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REVIEW

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Italian semen cryobank of autochthonous chicken and turkey breeds: a tool for preserving genetic biodiversity

Nicolaia Iaffaldano[a], Michele Di Iorio[a], Giusy Rusco[a], Emanuele Antenucci[a], Luisa Zaniboni[b], Manuela Madeddu[b], Stefano Marelli[b], Achille Schiavone[c], Dominga Soglia[c], Arianna Buccioni[d], Martino Cassandro[e], Cesare Castellini[f], Margherita Marzoni[g] and Silvia Cerolini[b]

[a]Dipartimento Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italia; [b]Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, via dell'Università 6, Lodi, Italia; [c]Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, Grugliasco, Italia; [d]Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali, Università degli Studi di Firenze, Firenze, Italia; [e]Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Università degli Studi di Padova, Viale dell'Università 16, Legnaro, Italia; [f]Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi di Perugia, Perugia, Italia; [g]Dipartimento di Scienze Veterinarie, Università-di Pisa, Pisa, Italia

ABSTRACT

The creation of genetic resource cryobanks provides a crucial link between *in situ* and *ex situ* techniques to improve the efficiency of conservation programs. Aim of the present review is to describe all the activities developed for the implementation of the first Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds. These activities can be classified into three main topics: (1) identification of species-specific semen freezing/thawing reference procedures; (2) drafting Standard Operative Procedures (SOP) for the implementation of the semen cryobank; (3) storage of semen doses from Italian chicken and turkey breeds to establish the cryobank. Several trials have been developed to identify a specie-specific semen cryopreservation protocol for chickens and turkeys. The major results are reviewed and a final reference protocol described. Taking into consideration the FAO guidelines for cryoconservation of animal genetic resources, SOP were drafted with the aim to provide technical guidance and logistical support on the choice of priority breeds, selection of birds for semen production, infrastructures and storage sites, birds and semen management, cryopreservation process and doses traceability. Lastly, the Italian Semen Cryobank was created. A total of 112 semen doses from 22 cockerels of three breeds, and 74 doses from 12 turkey males of three breeds were stored in the Cryobank. Breed specific semen quality parameters assessed before and after cryopreservation are reported. The described activities provide information and tools useful for the implementation of semen cryobanking in avian species and might be transferred also to other species after appropriate adaptations.

HIGHLIGHTS

- Implementation of the first Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds
- Drafting Standard Operative Procedures provides technical guidance and logistical support on the design and establishment of the cryobank
- Semen cryobank is a precious genetic reservoir and could be useful to safeguard genetic variability in small population *in vivo* conserved

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Introduction

In the last few decades, in Italy as well as in the rest of the world, the poultry sector has suffered a significant loss in terms of animal genetic resources (AnGR) and a progressive erosion of many native genotypes due to the massive use of high-performing commercial hybrids (Delany 2004; Fulton 2006). The evolution of intensive farming has threatened the survival of local chicken and turkey breeds; in fact, the populations of the native breeds are reared in very small numbers and may suffer from inbreeding and loss in genetic diversity (Zanon and Sabbioni 2001; Castillo et al. 2021). Safeguarding poultry biodiversity is a key objective in every developed country; almost no data are available on the wide avian genetic resources

CONTACT Dr. Michele Di Iorio michele.diiorio@unimol.it Dipartimento Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italia

reared in Italy and the urgent need of specific conservation programs for Italian poultry breeds is well recognised (Bittante 2011; Ozdemir et al. 2013). In this context, the project 'Conservation of biodiversity in Italian poultry breeds – TuBAvI' (www.pollitaliani.it) was developed from 2017 to 2020 with the financial support of Ministero delle Politiche Agricole Alimentari e Forestali (MIPAAF) and Fondo Europeo Agricolo per lo Sviluppo Rurale (FEASR). The TuBAvI project aimed to promote and support the conservation of the Italian poultry genetic resources providing new data and tools for the implementation of a wide comprehensive national conservation program.

Conservation techniques can be divided into *in situ* and *ex situ,* and the latter can be further divided into *in vivo* and *in vitro* (FAO 2012; Leroy et al. 2019). The *in situ* technique involves the maintenance of the living animals within the livestock production system in which they were developed, whilst the *ex situ in vivo* involves the maintenance of the living animals outside their original area. The *ex situ in vitro* technique involves the cryopreservation of genetic material in haploid form (semen and oocytes), diploid (embryos, somatic cells) or DNA sequences (FAO 2012; Mara et al. 2013). Cryopreserved material is usually stored in specific facilities defined as gene banks or cryobanks, which are generally maintained by universities, research centres, government or private companies (FAO 2012; Leroy et al. 2019). The *in situ* conservation technique has clear priority, even because the animals continue to evolve in their original habitat; however, this approach is often too expensive as it requires dedicated infrastructures and proper management (Patterson and Silversides 2003; Prentice and Anzar 2011). Therefore, the development of the *ex situ in vitro* technique to be used as a support of the *in vivo* one is ever-increasing. The creation of genetic resource cryobanks would provide a crucial link between both techniques to improve the efficiency of conservation programs (Prentice and Anzar 2011).

