376.

Curry, M.R., Redding, B.J., Watson, P.F. 1995. Determination of water permeability coefficient and its activation energy for rabbit spermatozoa. *Cryobiology,* 32: 175–181.

Cywa-Benko, K. 2002 Charakterystyka genetyczna i fenotypowa rodzimych rodów kur objętych programem ochrony bioróżnorodności [Genetic and phenotypic characteristic of native chicken breeds encompassed in biodiversity conservation program] *Roczniki Naukowe Zootechniki*, 15: 5–114 (in Polish, with English summary).

Dalimata, A.M., Graham, J.K. 1997. Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. *Theriogenology*, 48: 831–841.

Dalla Zotte, A. Rabbit farming for meat purposes.In Animal Frontiers pp 62-67. October 2014, Vol. 4, No. 4.

Dalvit, C., De Marchi, M., Zanetti, E., Cassandro, M. Genetic variation and population structure of Italian native sheep breeds undergoing in situ conservation. 2009. *J Anim Sci*, 87: 3837–3844.

Danchin-Burge, C., Verrier, E., Moureaux, S., Txier-Boicgard, M., Bibe, B. 2006. Sampling strategies and overview of the French National Cryobank. Proceedings of 8th World Congress on Genetics Applied to Livestock Production; Belo Horizonte, Brazil; 33:1–4.

Dawson, M.R. 1967. Lagomorph history and the stratigraphic record. In: C. Teichert and E.L. Yockelson, editors, Essays in paleontology and stratigraphy. R. C. Moore Commemorative Volume. Department of Geology, University of Kansas Spec. Publ. 2. University of Kansas Press, Lawrence. p. 287–316.

De Los Reyes, M., Saenz, L., Lapierre, L., Crosby, J., Barros, C. 2002. Evaluation of glucose as a cryoprotectant for boar semen.*Vet Rec,* 151: 477–480.

De Marchi M., Cassandro M., Targhetta C., Baruchello M., Notter D.R. 2005. Conservation of poultry genetic resource in the Veneto region of Italy. Animal Genetic Research, 37: 63-74.

Devireddy R.V., Swanlund D.J., Roberts K.P., Pryor J.L., Bischof J.C. 2000. The effect of extracellular ice and cryoprotective agents on the water permeability parameters of human sperm plasma membrane during freezing. *Hum Reprod*, 15: 1125-1135.

Dhali, A., Manik, R.S., Das, S.K., Singla, S.K., Palta, P. 2000. Effect of ethylene glycol concentration and exposure time on post-vitrification survival and in vitro maturation rate of buffalo oocytes. *Theriogenology*, 50: 521–530.

Di Iorio M., Manchisi, A., Rocco, M., Chrenek, P., Iaffaldano, N. 2014. Comparison of different extenders on the preservability of rabbit semen stored at 5°C for 72 hours. *Ital J Anim Sci*, 13: 710–714.

Dolti, P., Moghaddam, G.H., Ahmadian, H. 2016. Evaluation of the effects of cysteine and trehalose on long-term cryopreservation of ram semen. *JAAS Journal*, 4(1): 1–6.

Donga, Q., Sarah, Rodenburga, E., Huang, C., VandeVoorta, C.A. 2008. Effect of pre-freezing conditions on semen cryopreservation of rhesus monkey. *Theriogenology*, 70 (1): 61–69.

Donoghue, A.M., Wishart G.J., 2000. Storage of poultry semen. *Anim Reprod Sci*, 62: 213–232.

Douard, V., Blesbois, E., Hermier, D., Magistrini, M. 2004. Impact of changes in composition of storage medium on lipid content and quality of turkey spermatozoa. *Theriogenology*, 61: 1–13.

Du Bois, S., Len, J.A., Parlevliet, JM, Eilts, B.E. 2012. Effects of Cooling Time on Membrane Integrity and Motility of Frozen-Thawed Canine Spermatozoa using Two Different Commercial Egg Yolk–based Extenders at Two Different Cooldown Equilibration Times. *Reprod Dom Anim*, 47 (Suppl. 6), 278–280.

Du Plessis, L., Soley, J.T. 2011. Incidence, structure and morphological classification of abnormal sperm in the emu (Dromaius novaehollandiae). *Theriogenology*, 75: 589–601.

Ehling, C., Rath, D., Struckmann, C., Frenzel, A., Schindler, L., Niemann, H. 2006. Utilization of frozen–thawed epididymal ram semen to preserve genetic diversity in Scrapie susceptible sheep breeds. *Theriogenology*, 66: 2160–2164.

El-Badry, D.A., Abo El-Maaty, A.M., El Sisy, G.A. The Effect of Trehalose Supplementation of INRA-82 Extender on Quality and Fertility of Cooled and Frozen-Thawed Stallion Spermatozoa. J Equine Vet Sci, 48: 86–92.

El-Gaafary, M.N. 1994. Quality aid fertility of cooled rabbit semen supplemented with cyclic-AMP stimulators. *Anim Reprod Sci*, 34: 307–313.

El-Kelawy, H.M., Tawfeek, M.I., El-Gaafary, M.N., Ibrahim, H. 2012. Viability and fertilizing ability of extended rabbit semen stored at 5°C. in Proc. 10th World Rabbit Congress, Sharm El-Sheikh, Egypt. pp 285–289.

El-Sheshtawy, R.I, Sisy, G.A., El-Nattat, W.S. 2015. Effects of different concentrations of sucrose or trehalose on the post-thawing quality of cattle bull semen. *Asian Pac J Reprod*, 4 (1): 26–31.

Emiliani, S., Bergh, M.V.D., Vannin, A.S., Biramane, J., Englert, Y. 2000. Comparison of ethylene glycol 1, 1,2-propanediol and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. *Hum Reprod*, 15: 905–910.

Etches, R. 2010. Personal communication.

Facchin E., Zanirato M.G., Gualterio L., Valentini A. 1988. A.I. in rabbit breeding: note 1: A.I. service for meat rabbit breedings. Proc. 4th World Rabbit Congress, Budapest, Vol. 2, pp. 121–130.

Facchin E. 1995. Fecondazione artificiale, a che punto siamo. *Rivista di Coniglicoltura*, 10: 13–22.

FAO. 1984. Genetic aspects of conservation in farm livestock, by C. Smith. In Animal genetic resources conservation by management, data banks and training. Proceedings of the Joint FAO/UNEP Expert Panel Meeting, October 1983 Part 1, pp. 18–24. FAO Animal Production and Health Paper N° 44/1 Rome (http://www.fao.org/docrep/010/ah808e/ah808e00.htm).

FAO. 2007. The State of the World's Animal Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO. 2008. Secondary Guidelines for development of national farm animal resources management plans. Management of small populations at risk. In: www.fao.org.

FAO. 2009. Preparation of national strategies and action plans for animal genetic resources. FAO Animal Production and Health Guidelines. N°. 2. Rome (http://www.fao.org/docrep/012/i0770e/i0770e00.htm).

FAO. 2011b. Developing the institutional framework for the management of animal genetic resources. FAO Animal Production and Health Guidelines. N°. 6. Rome (available at http://www.fao.org/docrep/014/ba0054e/ba0054e00.htm).

FAO. 2012. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome.

FAO. 2015. The Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture, edited by B.D. Scherf & D. Pilling. FAO Commission on Genetic Resources for Food and Agriculture Assessments. Rome (http://www.fao.org/3/a-i4787e/index.html).

Farah, O.I., Cuiling, L., Jiaojiao, W., Huiping, Z. 2013. Use of Fluorescent Dyes for Readily Recognizing Sperm Damage. *J Reprod Infertil*, 14(3): 120–125.

Fargeas, E. 1995. Quelques résultats obtenus en insémination artificielle avec de la semence congelée. *Cuniculture*, 123: 103–106.

Ferdinand, A., 1992. Licht- und elektronenmikroskopische Untersuchungen zur Morphologie von Ganterspermatozoen. PhD Thesis, University of Veterinary Medicine, Hannover.

Fernandez-Gonzalez, L., Jewgenow, K. 2017. Cryopreservation of feline oocytes by vitrification using commercial kits and slush nitrogen technique. *Reprod Domest Anim*, 52, (S2): 230–234.

