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Conservation and valorisation of Italian chicken breeds

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To Marta and Baby

ABSTRACT

My research activities have been carried out within the CoVAL Project, a research program which examines the conservation of four Italian avian breeds (two chicken and two turkey breeds). The research has planned the development of several activities aiming the acquisition of a complete characterization of the breeds and the organization of a conservation program according FAO guidelines. Two conservation strategies have been considered: the primary *in situ* – that enables the conservation of the birds in their original background- and the complementary *ex situ in vitro* – with the aim to implement a semen cryobank. The project, funded by Regione Lombardia, lasted forty-two months, from December 2011 to June 2015.

The results reported in this thesis only concern the two chicken breeds, in particular *Milanino* breed, that presents high meat-production potentialities. Data have been systematically gathered studying various generations of birds.

The phenotypic characterization has highlighted the reproductive performance (fertility, hatchability) typical of each breed. Furthermore, variability of these traits has been studied depending on factors such as age, family, weight and storage period of eggs: prospects of selections and improvement have been pointed out.

The results of zootechnical trials have provided a procedural guideline for outdoor extensive farming system specific for the chicken breeds. The guideline provides information about rearing density and dietary protein level during the growing period. In *Milanino* breed the slaughter performance and the quality of meat have been studied at two different age of slaughter (150 and 180 days old chickens). *Milanino* chickens have disclosed a high slaughter performance and the analysis of their meat has pointed out a high protein and a small lipid percentage, which suggest a high nutritional value. In general, the results achieved prove the potentiality of *Milanino* breed for meat production

The *ex situ in vitro* strategy has been devoted to the identification of a reference procedure for the cryopreservation of chicken semen according FAO guidelines. Several trials have been carried out to improve cryopreservation success; in short, several gradients of cooling rates and the combined effect of permeant and non-permeant cryoprotectants have been studied. The final reference procedure involves the use of a very high cooling gradient and the innovative combination between a permeating cryoprotectant, dimethylacetamide, and a non-permeating one, trehalose. The cryopreservation reference procedure has been used for the conservation of the semen of *Milanino* and *Mericanel della Brianza* breeds, pointing out a lower cell damage post thawing in *Milanino* breed.

INDEX

INTRODUCTION

Analysis of the national poultry farming

Poultry sector, in Italy, constitutes 22% of the gross output that can be sold in the whole farm animal production system and 8% of the whole agricultural division; sales volume is 5.6 billion Euros (UNAITALIA, 2014). In fact poultry industry represents an important zootechnical sector that has been becoming totally independent and self-sufficient, and highly specialised. Poultry production mainly occurs in three Italian regions: Lombardy, Veneto and Emilia Romagna, while consumption spreads uniformly throughout Italy. Poultry farming involves about 570 million birds every year; in 2014, poultry meat production was 1,261,200 tons and table egg production was more than 12 billion units (UNAITALIA, 2014).

Intensive poultry production is the outcome of an appropriate combination between strong genetic selection and accurate environmental control. The selection has been focusing on a few types of chicken strains highly improved for meat and egg production, which have been spread globally in all those Countries where intensive farming has been developing. On one hand, such situation caused a total dependence of the poultry sector from the global companies supplier of breeding stock, while on the other hand it greatly increased the genetic uniformity of the birds reared in the intensive farming system and, therefore, the sanitary risk peculiar of the poultry farms was also greatly increased (FAO, 2008). Furthermore, the potential reduction of genetic variability in the pure lines under selection for decades is considered a potential restriction in realizing future selection programs based on new/different traits. Poultry breeds have been totally removed from the productive sector because have been

rapidly overtaken by the highly productive strains; poultry breeds remained the interest of fancy breeders (Marelli, 2008).
According to UNAITALIA statistic (2014), the national output of poultry meat comes almost entirely from intensive farming (93% of animals) and only a small share (7%) is from alternative rural systems. In contrast, Sommi and Pignatelli (2011) pointed out alternative poultry production being more substantial, consisting in 16% of national output. However, even this production is based on commercial strains and not local breeds. French poultry sector has chosen a different strategy from Italian sector: diversifying the products since the 1960s, and so production alternative to standard broilers consists in 26% of national output (Fanatico and Born, 2002).

Agro-Zootechnical biodiversity

Charles Darwin, in his well-known final paragraph of his masterpiece, explains in a notable way the incredible result of more than 3.5 billion years of evolution (Coyne, 2009):

> *"Thus, from the war of nature, from famine and death, the most exalted object of which we are capable of conceiving, namely, the production of the higher animals, directly follows. There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."*

Actually biological evolution endlessly produces biodiversity - universally recognized as the keystone of the conservation of ecosystem features – meant as both services supplied and increasing resilience against disturbing occurrences (Odum and Barrett, 2005).
Since the 1990s, considered the decade dedicated to Natural Environment, United Nations have opted for many efforts

devoted to protection of species and natural habitats. Landmark of the efforts has been the "Convention on Biological Diversity" - signed in 1992 in Rio de Janeiro and then endorsed in 193 Countries- whose goals can be adopted for all living organisms, both domestic and wild (Davis, 2001). Agro-zootechnical biodiversity has been first in line in the Convention agenda, contributing to the supply of all of the four ecosystem services that can be contemplated (Odum and Barrett, 2005):

1) *supply (or provisioning) services*, regarding food got from domestic animals, in specific local and traditional products bonded to territory;
2) *control (or regulating) services*, among which FAO highlights sanitary risk reduction when locally high genetic variability in animal population is present;
3) *cultural services*, meant to recover marginal areas by using hardy breeds as well as value breeding farms from an environmental and touristic perspective;
4) *back-up (or supporting) services*, as nutrient recycling, soil formation and primary animal production in free-range and organic systems.