In birds, the *ex situ in vitro* technique can rely only on semen cryopreservation, being the most suitable reproductive technology currently available for long storage of genetic resources (Long 2006; Blesbois 2011; Ehling et al. 2012). The cryopreservation of intact oocytes or embryos is not possible because of the characteristics of the megalecithal eggs (Long 2006; Blesbois et al. 2007; Blesbois 2011; Ehling et al. 2012). Technologies for the cryopreservation of gonadic tissues and primordial germ cells have been also developed during the last decade (Silversides et al. 2012; Liptoi et al. 2013; Nakamura et al. 2013; Nakamura 2016; Sztan et al. 2017). However, they are still very expensive and highly invasive compared to semen cryopreservation (Nakamura 2016; Nandi et al. 2016).

In some European countries (France, Spain, The Netherlands, Hungary, Slovak Republic) and in United States of America, several conservation programs of AnGR have been developed and a national semen cryobank of local chicken breeds and/or specific genetic lines is currently being created (Szalay 2004; Woelders et al. 2006; Blesbois et al. 2007; Blackburn 2009; Santiago-Moreno et al. 2011). Meanwhile in Italy, conservation projects for the safeguard of native poultry breeds have been based only on the *in situ* technique and have been developed with the support of regional and local public institutions. For the first time, TuBAvI project allowed to develop common conservation activities cohordinated at national level, including the planning and implementation of the first Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds. The Cryobank is mainly aimed to support the management of populations *in vivo* conserved and the potential reconstruction of breeds in case of extinction or drastic reduction of their population size. The aim of the present report is to describe the activities developed and the rules adopted for the creation of the Cryobank: (1) identification of freezing/thawing protocols for chicken and turkey semen; (2) drafting the Standard Operative Procedures (SOP) for the management of birds and semen from collection to storage; (3) storage in liquid nitrogen of semen doses in Italian chicken and turkey breeds.

Identification of the semen cryopreservation protocol in the *Gallus gallus* and *Meleagris gallopavo* species

The fundamental assumption for the realisation of a poultry semen Cryobank is the development of a successful freezing protocol. Even today, semen cryopreservation is still responsible for a severe loss in sperm integrity in the majority of domestic animals and the improvement of sperm cryosurvival and fertility after artificial insemination (AI) of frozen/thawed sperm is still the working focus in semen cryobanking for most mammalian and avian species. So far, the establishment of a sperm Cryobank for conservation of avian genetic resources has been usually associated with research trials to identify the most suitable freezing/thawing protocol able to preserve sperm integrity and fertilising ability (Woelders et al. 2006; Blesbois

et al. 2007; Santiago-Moreno et al. 2011; Thélie et al. 2019).

In order to identify a reference freezing/thawing protocol for chicken and turkey semen several trials have been developed to study the main factors involved in sperm cryosurvival: extenders, dilution rates, cryoprotectants (CPAs) and their concentration, freezing and thawing rates.

Although, chicken and turkey sperm share the same morphology i.e. both have peculiar physiological features such as a filiform shape, a long tail and a condensed nucleus, (Donoghue and Wishart 2000; Blesbois et al. 2005), however, the turkey sperm are generally recognised to be much more sensitive to freezing/thawing (Iaffaldano, et al. 2009; Di Iorio, Cerolini, et al. 2016; Iaffaldano, Di Iorio, Miranda, et al. 2016).

Specie-specific *in vitro* processing conditions have been tested and different protocols were finally identified according to the species.

Cryopreservation protocol for chicken semen – DiMethylacetamide (DM) is a permeant cryoprotectant successfully used for the cryopreservation of chicken semen packaged in pellets (Chalah et al. 1999; Tselutin et al. 1999; Zaniboni et al. 2014) and was also considered for straw packaging, according to FAO safety guidelines recommended in semen cryobanking (FAO 2012). Chicken semen packaged into straws was frozen in nitrogen vapours using very simple unexpensive floating racks, and the most suitable temperature gradient during freezing was studied. Rapid freezing obtained by exposure of straws to vapours 3 cm above the liquid nitrogen bath allowed to reach −40 °C within 1 min and was confirmed to be the most suitable gradient for freezing chicken semen packaged into straws (Madeddu et al. 2016). The cryoprotective action of DM was further improved in association with the non-permeant cryoprotectant trehalose, not with sucrose, playing a synergic action with DM able to improve sperm kinetic parameters and the recovery of progressive motile sperm from 15% to 24% after freezing/thawing (Mosca et al. 2016a). If combined with trehalose, DM concentration was reduced from 6% to 3% with no negative effect on sperm viability (32%), motility (24%) and progressive motility (1.4%) after freezing/thawing; however, trehalose alone did not provide an adequate cryoprotection and could not completely replace DM (Mosca et al. 2016b).