Floros, J.D., Newsome, R., Fisher, W., Barbosa-Cánovas, G.V., Chen, H., Dunne, C.P., Ziegler, G.R. 2010. Feeding the world today and tomorrow: The importance of food science and technology. *Compr Rev Food Sci F*. Volume 9, Issue 5.

Foote, R.H. 1972. Tris and organic buffers for the conservation of semen of various species. In: Animal Reproduction and Artificial Insemination, edited by A. C. Deda, pp 99–105, Edagricole.

Fox, R.R. 1961. Preservation of rabbit spermatozoa: fertility results from frozen semen. *Proc Soc Exp Biol Med*, 108: 663–665.

Fox, R.R., Burdick, J.F. 1963. Preservation of rabbit spermatozoa: ethylene glycol vs. glycerol for frozen semen. *Proc Soc Exp Biol Med* 113: 853–856.

Francham, R., Ballou, J.D., Briscoe, D.A. 2004. A Primer of Conservation Genetics, Cambridge University Press.

Fuller, B., Sharon, J., Paynter Watson, P. 2004. Cryopreservation of human gametes and embryos. In: Barry J Fuller, Nick Lane Erica E Benson, editors. Life in the frozen state, New York: Crc Press; pp. 505–539.

Gabarrou, J.F., Ge´raert, P.A., Picard, M., Bordas, A. 1997. Diet-induced thermogenesis in cockerels is modulated by genetic selection for high or low residual feed intake. *J Nutr*, 127: 2371–2376.

Gadea, J. 2003. Review: Semen extenders used in the artificial insemination of swine. Span. *J Agric Res*, 1: 17–27.

Gao, D., Critser, J.K. 2000. Mechanisms of cryoinjury in living cells. *ILAR J*, 41: 187–196.

Garner, D.L., Johnson L.A. 1995. Viability assessment of mammalian sperm using SYBR-14 and Propidium Iodide. *Biol Reprod*, 53: 276–284.

Garner, D.L., Thomas, C.A., Gravance, C.G. 1999. The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. *Reprod Dom Anim*, 34: 399–404.

Gee, G.F., Temple, S.A. 1978. Artificial insemination for breeding non-domestic birds. *Symp Zool Soc London*, 43: 51–72.

Gee, G.F. 1995. Artificial insemination and cryopreservation of semen from nondomestic birds. In: Bakst,M.R., Wishart, G.J. Eds.., Proc. 1st International Symposium on the Artificial Insemination of Poultry-Poultry Science Association, Savoy, IL, pp. 262–279.

Getachew, T. 2016. A Review Article of Artificial Insemination in Poultry. *World Vet J*, 6(1): 25–33.

Gibb, Z., Morris, L.H.A., Maxwell, W.M.C., Grupen, C.G. 2013. Dimethyl formamide improves the postthaw characteristics of sex-sorted and non sorted stallion sperm. *Theriogenology*, 79 (7): 1027–1033.

Gliozzi, T.M., Zaniboni, L., Cerolini, S. 2011. DNA fragmentation in chicken spermatozoa during cryopreservation. *Theriogenology*, 75: 1613–1622.

Gogol, P. 1999. Cryopreservation of plasma-deprived rabbit semen. *Ann Anim Sci*, 26: 85–92.

Götze, V.G., Paufler, S. 1976. Fertility results and prenatal mortality following artificial insemination with fresh and deep frozen semen in rabbits after normal induction of ovulation and superovulation. *Zuchthyg*, 11: 169–174.

Graham, E.F. 1975. The state of the art in cryobiology. Pages 182-192 In R. H. Carr (ed.), Applications of Cryobiology Technology Vol. 5. Scholium International Inc., Whitestone, N. Y.

Griffin, J.F.T., Hartigan, P.J., McGilligan, C.A., Nunn, W.R. 1974. Fertility of rabbits to artificial insemination with semen diluted in Nagase egg-yolk semen diluent. *Theriogenology*, 1: 51–54.

Groeneveld, E., Tinha, N.H., Kues, W., Vien, N.T. 2008. A protocol for the cryoconservation of breeds by low-cost emergency cell banks – a pilot study. *Animal*, 2: 1–8.

Guernec, A., Berri, C., Chevalier, B., Wacrenier-Cere, N., Le Bihan-Duval, E., Duclos, M.J. 2003. Muscle development, insulin-like growth factor-I and myostatin mRNA levels in chickens selected for increased breast muscle yield. *Growth Horm IGF Res*, 13: 8–18.

Hafez, E.S.E. 1984. Biologia e tecnologia della riproduzione nelle specie animali di interesse zootecnico. Ed. Grasso.

Hall, S.E., Negus, C., Johinke, D., Bathgate, R. 2017. Adjusting cryodiluent composition for improved post-thaw quality of rabbit spermatozoa. *PLOS one,* 1–14.

Hammadeh, M.E., Askari, A.S., Georg, T., Rosenbaum, P., Schmidt, W. 1999. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. *Int J Androl*, 22: 155–162.

Hammerstedt, R.H., Graham, J.K., Nolan, J.P. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl*, 11, 73–88.

Hanada, A., Nagase, H. 1980. Cryoprotective effects of some amides on rabbit spermatozoa. *J Reprod Fertil*, 60: 247–252.

Hanzawa, S., Niinomi, T., Miyata, T., Tsutsui, M., Tajima, A. 2010. Cryopreservation of chicken semen using N-methylacetamide as cryoprotective agent. *Japanese Poult Sci*, 47: J27–J32.

Harrison, R.A.P. 1983. The Acrosome, Its Hydrolases, and Egg Penetration. In: André J. (eds) The Sperm Cell. Springer, Dordrecht.

Hellemann, C., Krause, D., Weitze K.F. 1979a. Congelación de semen de conejo. I. Efecto de la glicerina, el DMSO y un surfactante sobre los acrosomas y la motilidad. *Arch Med Vet* 11: 27–31.

Hellemann, C., Gigoux, E. 1988. Deep freezing of rabbit. Effect of a surfactant on fertilizing capacity. *Zuchthyg*, 23: 33–37.

Hewitt, D.A., England, G.C. 1998. An investigation of capacitation and the acrosome reaction in dog spermatozoa using a dual fluorescent staining technique. *Anim Reprod Sci*, 51: 321–332.

Hiemstra, S.J. 2011.Cryopreservation strategies for farm animal genetic resources in Europe. Department(s) Livestock Research LR – Backoffice. Contribution in proceedings.

Hoagland, H., Pincus, G. 1942. Revival of mammalian sperm after immersion in liquid nitrogen. *J Gen Physiol*, 25: 337–344.

Hochi, S., Kozawa, M., Fujimoto, T., Hondo, E., Yamada, J., Oguri, N. 1996. In vitro maturation and transmission electron microscopic observation of horse oocytes after vitrification. *Cryobiology*, 33: 300–310.

Hoffmann, I. 2009. The global plan of action for animal genetic resources and the conservation of poultry genetic resources. *World Poult Sci J*, 65: 286–297.

Holt WV. 2000. Basic aspects of frozen semen storage. *Anim Reprod Sci*, 62: 3–22.

Hossain, M.S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P., Martinez, H.R. 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J Androl*, 13: 406–419.

Hsieh, L. 1996. Influence of extender, glycerol concentration and detergent on post-thaw motility of rabbit spermatozoa. *J Chin Soc Anim Sci*, 25: 175–181.

Hu, J.H., Li, Q.W., Li, G., Chen, X.Y., Yang, H., Zhang, S.S., Wang, L.Q. 2006. The cryoprotective effect on frozen–thawed boar semen ofegg yolk low density lipoproteins. *Asian-Aust J Anim Sci*, 19: 486–494.

Huang, J., Li, Q., Zhao, R., Li, W., Han, Z., Chen, X., Xiao, B., Wu, S., Jiang, Z., Hu, J., et al. 2008. Effect of sugars on maturation rate of vitrified-thawed immature porcine oocytes, *Anim Reprod Sci*, 106: 25–35.

Iaffaldano, N., Manchisi, A., Gambacorta, M., Di Iorio, M., Rosato, M.P. 2009. Effect of different sperm concentrations on the post-thaw viability and motility of turkey spermatozoa cryopreserved by the pellet method. *Ital J Anim Sci*, 8: 760–762.

Iaffaldano, N., Romagnoli, L., Manchisi, A., Rosato, M.P. 2011. Cryopreservation of turkey semen by the pellet method: Effects of variables such as the extender, cryoprotectant concentration, cooling time and warming temperature on sperm quality determined through principal components analysis. *Theriogenology*, 76: 794–801.

Iaffaldano, N., Di iorio, M., Rosato, M.P., Manchisi, A. 2014. Cryopreservation of rabbit semen using non-permeable cryoprotectants: effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or sucrose. *Anim Reprod Sci*, 151: 220–228.

Iaffaldano, N., Di Iorio, M., Miranda, M., Zaniboni, L., Manchisi, A., Cerolini, S. 2016a. Cryopreserving turkey semen in straws and nitrogen vapor using DMSO or DMA: effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. *Br Poult Sci*, 57: 264–270.

Iaffaldano, N, Di Iorio, M., Cerolini, S., Manchisi, A. 2016b. Overview of turkey semen storage: focus on cryopreservation – a review. *Ann Anim Sci*, 16 (4): 961–974.

Iaffaldano, N., Di Iorio, M., Manchisi, A., Gibertoni, P.P., Esposito, S. 2016c. Semen Quality of Threatened Native Population of Mediterranean Brown Trout (Salmo cettii, Rafinesque 1810) in the Biferno River (Molise Region - South Italy). *Turk J of Fish Aquat Sc*, 16: 259–266.

Jain, J.K., Paulson, R.J. 2006. Oocyte cryopreservation. *Fertil Steril*, 86 (Suppl. 3): 1037–1046.

Jia, X.X., Lu, J.X., Tang, X.J., Fan, Y.F., Huang, S.H., Ge, Q.L., Gao, Y.S. 2018. Genetic diversity of Jiangsu native chicken breeds assessed with the mitochondrial DNA D-loop region. *Br Poult Sci*, 58, Issue 5.

Jin, B., Mazur, P. 2015. High survival of mouse oocytes/embryos after vitrification without permeating cryoprotectants followed by ultra-rapid warming with an IR laser pulse. *Sci Rep-UK*, 5: 9271.

Jochová, M., Novák, K., Kott, T., Volek, Z., Majzlík, I., Tůmová, E. 2017. Genetic characterization of Czech local rabbit breeds using microsatellite analysis. *Livest Sci*, 201: 41–49.

Kang, S.J., Choi, J.W., Kim, S.Y., Park, K.J., Kim, T.M., Lee, Y.M., Kim, H., Lim, J.M., Han,J.Y. 2008. Reproduction of wild birds via interspecies germ cell transplantation. *Biol Reprod*, 79: 931–941.

Kashiwazaki, N., Okuda, Y., Seita, Y., Hisamatsu, S., Sonoki, S., Shino, M., Masaoka, T., Inomata, T. 2006. Comparison of glycerol, lactamide, acetamide and dimethylsulfoxide as cryoprotectants of Japanese white rabbit spermatozoa. *J Reprod Dev*, 52: 511–516.

Kato, Y., Nagao, Y. 2012. Effect of polyvinylpyrrolidone on sperm function and early embryonic development following intracytoplasmic sperm injection in human assisted reproduction. *Reprod Med Biol*, 11: 165–176.

Kato, Y., Nagao, Y. 2015. Changes in Sperm Motility and Capacitation Induce Chromosomal Aberration of the Bovine Embryo following Intracytoplasmic Sperm Injection. *PLoS ONE* 10(6): e0129285.

Kim, M.A., Park, T.S., Kim, J.N., Park, H.J., Lee, Y.M., Ono, T., Lim, J. M., Hau, J.Y. 2005. Production of quail (Coturnix japonica) germline chimeras by transfer of gonadal primordial germ cells into recipient embryos. *Theriogenology*, 63: 774–782.

Kimura, Y., Yanagimachi, R. 1995. Development of normal mice from oocytes injected with secondary spermatocyte nuclei. *Biol Reprod*, 53: 855–862.

Kiprianidis, V., Facchin, E. 1994. Monta naturale o inseminazione artificiale? Importanza del extender diluitore nella I.A. *Rivista di Coniglicoltura*, 11: 41–43.

Konc, J., Kanyó, K., Kriston, R., Somoski, B., Cseh, S. 2014. Cryopreservation of embryos and oocytes in human assisted reproduction. *Bio Med Research International*, Article ID 307268, 9 pages.

Kono, T., Suzuki, O., Tsunoda, Y. 1988 Cryopreservation of rat blastocysts by vitrification. *Cryobiology*, 25: 170–173.

Köster, E.M., Hefer, C.A. Nel, LH., Groenen, M.A.M. 2008. Genetic diversity and population structure of locally adapted South African chicken lines: Implications for conservation. *S Afr J Anim Sci*, 38 (4): 271–281.

Kotłowska, M., Dietrich, G., Wojtczak, M., Karol, H., Ciereszko, A. 2007. Effects of liquid storage on amidase activity, DNA fragmentation and motility of turkey spermatozoa. *Theriogenology*, 67(2): 276–286. Epub 11.

Kovácsné G.K. 2004. A sárga Magyar tyúk génmegőrzése és fajtafenntartása Mosonmagyaróváron. [Gene conservation and breeding of the Yellow Hungarian Chicken in Mosonmagyarovar. In Hungarian] *a Baromfi*, 7(1):21–24. www.mge-hu.com.

Kozdrowski, R., Dubiel, A., Noszczk-Nowak A. 2009. Comparison of Acetamide and Dimethylsulfoxide as cryoprotectants of European brown hare (Lepus europeus) spermatozoa. *J Repod Develop*, vol 55, n°1.

Kowalczyk, A., Łukaszewicz, E., Rzońca, Z. 2012. Successful preservation of capercaillie (Tetrao urogallus L.) semen in liquid and frozen states. *Theriogenology*, 77: 899–907.

Kowalczyk, A., Łukaszewicz, E. 2015. Simple and effective methods of freezing capercaillie (Tetrao urogallus L.) semen. *PLoS ONE*, 10: 1–11.

Kulíková, B., Di Iorio, M., Kubovičova, E., Kuželová, L., Iaffaldano, N., Chrenek, P. 2015. The cryoprotective effect of Ficoll on the rabbit spermatozoa quality. *Zygote*, 23 (5): 785–794.

Lake, P.E. 1978. The principles and practice of semen collection and preservation in birds. *Symp Zool Soc Lond*, 43: 31–49.

Lake, P.E., Stewart, J.M. 1978. Artificial insemination in poultry. Bulletin 213 – Ministry of Agriculture, Fisheries and Food, London, UK.

Lake, P.E. 1995. Historical perspective of artificial insemination technology. In Bakst, M.R., Wishart, G.J. (Eds), Proc. 1st International Symposium on the Artificial Insemination in Poultry. Poultry Science Association, Savoy, IL, pp. 1-20.

Le Bihan-Duval, E., Mignon-Grasteau, S., Millet, N., Beaumont, C. 1998. Genetic analysis of a selection experiment on increased body weight and breast muscle weight as well as on limited fat weight. *Br Poult Sci*, 39: 346–353.

Ledezma-Torres, R.A., Sandoval, L., Valladares, P., Mellado, M., Picòn, F.J., Ramìrez R., Snchèz, F. Effect of cooling o sperm motility before and after frozen-thawed stallion semen. *Afr J Biotechnol*, 13 (12): 1439–1443.

Le Gal, F. 1996. In vitro maturation and fertilization of goat oocytes frozen at the germinal vesicle stage. *Theriogenology*, 45: 1177–1185.

Lemma, A. 2011. Effect of Cryopreservation on Sperm Quality and Fertility. In: M. Manafi ed. Artificial Insemination in Farm Animals, InTech, chap. 12 pp. 191-216.

Leroy, G., Danchin-Burge, C., Verrier, E. 2011. Impact of the use of cryobank samples in a selected cattle breed: a simulation study. *Genet Sel Evol*, 43 (36): 1–8.

Lewis, P.D., Perry, G..C, Farmer, L.J., Patterson, R.L.S. 1997. Responses of two genotypes of chicken to the diets and stocking densities typical of UK and label rouge production systems: Performance. Behaviour and carcass composition. *Meat Sci*, 47: 501–516.