The research activities developed during the TuBAvI project have been focussed on the action of the CPAs on both sperm integrity, assessed *in vitro*, and embryo viability, assessed *in vivo*. DM was compared with N-Methylacetamide (NM), a new permeant cryoprotectant for chicken semen used with excellent fertility (77–84%) and hatchability (90%) results in the Yakido rare breed (Sasaki et al. 2010). Different DM and NM concentrations, from 2% to 9%, were tested and compared in the same trials. Both cryoprotectants showed a similar cryoprotective action on sperm integrity and function according a clear concentration dependent positive effect within the range 2–6% final concentration: sperm membrane integrity, motility and progressive motility recorded in frozen/thawed semen in presence of 6% CPA were 39%, 53% and 11% respectively (Zaniboni et al. 2021). A further increase to 9% CPA concentration was associated with a further improvement in sperm membrane integrity with DM, not NM (Mosca et al. 2019, 2020). In contrast, semen cryopreserved with DM and NM provided different results after artificial insemination according to the CPA. Despite similar fertility rates, the occurrence of embryo development was confirmed with NM-treated semen, not DMA, in following trials (Mosca et al. 2019; Zaniboni et al. 2021). In presence of NM, a negative relation between CPA concentration and fertility was found, being the highest fertility rate (9%) recorded with 2% NM, whereas the proportion of viable embryos/fertile eggs (recorded on day 7 of incubation) was not affected by CPA concentration and 49% overall mean was recorded (Zaniboni et al. 2021). In contrast in presence of DM, fertility and embryo viability were positively affected by the CPA concentration and the highest proportion of fertile eggs (9%) and viable embryos (47% on fertile eggs) required the 6% DM (Zaniboni et al. 2021). The results suggest a higher protective action, or a higher permeability to sperm membrane, of NM compared to DM, being able to provide fertilisation and embryo development at lower concentration.

The cryoprotective action of NM on sperm integrity and motility was also affected by the thawing temperature. Cold thawing, corresponding to 5 °C for 100 s, was less harmful compared with warm thawing, corresponding to 38 °C for 10 s, and the proportions of sperm with undamaged plasma membrane, motile and progressive motile sperm in frozen/thawed samples were 51%, 52% and 11% respectively (Mosca et al. 2020).

The concentration of the insemination dose of cryopreserved chicken semen was also studied to identify the lowest dose able to provide fertile eggs and viable embryos in order to optimise the use of frozen/thawed semen in artificial insemination protocols. A single insemination of 250, 500 and 750 $\times 10^6$ frozen/

thawed sperm provided a similar proportion of viable embryos (average 16% on egg set) from day 2 to day 4 after insemination, irrespective of the concentration dose. However, longer fertile period was recorded with 500 and 750×10^6 insemination dose of frozen/thawed sperm (Cerolini et al. 2019). The insemination of 250×10^6 sperm/dose is suggested in artificial insemination protocols with cryopreserved semen in order to optimise semen management and obtain maximum fertility with the lowest dose.

The following cryopreservation protocol was finally identified for *in vitro* processing of chicken semen:

- Dilution to 1.5×10^9 sperm/mL with Modified pre-Freezing Lake (MFL) diluent (Mosca et al. 2016a);
- Cooling at 4 °C for 20 min;
- Dilution at 1.0×10^9 sperm/mL with MFL diluent added with NM 2% final concentration;
- Equilibrium at 4 °C for 1 min;
- Packaging into straws (0.25 mL): 250×10^6 sperm/straw;
- Freezing by exposure of straws 3 cm above liquid nitrogen bath for 10 min;
- Transfer and storage of straws into liquid nitrogen cryotank;
- Thawing in a thermostatically controlled bath at 5 °C for 100 s.

Cryopreservation protocol for turkey semen - A promising freezing protocol for turkey semen was identified by testing different critical steps of *in vitro* processing, such as the choice of the permeant CPA and its concentration, and the freezing and thawing rates. The best protocol identified involved the use of 10% DMSO, semen freezing by exposure of straws at 10 cm above a liquid nitrogen bath and thawing at 50 °C for 10 s (Iaffaldano, Di Iorio, Miranda, et al. 2016). However, despite the protocol provided encouraging results, corresponding to 37% motility, 42% viability and 25% osmotic tolerance in frozen/thawed semen samples, further efforts were needed in order to further improve and standardise the cryopreservation protocol to be implemented in the Cryobank.

The research activities developed within the TuBAvI project were aimed to study the action of two dilution rates (1:2 and 1:4) and the effects of three non-permeant-cryoprotectants (sucrose, trehalose and Ficoll 70) at four different concentrations in combination with DMSO on *in vitro* post-thaw semen quality (Di Iorio, Rusco, Iampietro, Colonna, et al. 2020). The rationale of this study was to test the beneficial effect of the combined use between permeant and non-permeant cryoprotectants, according to literature reports (Blanco et al. 2011; Iaffaldano, Di Iorio, Cerolini, et al. 2016; Mosca et al. 2016a; Miranda et al. 2018). Among all combinations tested the best results in terms of sperm cryosurvival (32.6% total motility; 3.5% progressive motility; 42.5% membrane integrity; 24.4% osmotic resistance and 97.9% DNA integrity) were recorded with the use of 1 mM Ficoll and the 1:4 dilution rate. In order to validate the most effective freezing protocol, identified by *in vitro* assessments of semen quality, frozen/thawed semen was also used for artificial insemination to assess *in vivo* fertility and hatchability (Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020). Different concentrations of the insemination dose were tested in female turkey breeders showing a clear effect on fertility and hatching rates.