Li, R., Chen, H., Ni., M. 2002. Reflection on preservation and utilization of the genetic resources of domestic animal and poultry. *Ecol Domest Anim*, 23: 52–55.

Li, Q.Y., Hou, J., Chen, Y.F., An, X.R. 2010. Full-term development of rabbit embryos produced by ICSI with sperm frozen in liquid nitrogen without cryoprotectants. *Reprod Domest Anim*, 45: 717–722.

Liebermann, J., Nawroth, F., Isachenko, V. et al. 2002. Potential importance of vitrification in reproductive medicine. *Biol Reprod*, 67: 1671–1680.

Liebermann, J., Tucker M.J, Sills E.S. 2003. Cryoloop vitrification in assisted reproduction: analysis of survival rates in > 1000 human oocytes after ultra-rapid cooling with polymer augmented cryoprotectants.*Clin Exp Obstet Gynecol*, 30 (2-3): 125-129.

Liu, E., Kitajima, S., Wiese, E., Reifenberg, K., Morimoto, M., Watanabe, T., Fan, J. 2007. Re-establishment of complement C6-deficient rabbit colony by cryopreserved sperm transported from abroad. *Exp Anim*, 56: 167–171.

Liu, F., Long, H.P., Wang, Z.G., He, Z.W. 2014. Import of Animal Genetic Resources in China: The Spatiotemporal Change and Drivin Forces. In: International Conference on Economics and Management Engineering (ICEME, 2014).

Liu, J., Tanrikut, C., Wright, D.L., Lee, G.W., Toner, M., Biggersa, J.D. and Thomas L. Toth. 2016. Cryopreservation of human spermatozoa with minimal nonpermeable cryoprotectant. *Cryobiology*, 73(2): 162–167.

Loaiza-Echeverri, A.M., Cruz, B.C., das Neves Snoeck, P.P, Mora, L.C.O., Parzewski, B., Neves, M.M., Machado Neves, M., Heneine, L.G.D., Henry, M. 2015. Low density lipoproteins added to an extender frozen or lyophilized are evenly efficient in cryoprotecting ovine sperm cells than when 16% whole egg yolk was added. Semina: Ciências Agrárias, Londrina, v. 36 (3): 1335-1346.

Long, J.A. 2006. Avian semen cryopreservation: what are the biological challenges? *Poult Sci*, 85: 232–236.

Long, J.A., Bongalhardo, D.C., Peláez, J., Saxena, S., Settar, P., O' Sullivan, N.P., Fulton J.E. 2010. Rooster semen cryopreservation: Effect of pedigree line and male age on postthaw sperm function. *Poult Sci*, 89: 966–973.

López-Gatius, F., Sances, G., Sancho, M., Yániz, J., Santolaria, P., Gutiérrez, R., Núñez, M., Núñez, J., Soler, C. 2005. Effect of solid storage at 15°C on the subsequent motility and fertility of rabbit semen. *Theriogenology*, 64: 252–260.

Lovelock, J.E., Bishop, M.W. 1959. Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature*, 183: 1394–1395.

Lukaszewicz, E, 2002. An effective method for freezing White Italian gander semen . *Theriogenology*, 58: 19–27.

Lush, J.L. 1994. The genetic nature of differences between groups. In: The Genetics of Populations (Chapman, A.B., Shrode, R.R. and Crow, J.F., Eds.), Iowa State University, Ames, Iowa, USA, pp. 248-268.

Maclellan, L.J., Carnevale, E.M., Coutinho da Silva, M.A., Scoggin, C.F., Bruemmer, J.E., Squires, E.L. 2002. Pregnancies from vitrified equine oocytes collected from super-stimulatedand non-stimulated mares. *Theriogenology*, 58: 911–919.

Madeddu, M., Mosca, F., Abdel Sayed, A., Zaniboni, L., Mangiagalli, M.G., Colombo E., Cerolini, S. 2016. Effect of cooling rate on the survival of cryopreserved roostersperm: Comparison of different distances in the vapor abovethe surface of the liquid nitrogen. *Anim Reprod Sci*, 171: 58–64.

Maeda, T., Liu, E., Nishijima, K., Tanaka, M., Yamaguchi, S., Morimoto, M, Watanabe, T., Fan J., Kitajima, S. 2012. Effect of the primary cooling rate on the motility and fertility of frozen-thawed rabbit spermatozoa. *World Rabbit Sci*., 20: 65–70.

Maertens, L., Luzy, F. 1995. Note concerning the effect of PMSG stimulating on the mortality rate at birth and the distribution of litter size in artificially inseminated does. *World Rabbit Sci*, 3: 57–61.

Maj, D., Bieniek, J., Łapa, P., Sternstein, I. 2009. The effect of crossing New Zealand White with Californian rabbits on growth and slaughter traits. *Archiv Tierzucht* 52 (2): 205–211, ISSN 0003-9438.

Makarevich, A.V., Chrenek, P., Olexiková, L., Popelkova, M., Turanova, Z., Ostro, A., Pivko, J. 2008. Post-thaw survival, cell death and actin cytoskeleton in gene-microinjected rabbit embryos after vitrification. *Theriogenology*, 70, 675–681.

Makhafola, M.B., Lehloenya, K.C., Mphaphathi, M.L. Dinnyes, A., Nedambale, T.L. 2009. The effect of breed on the survivability and motility rate of cryopreserved cock semen. South *Afr J Anim Sci*, 39 (Supplement 1). 10 th World Conference on Animal Production.

Mammuccini, M.G. 2006. Presentazione al manuale “L’allevamento della Valdarnese Bianca” Ed. ARSIA (Agenzia Regionale per lo Sviluppo e l’Innovazione del settore Agricolo-forestale).

Manjunath, P., Nauc, V., Bergeron, A., Menard, M., 2002. Major proteins ofbovine seminal plasma bind to the low-density lipoprotein fraction ofhen’s egg yolk. *Biol Reprod*, 67: 1250–1258.

Mantovani, R., Rota, A., Falomo, M.E., Bailoni, L., Vincenti, L. 2002. Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypoosmotic swelling test. *Reprod Nutr Dev*,42: 217–226.

Marelli, S.P., 2008. Genetica e selezione. In: Avicoltura e Coniglicoltura, ed. Point Veterinaire Italie, La Fenice Grafica, Lodi, Italy: pp.32–33.

Mariante, A.S, Albuquerque, M.S.M, Egito A.A, McManus C, Lopes A, Paiva S.R. 2009. Present status of the conservation of livestock genetic resources in Brazil. *Livest Sci*, 120: 204–212.

Marmar, J.L. 1998. The emergence of specialized procedures for the acquisition, processing, and cryopreservation of epididymal and testicular sperm in connection with intracytoplasmic sperm injection. J Androl, 19: 517–526.

Martin, R.D. (ed.). 1975. Breeding endangered species in captivity. Academic Press, N.Y. and London.

Martín-Bilbao, M. 1993. Congelación de semen de conejo. Efecto de algunos agentes crioprotectores sobre la viabilidad espermática, V Jornadas sobre Producción Animal. ITEA 12 (Suppl. II), 486–488.

Matassino, D. 2010. Experience CONSDABI - SUB NFPI - FAO [Mediterranean biodiversity] already nfp.I-fa o [1994-2009]. In : La salvaguardia della biodiversità animale-Iniziative generali ed azioni intraprese in Italia a tutela delle razze minacciate a cura di F. Panella, fondazione iniziative zooprofilattiche e zootecniche – Brescia, 2011.

McCall, M.R., Freiz, B. 1999. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic Biol Med*, 26: 1034–1053.

McNitt J.I., Lukefahr S.D., Cheeke P.R., Patton N.M. 2013. Rabbit Production, 9th Edition. CABI Publishing.

McRorie, R.A., Williams, W.L. 1974. Biochemistry of mammalian fertilization. *Annu Rev Biochem*, 43, 777–802.

Mehrabani, D., Tajedini, M., Tamadon, A., Dianatpour, M., Parvin, F., Zare, S., Rahmanifar, F. 2016. Establishment, characterization and cryopreservation of Fars native goat fetal fibroblast cell lines. *Asia Pac J Reprod*, 5(3): 247–251.

Mihók S. 2004. Őshonos és réghonosult baromfifajok fenntartása a Debreceni Agrártudományi Centrumban. [Conservation of indigenous and adapted poultry species in the Centre of Agricultural Science of the Debrecen University. In Hungarian]. *A Baromfi*, 7 (2): 8–13.

Miller, M.M., Bacon, L.D., Hala, K., Hunt, H.D., Ewald, S.J., Kaufman, J., Zoorob, R., Briles, W.E. 2004. Nomenclature for the chicken major histocompatibility (B and Y) complex. *Immunogenetics*, 56: 261–279.

Minelli, A., Moroni, M., Castellini, C. 2001. Isolation of the IGF-I protein complex from rabbit seminal plasma: effects on sperm mobility and viability. *J Exp Zool*, 290: 279–290.

Ministero delle Politiche Agricole, Alimentari e Forestali. 2014. D.M. 19536 del 01/10/2014, Istituzione del Registro anagrafico delle razze avicole autoctone. Available at: http://www.aia.it/aia_website/it/settori/areatecnica/ ufficiosviluppo/registroanagrafico-delle-razze avicole- autoctone.

Miranda, M., Kulíková, B., Vašíček, J., Olexiková, L., Iaffaldano, N., Chrenek, P. 2018. Effect of cryoprotectants and thawing temperatures on chicken sperm quality. *Reprod Dom Anim*, 53(1): 93–100.

Mocé, E., Vicente J.S. 2002. Effect of cooling and freezing, the two first steps of a freezing protocol, on the fertilizing ability of the rabbit sperm. *Reprod Nutr Dev*, 42: 189–196.

Mocé, E., Lavara, R., Vicente, J.S. 2003a. Effect of an asynchrony between ovulation and insemination on the results obtained after insemination with fresh or frozen sperm in rabbits. *Anim Reprod Sci,* 75: 107–118.

Mocé, E., Vicente, J.S., Lavara, R. 2003b. Effect of freezing–thawing protocols on the performance of semen from three rabbit lines after artificial insemination. *Theriogenology*, 60: 115–123.

Mocé, E., Vicente, J.S., Lavara, R. 2003c. Diferencias en la congelabilidad del semen de dos lineas de conejo X Jornadas sobre Produccion Animal. ITEA 24 (Suppl. I), 336–338.

Mocé, E., Graham, J.K. 2005. Effect of egg yolk, cooling/thawing rates and cholesterol on cryosurvival of rabbit sperm. In: Bremmer,W.J. (Ed.), 30th Annual Meeting of the ASA. J. Androl. Supplement, 79, abstract 121.

Mocé, E., Vicente, J.S. 2009. Rabbit sperm cryopreservation: a review. *Anim Reprod Sci*, 110: 1–24.

Mocé, E., Lavara, R., Vicente, J.S. 2010. Effect of cooling rate to 5°C, straw size and farm on fertilizing ability of cryopreserved rabbit sperm. *Reprod Domest Anim*, 45: e1–7.

Mocé, E., Blanch, E., Talaván, A., Viudes de Castro, M.P. 2014. Reducing the time rabbit sperm are held at 5°C negatively affects their fertilizing ability after cryopreservation. *Theriogenology*, 82: 1049–1053.

Moreno, D., Bencharif, D., Amirat-Briand, L., Neira, A., Destrumelle, S., Tain-turier, D., 2013. Preliminary results: the advantages of low-densitylipoproteins for the cryopreservation of equine semen. *Equine Vet Sci*, 33: 1068–1075.

Morisson, M., Bordas, A., Petit, J.M., Jayat-Vignoles, C., Julien, R., Minvielle, F. 1997. Associated effects of divergent selection for residual feed consumption on reproduction, sperm characteristics, and mitochondria of spermatozoa. *Poult Sci*, 76: 425–431.

Morrell, J.M. 1995. Artificial insemination in rabbits. *Br Vet J*, 151(5): 477–488.

Morrell, J.M., Wallgren, M. 2014. Alternatives to Antibiotics in Semen Extenders: A Review. *Pathogens*, 3: 934–946.

Morris, G.J., Goodrich, M., Acton, E., Fonseca, F. 2006. The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation. *Cryobiology* 52, 323–334.

Morton, D.B., 1976. Lysosomal enzymes in mammalian spermatozoa. In Lysosomes in biology and pathology. Vol. 5. Dingle and R.T. Dean (eds) New York, American Elsevier Publishing Co., Inc., pp. 203-205.

Mosca, F., Madeddu, M., Mangiagalli, M.G., Colombo, E., Cozzi, M.C., Zaniboni, L., Cerolini, S. 2015. Bird density, stress markers and growth performance in the Italian chicken breed Milanino. *J Appl Poult Res*, 24: 529–535.

Mosca, F., Madeddu, M., Sayed, A.A., Zaniboni, L., Iaffaldano, N., Cerolini, S. 2016. Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm. *Cryobiology*, 73: 343–347.

Mota Filho, A.C., Telesa, C.H.A., Jucáa, R.P., Cardosob, J.F.S., Uchoaa, D.C., Campelloc, C.C., Silvad, A.R., Silva L.D.M. 2011. Dimethylformamide as a cryoprotectant for canine semen diluted and frozen in ACP-106C. *Theriogenology* 76: 1367–1372.

Moussa, M., Martinet, V., Trimèche, A., Tainturier, D., Anton, M., 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen–thawed bull semen. *Theriogenology*, 57: 1695–1706.

Moustacas, V.S., Zaffalon, F.G., Lagares, M.A., Loaiza-Eccheverri, A.M., Varago, F.C., Neves, M.M., Heneine, L.G.D., Arruda, R.P., Henry, M. 2011. Natural, but not lyophilized, low density lypoproteins were an acceptable alternative to egg yolk for cryopreservation of ram semen. *Theriogenology*, 75: 300–307.

Mphaphathi, M. L., Seshoka, M. M., Luseba, D., Sutherland, B., Nedambale, T. L. 2016. The characterisation and cryopreservation of Venda chicken semen. *Asian Pac J Reprod*, 5 (2): 132–139.

Nagy, S.Z., Sinkovics, G.Y., Kovács, A. 2002. Viability and acrosome integrity of rabbit spermatozoa processed in a gelatin-supplemented extender. *Anim Reprod Sci*, 70: 283–286.

Nakamura, Y. 2016. Poultry genetic resource conservation using primordial germ cells. *J Reprod Develop*, 62 (5): 431–437.

Nandi, S., Whyte, J., Taylor, S., Sherman, A., Nair, V., Kaiser, P., McGrew, M.J. 2016. Cryopreservation of specialized chicken lines using cultured primordial germ cells. *Poult Sci*, 95: 1905–1911.

Neil, L.O.', Paynter, S.J., Fuller, B.J., Shaw, R.W. 1997. Vitrification of Mature Mouse Oocytes: Improved Results Following Addition of Polyethylene Glycol to a Dimethyl Sulfoxide Solution. *Cryobiology*, 34 (3): 295–301.

Nynca, J., Dietrich, G.J., Dobosz, S., Grudniewska, J., Ciereszko, A. 2014. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. *Aquaculture*, 433: 62–65.

Obata, T., Takeda, H., Lzaike, Y. Preservation of Animal Genetic Resources in Japan .*JARQ*, 26: 1–6.

OECD-FAO. 2016. Agricultural Outlook 2017-2026 Di OECD, Food and Agriculture Organization of the United Nations.

Ohboshi, S.N., Fujihara, T., Yoshida, Tomogane, H. 1997. Usefulness of polyethylene glycol for cryopreservation by vitrification of in vitro-derived bovine blastocysts. *Anim Reprod Sci*, 48 (1): 27–36.

Okuda, Y., Seita, Y., Hisamatsu, S., Sonoki, S., Shino, M., Masaoka, T., Inomata, T., Kamijo, S.I., Kashiwazaki, N. 2007. Fertility of spermatozoa cryopreserved with 2% acetamide or glycerol through artificial insemination in the Japanese white rabbit. *Exp Anim*, 56: 29–34.

Olivares, M., Viudes de Castro, M.P., Lavara, F., Lavara, R., Vicente, J.S. 2005. Effect of gelatine or acetamide in post-thawing rabbit sperm parameters. In: Vazquez, J.M. (Ed.), 9th Annual Conference ESDAR. Reprod. Domest. Anim. 40, vol. 382, abstract P164.