Hens were inseminated both using fresh and frozen semen twice every 7 days. The best results of fertility and hatchability (inseminating dose of 400×10^6 sperm/hen) were 87% and 71% respectively using frozen semen compared to 90.8 and 75.6% respectively using fresh semen (Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020).

The following cryopreservation protocol was finally identified for *in vitro* processing of turkey semen:

- Pre-dilution to 6×10^9 sperm/mL with Lake diluent (Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020);
- Cooling to 4 °C for 25 min;
- Dilution 1:1 (v:v) at 3×10^9 sperm/mL with freezing Lake extender + 20% DMSO + 1 mM Ficoll;
- Packaging in straws (0.25 mL): 750×10^6 sperm/straw;
- Equilibration at 4 °C for 20 min;
- Freezing by exposure of straws 10 cm above liquid nitrogen bath for 10 min;
- Transfer and storage of straws into liquid nitrogen cryotank;
- Thawing in a thermostatically controlled bath at 50 °C for 10 s.

Standard Operative Procedures (SOP) for the implementation of the Semen Cryobank

In order to realise the first Italian Semen Cryobank of autochthonous chicken and turkey breeds, Standard Operative Procedures have been discussed taking into consideration the FAO guidelines on the management of small populations and guidelines on animal genetic resources cryobanking (FAO 2009, 2012). The main aspects dealt with were: choice of priority breeds, selection and management of semen donor males,

semen management and cryopreservation processing, infrastructures and storage sites.

The breed priority for *ex situ in vitro* conservation is based on the knowledge of the status of the breed or breeding population's potentiality. However, very few data were available about genetic, breeding and farming characteristics of Italian poultry breeds. In order to fill this gap, TuBAvI project developed several activities for breed characterisation, including the census of Italian poultry breeds still reared in farming systems (Castillo et al. 2021; Franzoni et al. 2021) and the study of genetic diversity in the *in vivo* populations (Cendron et al. 2020; Soglia et al. 2021).

The TuBAvI census confirmed the presence of 18 chicken breeds and 7 turkey breeds in the different Italian regions of the Country (Castillo et al. 2021), corresponding to the large majority of the breeds included in the Poultry Breed Herd Book (www.anci-aia.it). However, the total population size was less than 500 birds in the majority of the breeds and was above 1000 birds in only 4 chicken breeds (Bionda piemontese, Livorno, Padovana, Polverara) (Castillo et al. 2021). In agreement with the 7 FAO risk categories, established according the overall population size, the number of breeding females and the trend in population size (FAO 2003), most of the Italian chicken and turkey breeds can be classified in the critical category and a small number in the endangered category. Therefore, all Italian chicken and turkey breeds are considered as priorities for *ex situ in vitro* germplasm conservation.

The study on genetic diversity between and within breeds revealed a large variation among breeds in the level of genomic inbreeding, investigated using Run of Homozygosity (ROH) data, and very few breeds showed a low level with $F_{ROH} < 0.1$ (Cendron et al. 2020). The average population molecular kinship was 53% and the mean inbreeding rate 56% with self-coancestry of 78% as a consequence of the reduced population size and genetic drift; the Livorno, Robusta Maculata, Robusta Lionata, Pepoi and Ermellinata di Rovigo breeds were recognised the most endangered populations (Soglia et al. 2021). The need to implement the *ex situ in vitro* technique to support *in vivo* breed conservation was then confirmed.

Semen donors will be selected according to different characteristics: (a) morphology; (b) genetic diversity; (c) health status; (d) semen quality. The morphological characterisation will be performed recording qualitative and quantitative traits, according to FAO guidelines for phenotypic characterisation (2012). Phenotypic traits has to refer to the breed standard, as reported in the Poultry Breed Herd Book, and birds carrying undesired traits will be discarded. In addition, male donors should have the lowest possible degree of kinship, assessed according pedigree records, if available. In breed populations where bird genotyping with microsatellite markers is planned to apply mating scheme, selection of birds with high individual genetic variability will be also considered. The health status of birds is of relevant importance in order to avoid the vertical transmission of diseases to the future generations. Semen donors have to be vaccinated against the most severe infective poultry diseases (Marek and Newcastle diseases), and tested to be free of the following diseases: mycoplasmosis (*M. gallisepticum, M. synoviae, M. meleagridis*), salmonellosis, pullorum disease/fowl typhoid (*S. pullorum and gallinarum*) and avian influenza.

The plan to implement the Cryobank is mainly aimed to support the management of populations *in vivo* conserved. According to FAO guidelines n. 12 (2012), from 3 to 20 birds within breed will be selected and at least 20 semen doses per bird will be stored; the number of semen doses per bird could be modified according to the number of semen donors available in order to collect almost 400 semen doses per breed. Semen doses will be collected from the majority of the Italian chicken and turkey breeds during the lifetime of TuBAvI-2 project.

Semen management is described in section 'Creation of the semen Cryobank'; the protocol for cryopreservation of chicken and turkey semen and the concentration dose of frozen/thawed semen for artificial insemination are described in section 'Introduction'.

According to FAO guidelines (2012), the cryobank could be constructed in existing infrastructures, to carry out all of the activities, such as: (1) collection of the semen, (2) semen processing and freezing and (3) sperm dose storage. It is necessary to have different buildings suitable for carrying out these activities although, having all infrastructures in the same place is not strictly necessary.

In most cases the facilities will belong to commercial organisations (AI centre, university, research centres, industry), but in specific cases they could be private facilities that belong to individual breeders or non-profit organisations (ERFP, 2003).

The semen collection must be performed in an animal holding facility while the semen evaluation and freezing occurs in a specific laboratory.

The infrastructure and expertise required for the creation of the Cryobank are provided by the University of Milan and the University of Molise, being

both TuBAvI partners, and the same universities will be also the storage sites of semen doses.

Creation of the semen Cryobank

Materials and methods

Data were processed according to descriptive statistics using the MS Excel software. Mean values and their variability (SD) are presented.

Birds management and semen collection

Birds from different breeds within the *Gallus gallus* and *Meleagris gallopavo* species have been used to start-up the Italian Semen Cryobank. The chicken breeds were: Bianca di Saluzzo (BS, $n = 18$), Biondа Piemontese (BP, $n = 25$), Mericanel della Brianza (MB, $n = 9$); the turkey breeds were: Romagnolo (RO, $n = 6$), Ermellinato di Rovigo (ER, $n = 2$), Bronzato Comune (BC, $n = 5$). Cockerels were housed indoor in individual cages (40×50 cm) in controlled environment at the Animal Production Centre, University of Milan (Lodi). Turkeys were raised in outdoor pens in a private breeding farm (Masseria Paglicci, Rignano Garganico, Foggia). All birds were fed *ad libitum* a standard commercial breeder diets (16% CP, 2800 kcal ME/kg) and drinking water. Birds were fasted before semen collection.

Semen was routinely collected twice weekly with the consolidated technique of the abdominal massage (Burrows and Quinn 1935), after a training period ranging from 2 to 4 weeks. A first macroscopic assessment of semen quality was performed soon after collection and only ejaculates with homogeneous white opalescent appearance and high viscosity were kept for further analyses and *in vitro* processing. The handling of animals and semen collection was conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU.

Semen quality assessment and cryopreservation

The quality of the ejaculates was assessed soon after collection by the measurement of volume, concentration, sperm membrane integrity (SMI), total motility (TM) and progressive motility (PM). Volume was measured with calibrated micropipette. Sperm concentration was measured after 1:200 dilution in 0.9% NaCl using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm (Brillard and McDaniel 1985). In the turkey, sperm membrane integrity (SMI) was assessed by means of the Muse® Cell Analyser (Luminex corporation, 12212 Technology Blvd Suite 130, Austin, TX 78727, United States) following the manufacturer's protocol. Semen samples were extended in PBS to reach a concentration ranging from 1×10^5 to 1×10^7 spermatozoa/mL. Then, 20 μL of this suspension was mixed with 780 μL (dilution factor 1:40) of Muse Count & Viability Kit® in an Eppendorf tube (Luminex corporation) and incubated for 5 min at room temperature in the dark. Subsequently, the sperm suspension was analysed by flow cytometry. Then, the Software Module performed calculations and displayed data in two dot plots: (1) nucleated cells; a membrane-permeant DNA staining dye that stained all cells that had a nucleus. This plots function is to identify cells with a nucleus from debris and non-nucleated cells. (2) Viability; a DNA-binding dye stains cells that had lost their membrane integrity and allowed the dye to stain the nucleus of dead and dying cells. This parameter discriminates viable (live cells that do not stain) from non-viable (dead or dying cells that stain). In the chicken, sperm membrane integrity was measured using the SYBR14/PI (propidium iodide) dual staining procedure (LIVE/DEAD SpermViability Kit, Molecular Probes, Invitrogen), as described by Iaffaldano et al. (2011) with minor modifications (Mosca et al. 2020). Total sperm motility (TM) and progressive motility (PM) were assessed by means of a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) using the Sperm Class Analyser (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Semen samples were diluted with 0.9% NaCl to 100×10^6/mL concentration, incubated for 5 min at 38 °C (turkey semen) or 20 min at room temperature (chicken semen) and then a semen aliquot was analysed under the microscope at 100× total magnification to record the proportion of motile (%) and progressive motile sperm (%).