Ollivier, L., Renard, J.P. 1995. The costs of cryopreservation of animal genetic resources. In Book of abstracts of the 46th Annual Meeting of the European Association for Animal Production, p. 57. Wageningen, the Netherlands. Wageningen Press.

Ono, T., Yokoi, R., Aoyama, H. 1996. The transfer of male or female primordial germ cells of quail into chick embryonic gonads. *Exp Anim*, 45: 347–352.

O'Shea, T., Wales, R.G. 1969. Further studies of the deep freezing of rabbit spermatozoa in reconstituted skim milk powder. *Aust J Biol Sci*, 22: 709–719.

Otoi, T., Yamamoto, K., Koyama, N., Tachikawa, S., Suzuki, T. 1995. The development of immature bovine oocytes cryopreserved by 1,2-propanediol. *J Reprod Develop*, 41: 361–366.

Özdemir, D., Özdemir, E.D., De Marchi, M., Cassandro, M. 2013. Conservation of Local Turkish and Italian Chicken Breeds: A Case Study. *It J Anim Sci*, 12, e49.

Pagiola, S., Kellenberg, J., Vidaeus, L., Srivastava, J. 1997.Mainstreaming Biodiversity in Agricultural Development. Toward Good Practice. Global Overlays Program.

Partyka, A.; Łukaszewicz, E., Niżański, W. 2011a. Flow cytometric assessment of fresh and frozen-thawed Canada goose (Branta canadensis) semen. *Theriogenology*, 76: 843–850.

Penfold, M., Wildtz, D. E., Hezog, T. L. Lynch, W., Ware, L., Derickson, S. E, Menfort, S.A. 2000. Seasonal pattern of LH, testosterone and semen quality in the Northern pintail Duck. (Anas ecuta). *Reprod Fertil Develop*, 12: 229–235.

Pizzi, F., Turri, F., Gliozzi, T. M., Gandini, G. 2016. Implementation and cost analysis of a regional farm animal cryobank: An Italian case study. It J Anim Sci, 15: 207–210.

Palermo, G., Joris, H., Devroey, H., Van Steirteghem, A.C. 1992. Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte. *Lancet*, 340: 17–18.

Parks, J.E., Graham, J.K. 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, 38: 209–222.

Parrish, J.J., Foote, R.H. 1986. Fertility of cooled and frozen rabbit sperm measured by competitive fertilization. *Biol Reprod*, 35: 253–257.

Partyka, A., Niżański, W., Ochota, M. 2012. Methods of assessment of cryopreserved semen. In: Katkov II (ed) Current Frontiers in Cryobiology. In Tech Open Access Publisher, Rijeka, Croatia, pp 547-574.

Paufler, S. 1985. Reproduction: in "A compendium of rabbit production". Ed. GTZ Gmbh-Dag Hammarskiold, Eschborn, D 6236.

Péris, S.I., Morrier, A., Dufour, M., Bailey, J.L. 2004. Cryopreservation of ram semen facilitâtes sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. *J Androl*, 25: 224–233.

Péris, S.I., Bilodeau, J.F., Dufour, M., Bailey, J.L. 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. *Mol Reprod Dev*, 74: 878–892.

Pesch, S., Bergmann, M. 2006. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron*, 37: 597–612.

Pierson, E.E., Mc Daniel, G.R., Krista, L.M. 1988. Relationship between fertility duration and in vivo sperm storage in broiler breeder hens. *Br Poult Sci*, 29: 199–203.

Pisenti, J.M., Delany, M.E., Taylor, R.L., U.K, Jr., Abbott, H. Abplanalp, J.A. Arthur, M.R. Bakst, C. Baxter-Jones, J.J. Bitgood, F.A. Bradley, K.M. et al. 1999. Avian Genetic Resources at Risk: An Assessment and Proposal for Conservation of Genetic Stocks in the USA and Canada. Report No. 20. University of California Division of Agriculture and Natural Resources, Genetic Resources Conservation Program, Davis, CA USA.

Plachy, J., Vilhelmova, M., Karakoz, I., Schulmannova, J. 1989. Prague inbred lines of chickens: A biological model for MHC research. *Folia Biol (Praha)*, 35: 177–196.

Polge C., 1951. Functional survival of fowl sperm after freezing at -70 °C. *Nature*, 167:949-950.

Porcu, E., Venturoli, S. 2006. Progress with oocyte cryopreservation. *Curr Opin Obstet Gyn*, 18: 273–279.

Prapaiwan, N., Tharasanit, T., Punjachaipornpol S., Yamtang, D., Roongsitthichai, A., Moonarmart, W., Kaeoket, K., Manee-in, S. 2016. Low-density Lipoprotein Improves Motility and Plasma Membrane Integrity of Cryopreserved Canine Epididymal Spermatozoa. *Asian Australas J Anim Sci*., 29 (5): 646–651.

Prentice, J.R., Anzar, M. 2011. Cryopreservation of Mammalian Oocyte for Conservation of Animal Genetics. *Vet Med Int*, Article ID 146405, 11 pages.

Psenicka, M., Saito, T., Linhartová, Z., Gazo, I. 2015. Isolation and transplantation of sturgeon early-stage germ cells. *Theriogenology*, 83: 1085–1092.

Rakha, B.A, Ansari, M.S., Akhter, S.,Zafar, Z., Hussain, I., Santiago Moreno, J., Blesbois, E. 2017. Cryopreservation of Indian red jungle fowl (Gallus gallus murghi) semen with polyvinylpyrrolidone. *Cryobiology*, 78: 27–33.

Rall, W.F., Fahy, G.M. 1985. Ice-free cryopreservation of mouse embryos at – 196°C by vitrification. *Nature*, 313: 573–575.

Ravie, O, Lake, P.E. 1985. The phospholipid-bond fatty acids of fowl and turkey spermatozoa. *Anim Reprod Sci*, 9: 189–192.

Ribas-Maynou, J., Garcı´a-Peiro, A., Abad, C., Amengual, M.J., Navarro, J., Benet, J. 2012. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Human Reprod*, 27(3): 652–658.

Ricard, F.H., Marche´, G., Lebihan-Duval, E. 1994. Essai d'ame´lioration par se´lection de la qualite´ de la carcasse du poulet de chair. *INRA Prod. Anim*, 7: 253–261.

Rijsselaere, T.; Van Soom, A.; Tanghe, S.; Coryn, M.; Maes, D., de Kruif, A. 2005. New techniques for the assessment of canine semen quality: A review. *Theriogenology*, 64: 706–719.

Roca, J., Martínez, S., Vázquez, J.M., Lucas, X., Parrilla, I., Martínez, E.A. 2000. Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15°C. *Anim Reprod Sci*, 64: 103–112.

Rohloff, D., Laiblin, C. 1976. Deep-freezing of rabbit spermatozoa. *Berl Munch Tierarztl Wochenschr*, 89: 181–183.

Romanov, M.N., Wezyk, S., Cywa-Benko, K., Sakhatsky, N.I. 1996: Poultry Genetic Resources in the Countries of Eastern Europe – History and Current State. *Poultry Avian Biol Rev*, 7: 1–29.

Rosato, M.P., Iaffaldano, N. 2011. Effect of Chilling Temperature on the Long-Term Survival of Rabbit Spermatozoa held Either in a Tris-Based or a Jellified Extender. *Reprod Dom Anim*, 46: 301–308.

Rosato, M.P., Centoducati, G., Santacroce, M.P., Iaffaldano, N. 2012. Effects of lycopene on in vitro sperm quality and lipid peroxidation in refrigerated and cryopreserved turkey spermatozoa. *Br Poult Sci*, 53: 545–552.

Rosato, M.P., Iaffaldano, N. 2013. Cryopreservation of rabbit semen: comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. *Theriogenology*, 79, 508–516.

Sabbioni, A., Zanon, A., Beretti, V., Superchi, P., Zambini, E.M., 2006. Carcass yield and meat quality parameters of two Italian autochthonous chicken breeds reared outdoor: Modenese and Romagnola. Paper n. 204 in Proc. 12th Eur. Poultry Conf., Verona, Italy.

Saint Jalme, M., Lecoq, R., Seigneurin, F., Blesbois, E., Plouzeau, E. 2003. Cryopreservation of semen from endangered pheasants: the first step towards cryobank for endangered avian species. *Theriogenology*, 59: 875–888.