After the quality assessment, ejaculates were processed for cryopreservation according to the protocol described in section 'Introduction'. Sperm membrane integrity, motility and progressive motility were measured in frozen/thawed semen also. After at least 7 days storage, semen was thawed according the procedure described in section 'Introduction' and quality parameters assessed as previously described.

An appropriate code system was adopted to guarantee the traceability of semen doses. The code system has originated as follows: each straw was given a code (ID) associated with male information (i.e. breed, age, origin, etc.); straws from each male were collected in the same cryo-goblet marked with the semen donor

Table 1. Quality of fresh semen (means ± SD) in chicken and turkey Italian breeds: Romagnolo (RO), Bronzato Comune (BC), Ermellinato di Rovigo (ER), Bianca di Saluzzo (BS), Bionda Piemontese (BP), Mericanel della Brianza (MB).

	Semen parameters*				
Breed	Vol	Conc	TM	PM	SMI
Gallus gallus					
BS	330 ± 110	2.89 ± 0.62	62.75 ± 17.18	13.45 ± 0.07	65.77 ± 1.05
BP	250 ± 170	3.77 ± 0.76	86.07 ± 12.17	19.05 ± 5.73	84.21 ± 11.56
MB	106 ± 13	2.54 ± 0.49	87.60 ± 7.19	26.60 ± 10.02	96.01 ± 2.62
Meleagris gallopavo					
RO	190 ± 88	5.81 ± 1.31	76.64 ± 6.24	21.47 ± 7.04	95.90 ± 1.03
BC	150 ± 50	5.51 ± 1.08	76.20 ± 8.10	22.31 ± 7.23	93.40 ± 1.42
ER	112 ± 18	6.27 ± 0.74	73.81 ± 0.52	15.35 ± 1.34	88.73 ± 3.31

*Vol: volume (μL); Conc: concentration (× 10^9/mL); TM: total motile sperm (%); PM: progressive motile sperm (%); SMI: sperm membrane integrity (%).

Table 2. Quality of frozen/thawed semen (means ± SD) in chicken and turkey Italian breeds: Romagnolo (RO), Bronzato Comune (BC), Ermellinato di Rovigo (ER), Bianca di Saluzzo (BS), Bionda Piemontese (BP), Mericanel della Brianza (MB).

	Semen parameters*		
Breed	TM	PM	SMI
Gallus gallus			
BS	17.45 ± 7.37	0.83 ± 1.83	15.55 ± 5.61
BP	16.98 ± 5.21	1.50 ± 1.83	17.24 ± 5.61
MB	30.70 ± 13.81	4.97 ± 2.38	33.55 ± 13.52
Meleagris gallopavo			
RO	22.38 ± 0.10	2.57 ± 1.38	51.50 ± 7.40
BC	23.72 ± 3.10	1.88 ± 0.68	44.33 ± 1.42
ER	14.64 ± 1.28	0.89 ± 0.31	37.38 ± 4.46

*TM: total motile sperm (%); PM: progressive motile sperm (%); SMI: sperm membrane integrity (%).

ID and the date of freezing. An excel database was created to organise all the data related to the stored semen doses.

Results

Training to semen collection was rather difficult in cockerels and ejaculation was absent or very poor in many birds. The males selected for semen production were only 7 (28% total males) in the BP breed, 6 (33% total males) in the BS breed and 6 (67% total males) in the MB breed. In contrast, toms were easily trained to semen collection and almost all birds have been used for semen production.

Semen quality parameters recorded in fresh semen of all breeds are reported in Table 1. In chicken breeds, mean ejaculate volumes and concentrations were within the standard range peculiar of the species (Marzoni 2008), with the exception of a low ejaculate volume in the MB breed, probably related to the small size of the birds, having a mean body weight of 900 g. TM, PM and SMI showed higher mean values in the ejaculates of BP and MB breeds compared to the ejaculates of BS breed. In turkey breeds, ejaculate volume was within the standard range values peculiar of the species, whereas lower sperm concentration was recorded (Marzoni 2008). Higher values of semen volume were found in the RO breed, whilst a higher sperm concentration was recorded in the ER breed. TM values were very similar in ejaculates of all turkeys. Similar values of PM and SMI were found in ejaculates of RO and BC breeds that resulted as lower in ER breed.

As expected, the semen cryopreservation process caused a significant deterioration in sperm quality, in agreement with our previous studies both in chickens (Madeddu et al. 2016; Mosca et al. 2016a, 2019) and turkeys (Iaffaldano, Di Iorio, Miranda, et al. 2016, Di Iorio, Rusco, Iampietro, Colonna, et al. 2020; Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020). In chickens, quality parameters recorded in frozen/thawed semen of BS and BP breeds were similar, even if the quality of fresh semen was different between breeds, whereas higher values in semen quality parameters were recorded in the MB breed (Table 2). Severe loss in SMI, TM and PM was observed in all chicken breeds and the most severe damage, corresponding to the loss of almost 90%, was recorded in the proportion of PM. In turkeys, a general loss of 70% of TM and over 90% of PM was observed (Table 2).

The census of the semen Cryobank, started within the TuBAvI project is shown in Table 3.

Discussion

In recent years, the interest in the conservation of animal biodiversity has intensified due to the risk of extinction of many native breeds, including in particular avian species. The awareness of the importance in conserving AnGR is demonstrated by the increasing numbers of conservation programmes developed in recent decades in Europe and worldwide (FAO 2015).

Gene banks represent the primary tool for conservation of AnGR according the *in vitro* technique. It has been estimated at global level that at least 128 countries have or are starting gene banks for preserving livestock genetic resources (FAO 2015). Paiva et al. (2016) reported that the global collection of AnGR probably exceeds 67,000 animals and about 4 million types of germplasm/tissue, and among these a consistent proportion is represented by semen cryobanks.

Semen cryobanking is aimed to several relevant purposes: (a) reconstruction of breeds in case of extinction because of catastrophic events (i.e. disease, climatic adversities), (b) as a back-up to quickly modify the selection process of populations and/or in case of

Table 3. Semen doses stored within the Italian Semen Cryobank of autochthonous chicken and turkey breeds at the University of Molise and Milan.

Species	Breeds	N° of donors	N° of doses
Gallus gallus	Bianca di Saluzzo	6	32
	Bionda Piemontese	7	67
	Mericanel della Brianza	9	13
Meleagris gallopavo	Romagnolo	5	50
	Bronzato	2	13
	Ermellinato di Rovigo	5	11

genetic problems in the *in vivo* conserved populations, (c) to increase effective population size and reduce genetic drift, (d) research investigations (Gandini and Oldenbroek 2007).

Moreover, semen cryobanks can also supply high quality and safe semen as reservoir of genetic traits of interest (performance, resistance to diseases and/or parasites, behavioural traits related to adaptability and welfare) to be introduced in commercial productions or reintroduced after their loss during selective procedures in hybrids' creation.

The semen cryobank realised within our project is yet in the embryonic phase, however we aim to obtain an open cryobank that could serve as a service for breeders by providing them with semen doses from males with a high genetic value and at the same time contributing to the control of inbreeding on farms.

In accordance to FAO guidelines (FAO 2012), when the realisation of a semen Cryobank is approached the following three principles should be considered: (1) to conserve small amounts of germplasm from many donor animals rather than large amounts from few donors; (2) to choose donors that are as genetically and phenotypically different in order to represent the genetic diversity of the population as much as possible; (3) to store the breeds as pure lines rather than gene pools to allow the use of the unique combinations of traits and the flexibility of stock combination. The Italian Semen Cryobank of autochthonous chicken and turkey breeds is going to be a precious genetic tank to support the management of *in vivo* conserved populations. Integrated live and cryopreserved schemes are aimed to re-establish the populations in case of physical disaster or genetic problems, and are highly recommended if the effective population size does not exceed 50 (Meuwissen 2017). Conservation programs of Italian poultry populations, characterised by high risk status, will take advantage of the potential application of integrated live and cryopreserved schemes.

Semen cryobanks result as more convenient in the long-term than *in vivo* conservation programmes. In the late 1990s, it was announced that gene banking was more expensive than maintaining *in vivo* populations (FAO 1998). However, it has been proven over a 20-years' time horizon that gene banks reduce the conservation costs of an avian population by over 90% compared to *in vivo* conservation (Silversides et al. 2012).

The main challenge for the creation of a sperm Cryobank was the identification of an effective cryopreservation protocol that is able to guarantee both at least 25–30% of live/motile spermatozoa after the freezing/thawing process and sperm fertilisation followed by embryo development. The identification of an effective freezing protocol for avian semen is particularly difficult because avian sperm are more sensitive to freezing damages than mammalian sperm, as a consequence of their unique morphological features. In fact, avian sperm are characterised by a filiform shape due to the cylindrical head not much wider than the tail, a more condensed nucleus, almost no cytoplasmic volume and a very long tail approximately eight times the head length (Donoghue and Wishart 2000; Long 2006). In birds, semen cryopreservation is not a standardised procedure and its success is still greatly variable and dependent on the species, the genetic types/breeds within the species and the *in vitro* processing.

Cryopreservation *in vitro* processing involves several steps and each one affects sperm structure and functions (Garner et al. 1999; Bailey et al. 2003; Iaffaldano, Di Iorio, Cerolini, et al. 2016; Iaffaldano, Di Iorio, Miranda, et al. 2016). Deleterious effects are the result of osmotic stress and temperature changes produced during cooling, freezing and rewarming, being ice crystal formation the main biophysical mechanism responsible for cell death (Swain and Smith 2010).