Santiago-Moreno, J., Castaño, C., Coloma, M. A., Gómez-Brunet, A., Toledano-Díaz, A., López-Sebastián, A., Campo, J.L. 2009b. Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range poultry. *Poult Sci* 88: 2661–2669.

Santiago-Moreno, J, Castano, C, Toledano-Diaz, A, Coloma, MA, Lopez-Sebastian, A, Prieto, MT, Campo, J.L. 2011. Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: Optimization of freezing rate and equilibration time. *Poult Sci*, 90: 2047–2053.

Santiago-Moreno, J., Esteso, M., Villaverde-Morcillo, S., Toledano-Díaz, A., Castaño, C., Velázquez, R., Martínez, J. 2016. Recent advances in bird sperm morphometric analysis, and its role in male gamete characterization and reproduction technologies. *Asian J Androl*, 18 (6): 882–888.

Saragusty, J., Arav, A. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*. https://doi.org/10.1530/REP-10-0236

Sasaki, K., Tatsumi, T., Niinomi, T., Imai, T., Naito, M., Tajima, A., & Nishi, Y. 2010. A method for cryopreserving semen from Yakido roosters using N-Methylacetamide as a cryoprotective agent. *J Poult Sci*, 47: 297–301.

Sawada, Y., Chang, M.C. 1964. Motility and fertilizing capacity of rabbit spermatozoa after freezing in a medium containing dimethylsulfoxide. *Fertil Steril*, 15, 222–229.

Seigneurin, F., Grasseau, I., Chapuis, H., Blesbois, E. 2013. An efficient method of guinea fowl sperm cryopreservation. *Poult Sci*, 92 :2988–2996.

Sexton, T.J. 1979. Preservation of poultry semen: a review. Pages 159-170 In H. W. Hawk (ed.) Animal Reproduction, Beltsville Agr. Res. Center Symp.

Sexton, T.J. 1981. Development of a commercial method for freezing turkey semen: 1. Effect of prefreeze techniques on the fertility of frozen and frozen-thawed semen. *Poult Sci*, 60: 1567–1572.

Shahverdi, A., Sharafi, M., Gourabi, H., Amiri Yekta, A., Esmaeili, V., Sharbatoghli, M., Janzamin, E., Hajnasrollahi, M., Mostafayi, F. 2015. Fertility and flow cytometric evaluations of frozen-thawed rooster semen in cryopreservation medium containing low-density lipoprotein. *Theriogenology*, 83: 78–85.

Si, W., Hildebrandt, T.B., Reid, C., Krieg, R., Ji, W., Fassbender, M., Hermes, R. 2006. The successful double cryopreservation of rabbit (Orictolagus cuniculus) semen in large volume using the directional freezing technique with reduced concentration of cryoprotectant. *Theriogenology*, 65: 788–798.

Silva, C.G., Cunha, E.R., Blume G.R., Malaquias, J.V., Báo, S.N., Martins, C.F. 2015. Cryopreservation of boar sperm comparing different cryoprotectants associated in media based on powdered coconut water, lactose and trehalose. *Cryobiology*, 70: 90–94.

Siudzińska, A., Łukaszewicz, E. 2008. The effect of breed on freezability of semen of fancy fowl. *Anim Sci P*, 26 (4): 331–340.

Smith, A.U., Polge, C. 1950. Survival of spermatozoa at low temperatures. *Nature* 166, 668–669.

Smyth, J.R. 1968. Poultry. Pages 258-300 In E. J. Perry (ed.). The Artificial Insemination of Farm Animals. Rutgers University Press, New Brunswick, N. J.

Song, Y., Silversides, F.G. 2007b. Production of offspring from cryopreserved chicken testicular tissue. *Poult Sci*, 86: 1390–1396.

Stranzinger, G.F., Maurer, R.R., Paufler, S.K. 1971. Fertility of frozen rabbit semen. *J Reprod. Fertil*, 24: 111–113.

Srurkie P.D. Avian physiology Springer, 1986.

Szalay, I. 2004. A régi magyar baromfifajták tenyésztése és génvédelme a KÁTKI-ban. [Breeding and conservation of old Hungarian poultry breeds in KATKI. In Hungarian]. A Baromfi 7(3): 22–25.

Sztein, J.M., Noble, K., Farley, J.S, Mobraaten, L.E. 2001. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. *Cryobiology*, 42: 28–39.

Szumowski, P., Theret, M., Denis, B. 1976. Semen and artificial insemination of pigeons. Proc. VlIIth Int. Congr. Anim. Reprod. Artificial Insemination 256.

Suc, N.Q., Binh, D.V., Ha, L.T.T., Preston, T.R. 1996: Effect of housing system (cage versus underground shelter) on performance of rabbits on farms. *LRRD*, 8: Article #34.

Surai, P.F., Blesbois, E., Grasseau, I, Chalah, T., Brillard, J.P., Wishart, G.J., Cerolini, S., Sparks, N.H.C. 1998. Fatty acid composition, glutathione peroxidase and superoxide dismutase activity and total antioxidant activity of avian semen. *Comp Biochem Physiol*, Part B 120: 527–533.

Surai, P.F., 1999. Vitamin E in avian reproduction. *Poult Avian Biol Rev*, 10: 1–60.

Swain, J., Smith, G. 2010. Cryoprotectants. In R. Chian & P. Quinn (Eds.), Fertility Cryopreservation (pp. 24-38). Cambridge: Cambridge University Press.

Tacconi, L. 2000. Biodiversity and Ecological economics: participation, value and resource management. Earth scan publication. Ltd London.

Tajima A. 2013. Conservation of avian genetic diversity. *Jpn Poultry Sci*, 50: 1–8.
Theau-Clément, M., Poujardieu, B., Tartié, V., Bencheikh, N., Mercier, P., Bellereaud, J. 1996. Utilisation de semence congelée pour l'insemination artificielle. *Cuniculture*, 131: 222–226.

Thuy, L.T., Vang, N.D., Giao, H.K 2013. Farm animal genetic resources and their conservation in Vietnam.

Váradi, E., Végi, B., Liptói, K., Barna, J. 2013. Methods for cryopreservation of guinea fowl sperm. *PLOS one*, 8 (4): e62759.

Vasconcelos, A.C.N., Felizardo, V.O., de Carvalho, A.F.S., Garcia, R.R.F, Ramos, S.E., Murgas, L.D.S. 2015. Cryopreservation of *Prochilodus lineatus* semen: effect of cryoprotectants combination, *Bol Inst Pesca São Paulo*, 41(esp.): 817–824.

Vašíčková, K., Ondruška, Ľ., Baláži, A., Parkányi, V., & Vašíček, D. (n.d.). Genetic characterization of Nitra rabbits and zobor rabbits. *Slovak J Anim Sci*, 49 (3): 104–111.

Vicente J.S., Viudes de Castro M.P. 1996. A sucrose-DMSO extender for freezing rabbit semen. *Reprod Nutr Dev*, 36: 485–492.

Viudes de Castro M.P., Vicente J.S. 1997. Effect of sperm count on the fertility and prolificity rates of meat rabbits. *Anim Repr Sci*, 46: 313–319.

Viudes de Castro M.P., Vicente J.S., Lavara R. 1999. Effet du nombe de spermatozoïdes sur la fertilité de la semence conservée 24 heures chez le lapin. *Ann Zootech*, 48: 407–412.

Wales, R.G., O'Shea, T. 1968. The deep freezing of rabbit spermatozoa. Aust. J. Biol. Sci. 21, 831–833.

Watson, P.F. 1990. Artificial insemination and the preservation of semen. In: Lamming G.E. (Ed.), Marshall's Physiology of Reproduction. Vol. 2. Reproduction in the Male. Churchill Livingstone Editorial, Edinburgh, pp. 747–869.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*, 7: 871–891.

Weigend, S., Romanov, M.N. 2001. Current strategies for the assessment and evaluation of genetic diversity in chicken resources. World Poult Sci J. 57. 282–288.

Weitze, K.F., Helleman, C., Krause, D. 1976. Insemination with rabbit semen frozen in plastic straws. In: Tischner, Pilch, J. (Eds.), Proceedings of the 8th Intl. Congr. Anim. Reprod. A.I., vol. 4, pp. 1100–1103.

Whittingham, D.G, Leibo, S.P., Mazur, P. 1972. Survival of mouse embryos frozen to -196° and -269°C. *Science*, 178: 411–414.

Wishart, G.J. 2009. Semen quality and semen storage. In: Biology of breeding poultry. Poultry Science Symposium Series, Hocking P. (ed.). London, UK, pp. 151–178.

Woelders, H., Zuidberg, C.A., Hiemstra, S.J. 2006. Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. *Poult Sci*, 85: 216–222.

Wowk, B., Darwin, M., Harris, S.B., Russell, S.R., Rasch, C.M. 1999. Effects of solute methoxylation on glass-forming ability and stability of vitrification solutions. *Cryobiology*, 39 (3): 215–227.

Wright, S. 1931. Evolution in Mendelian populations. *Genetics*, 16: 97–159.

Yeoman, R.R, Gerami-Naini, B., Mitalipov, S., Nusser K.D., Widmann-Browning A.A, Wolf D.P. 2001. Cryoloop vitrification yields superior survival of Rhesus monkey balstocysts. *Human reproduction*, 16 (9): 1965–1969.

Zanetti, E., De Marchi, M., Dalvit, C., Cassandro, M. 2010. Genetic Characterization of Local Italian Breeds of Chickens Undergoing In-Situ Conservation. J Poultry Sci, 89: 420–427.

Zaniboni, L., Cassinelli, C., Mangiagalli, M.G., Gliozzi, T.M., Cerolini, S. 2014. Pellet cryopreservation for chicken semen: Effects of sperm working concentration, cryoprotectant concentration, and equilibration time during in vitro processing. *Theriogenology*, 82: 251−258.

Zaniboni, L., Cerolini, S. 2008. L'apparato riproduttore maschile. In: Avicoltura e Coniglicoltura. Editors Cerolini S., Marzoni M., Romboli I., Schiavone A., Zaniboni L, Le Point Veterinaire Italie, Milano: 93−105.

Zaniboni, L., Cerolini, S. 2009. Liquid storage of turkey semen: Changes in quality parameters, lipid composition and susceptibility to induced in vitro peroxidation in control, n-3 fatty acids and alpha-tocopherol rich spermatozoa. *Anim Reprod Sci*, 112: 51–65.

Zanon, A., Sabbioni, A. 2001. Identificazione e salvaguardia genetica delle razze avicole italiane. *Annali Facoltà Medicina Veterinaria di Parma*, 21: 117–134.

Zeferino, C.P., Moura A.S.A.M.T., Fernandes, S., Kanayama, J.S., Scapinello, C., Sartori, J.R. 2011. Genetic group×ambient temperature interaction effects on physiological responses and growth performance of rabbits. Livest Sci, 140: 177–183.

Zhou, Y., Fu, X., Zhou, G., Jia, B., Fang, Y., Hou, Y, Zhu, S. 2014.An efficient method for the sanitary vitrification of bovine oocytes in straws. *J Anim Sci Biotechno*, 5:19.

Zhu, Z., Fan, X., Lv, Y., Zhang, N., Chuning, F., Pengfei, Z., Wenxian, Z. 2015. Vitamin E Analogue Improves Rabbit Sperm Quality during the Process of Cryopreservation through Its Antioxidative Action. *PLoS ONE*, 10(12): e0145383.

Zhu, Z., Wenxian, Z., Xiaoteng, F., Yang, P., et al. 2017. Trehalose improves rabbit sperm quality during cryopreservation. *Cryobiology*, 75: 45–51.

Zindl, C. Cryopreservation of Mexican gray wolf (Canis lupus baileyi) semen - evaluation of different times and rates of pre-freeze cooling and Equex pasta-supplementation - in comparison with semen of the domestic dog and generic gray wolf (Canis lupus).PhD thesis, durch die Tierärztliche Hochschule Hannover, 2006.

Zubair, M., Lodhi, L.A., Ahmad, E., Muhammad, G. 2013. Hypo osmotic swelling test as screening for evaluation of semen of bull. Journal of Entomology and Zoology Studies. *JEZS*, 124(16), 124–128.

## List of figures

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| ABBREVIATION | MEANING |
|---|---|
| **AI** | artificial insemination |
| **ALH** | amplitude of lateral head displacement |
| **ANCI** | Italian rabbit breeders association |
| **ANGRs** | Animal genetic resources |
| **ANOVA** | analysis of variance |
| **AO** | Acridine Orange |
| **ATP** | adenosine triphosphate |
| **BCF** | beat cross frequency |
| **Calcein-AM** | acetomethoxy derivate of calcein |
| **Carboxy-SNARF** | carboxy-seminaphthorhodafluor |
| **CASA** | computer assisted semen analysis |
| **CFDA** | carboxyfluorescein diacetate |
| **COMET** | single-cell DNA gel electrophoresis assay |
| **CPA** | cryoprotectant |
| **EthD-1** | ethidium homodimer |
| **P-CPA** | Permeating cryoprotectant |
| **N-CPA** | Non permeating cryoprotectant |
| **DAPI** | 4,6-diamidino-2-phenylindole |
| **DMA** | dimethylacetamide |
| **DMF** | Dimethylformamide |
| **DMSO** | dimethylsulfoxide |
| **DNA** | deoxyribonucleic acid |
| **ds** | double strand |
| **dUTP** | deoxy nucleotidyl transferase |
| **EG** | ethylene glycole |
| **FC** | Flow cytometry |
| **FITC** | fluorescein isothiocyanate |
| **FPM** | Forward progressive motility |
| **GLM** | general linear model |
| **HOST** | Hypoosmotic swelling test |
| **ICSI** | intracytoplasmic sperm injection |
| **IVF** | in vitro fertilization |
| **LIN** | linearity of the curvilinear path |
| **LN2** | liquid nitrogen |
| **MA** | N-Methylacetamide |
| **MF** | Methylformamide |
| **mmt** | Million metric tonnes |
| **n-6** | Omega-6 |
| **n-9** | Omega-9 |
| **PEG** | Polyethylen glycol |
| **PGCs** | Primordial germ cells |
| **PI** | Propidium iodide |

| | |
|---|---|
| **PS** | Phosphatydil serine |
| **PSA** | Pisum sativum agglutinin |
| **PNA** | peanut agglutinin from Arachis hypogaea |
| **PUFA** | polyunsaturated fatty acids |
| **PVP** | Polyvinilpirrolidone |
| **STR** | straightness of the average path |
| **TEM** | transmission electron mycroscope |
| **TM** | Total motility |
| **VAP** | average path velocity |
| **VCL** | curvilinear velocity |
| **VSL** | straight line velocity |

## LIST OF PUBLICATIONS

### *Publications at Conference/Congress*

Nicolaia Iaffaldano, Michele Di Iorio, **Marsia Miranda**, Mario Petrecca, Silvia Cerolini, Pier Paolo Gibertoni, Stefano Esposito. Effect of equilibration time for semen cryopreservation of endangered Mediterranean brown trout (Salmo cettii) inhabiting the Biferno river (Molise region, South Italy). *Ital J Anim Sci vol.16:s1, 2017*

Nicolaia Iaffaldano, Michele Di Iorio, **Marsia Miranda**, Paola Principe, Michele Schiavitto, Angelo Manchisi, Silvia Cerolini. Comparison of two basic extenders on the in vitro post-thaw quality of rabbit semen. *Ital J Anim Sci vol.16:s1, 2017*

### *Publications in International Journals with I.F.*

N. Iaffaldano, M. Di Iorio, **M. Miranda,** L. Zaniboni, A. Manchisi & S. Cerolini (2016). Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. *British Poultry Science*, 57:2, 264-270, DOI: 10.1080/00071668.2016.1148261

**M. Miranda,** B. Kulíková, J. Vašíček, L. Olexiková, N. Iaffaldano, P. Chrenek. (2018). Effect of cryoprotectants and thawing temperatures on chicken sperm quality. *Reprod Dom Anim*. 53(1): 93–100, DOI: 10.1111/rda.13070.

M. Di Iorio, M.A. Colonna, **M. Miranda,** P. Principe, M. Schiavitto, S. Cerolini, A. Manchisi, N.Iaffaldano. (2017). The initial cooling time before freezing affects the post-thaw quality and reproductive performance of rabbit semen. *Animal Science Journal*, submitted.