The specie-specific freezing/thawing protocols identified for the implementation of the Italian Semen Cryobank have been developed according to the results of several investigations aimed to study the effect on sperm quality and fertilising ability of many steps involved in *in vitro* processing: mainly the extender composition (Cerolini et al. 2007; Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020), the type and concentration of the permeant CPA (Iaffaldano, Di Iorio, Miranda, et al. 2016; Mosca et al. 2019; Zaniboni et al. 2021) and the addition of non-permeant CPA (Mosca et al. 2016a,b; Di Iorio, Rusco, Iampietro, Colonna, et al. 2020), the freezing and thawing rates (Madeddu et al. 2016; Iaffaldano, Di Iorio, Miranda, et al. 2016; Mosca et al. 2020). In the chicken, despite a high proportion of viable and motile sperm recovered after thawing (Mosca et al. 2020), a low

proportion of fertile eggs and viable embryos were recorded after AI of thawed semen (Mosca et al. 2019; Zaniboni et al. 2021). Fertility of chicken sperm cryopreserved in presence of NM is highly variable and the range 0–100% is reported according to the breed/line (Sasaki et al. 2010; Lee et al. 2012; Kim et al. 2014; Shanmungam et al. 2018; Pranay Kumar et al. 2018). The storage of germplasm from many different chicken breeds within the Italian Cryobank is of great scientific interest allowing a wide investigation on the relation between breeds and sperm sensitivity to cryopreservation that might provide new strategies to improve integrity and fertilising ability of frozen/thawed semen. In the turkey, high proportions of viable and motile sperm were recorded after thawing (Iaffaldano, Di Iorio, Miranda, et al. 2016; Di Iorio, Rusco, Iampietro, Colonna, et al. 2020) and also high proportions of fertile eggs and hatched birds after AI of thawed semen (Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020). For the first time, a successful cryopreservation protocol is proposed for semen cyobanking in the *Meleagris gallopavo* species and the positive results are expected to be confirmed in the Italian breeds. Very few data on fertility rate of frozen/thawed turkey semen packaged into straws were previously reported and reviewed (C¸iftci and Aygün 2018).

The draw up of SOP provided technical guidance and logistical support for the establishment of the Cryobank. The guidelines represents an handbook for the implementation of the semen Cryobank of Italian chicken and turkey breeds and the final official document will be published in the TuBAvI project website (www.pollitaliani.it). The SOP have been prepared following recommendations of the FAO Guidelines 'Cryoconservation of AnGR' (2012) that have been supplemented according the activities and results developed within TuBAvI project. The SOP identified for the Italian Semen Cryobank might provide interesting hints and support the development of semen cryobanking for poultry breeds by other universities, research centres or private companies.

A critical point emerged from the beginning of semen cryobanking was the limited number of birds available within breed populations. The results of the recent TuBAvI census showed that most of the Italian chicken breeds ($n = 10$) and all Italian turkey breeds ($n = 7$) have a total population of less than 500 birds (Castillo et al. 2021), and the size of the populations could be also more critical when referred only to breeding birds. The very small population size of Italian poultry breeds inevitably reduces the amount of genetic resources available for the selection of male breeder semen donors and, as a consequence, for semen storage in the Cryobank. Furthermore, the proportion of birds successfully trained to the handling required for semen collection was rather low in BS (33%) and BP (28%) chicken breeds, less critical in MB chicken breed and unexpectedly successful in turkey breeds. FAO guidelines (FAO 2012) suggests to collect semen from a minimum of 25 donors per breed and the required number of straws for the reconstitution process (five generations backcross) of a chicken breed was calculated to correspond to 513 (FAO 2012). However in critical conditions due to very small populations and/or limited budget, the recommendation is to collect as much germplasm as possible and collecting germplasm from fewer than 25 animals from each available breed may be preferred over collecting germplasm from 25 animals from a lower number of breeds.

According to FAO recommendations and the general high risk of biodiversity erosion in Italian poultry breed populations, the Italian Semen Cryobank of autochthonous chicken and turkey breeds will be developed storing germplasm of as much as possible breeds even if the number of birds within breed will be limited. The storage of semen doses from 15 chicken breeds and 4 turkey breeds, representing the large majority of the breeds included in the Italian Herd Book, has been planned in the next 3 years and birds will be preferably selected within the populations maintained within Public Poultry Conservation Centres.

Conclusions

The establishment of the first Italian Semen Cryobank of autochthonous chicken and turkey breeds represents a precious tool for the safeguard and conservation of biodiversity in the Italian poultry breed populations still present in farming systems. The Italian Semen Cryobank will allow the application of integrated *in vivo* and cryopreserved schemes to overcome genetic problems and prevent the extinction of breeds. The conservation of local breeds support the development of rural economies in marginal agricultural areas and niche markets for high-quality poultry products.

Efforts have been already planned to fully implement the Cryobank and store genetic resources from several different breeds. The plan includes the storage of semen doses from 15 chicken breeds and 4 turkey breeds representing the large majority of the breeds

included in the Italian Poultry Herd Book. The activities reported in the present paper for the establishment of the Italian avian Semen Cryobank represent general guidelines that might be transferred to similar initiatives in birds and/or in different domestic animals.

Ethical approval

The handling of animals and semen collection was conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The Datasets Used Are Available From The Corresponding Author On Reasonable Request.

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