In Zootechnics, biodiversity is mostly related to diversity within the group of domestic breeds. Among 50,000 known species of birds and mammals, only about forty are categorized as domestic or domesticable, and only 5 (cows, goats, sheep, chickens, pigs) are bred all over the world. Of these species many breeds exist, together with subspecific groups that are not-easily classifiable and are identified as "animal populations separated from others of the same species for common inheritable traits" (Matassino, 2010).

These genetic resources form the group of "Domestic Animal Diversity" (DAD) which is essential for fulfilling the constant increase of the demand of animal products and agricultural services. Almost the half (46%) of global domestic diversity occurs in Europe, and Italy is one of the richest Countries in biodiversity in Europe and in the world. The existence of

complex and long-time farming tradition environments has produced, over the centuries, the selection of many breeds.
In the Countries in which intensive animal production systems prevail – especially in North America and Europe – about 40% of the breeds is at risk of extinction and 30% of breeds existing at the beginning of last century are considered permanently extinct (Hammond and Leitch, 1996). In Italy, since 1950s, we have been witnessing the dropping out of multi-purpose breeds, typical of local breeds, in favour of mono-purpose breeds, typical of selected international breeds. As a consequence of this change, the number of local breeds has been progressively decreasing and a severe reduction of the population size has been undergoing (FAO, 2008).
A vast genetic variability in farm animal species is essential both to diversify products and satisfy market demands, and to guarantee the development of the production systems, these days more and more facing environmental and sanitary problems. Furthermore, local breeds are the outcome of a long-lasting process of domestication and adaptation to the natural environment typical of a specific ecosystem, and also considered a socio-economic, cultural and ecological value (FAO, 2011).
The need for a worldwide strategy aimed to genetic resource preservation is determined by the global acknowledgment of the current threat of genetic erosion in domestic animal biodiversity (FAO, 2011). Animal biodiversity conservation is recognized as an essential action and it has been widely promoted by many international Organizations. FAO has been fostering various actions since 1980 and in 2007 it finalised the DAD-IS (Domestic Animal Diversity Information System); in 1992 UN ratified the importance of genetic animal resources in Agenda 21 and in CBD (Convention on Biological Diversity); European Union has funded the EFABIS project (European Farm Animal Biodiversity Information System) aimed to merge the existing databases contributing to DAD-IS development in its present structure. The year 2010 has been declared 'international biodiversity year'

by UN and the focus was on making public opinion aware and promoting governmental commitments globally and locally to the topic of biodiversity.

In Italy, the *Dipartimento delle Politiche di Sviluppo - Ministero delle Politiche Agricole, Alimentari e Forestali* - published the "National Plan for the safeguard of Biodiversity in farm animals" (PNB) in 2008, and the "Guidelines for conservation and characterization of farm animal genetic resources" (LGBAA) in 2013. One of the purposes of PNB has been the supporting of the introduction of a national strategy for biodiversity conservation in the agricultural sector, capable of efficaciously re-introduce in the animal production system the majority of the native breeds with the consequence to benefit the environment, the sustainable agriculture and the rural development. Most of local populations at risk of extinction, in particular sheep and goats, are characterised by high adaptive ability to harsh environmental conditions where high-productive breeds could not be farmed. In other cases, the survival of these breeds is related to local agricultural tradition and culture, which have guarantee the protection of many local populations until today. Only recently consumer changes in taste and demand have been offering new opportunities for local products and, as a result, for alternative farming systems based on native breeds. The link between farming environment, local breeds and local products needs to be highlighted and strengthened to develop niche markets representing the safest long term strategy for local breed conservation (FAO, 2008).

In birds, safeguard of biodiversity is recognized an urgent need because the great level of standardization and specialization achieved in the poultry sector based on the exclusive global rearing of commercial strains (Marelli, 2008). 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, this course has determined some negative effects on animals: biodiversity reduction, wellness and wealth deterioration. Globally (FAO, 2008), 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.

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 been adapting 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 re-introduce agricultural activity in marginal rural areas.

A recent census in Italian poultry breeds has pointed out that 60% of the 90 breeds that are traditionally known must be considered extinct, almost 30% is endangered, and only less than

10% is not at risk (Zanoni and Sabbioni, 2001). The chicken breeds from Lombardia region listed in the census are four: *Brianzola*, *Maestà 57*, *Mericanel della Brianza* and *Milano*; among these only the *Mericanel* is still diffused, while the other breeds are extinct. *Brianzola* is a primitive breed permanently disappeared, while *Maestà 57* and *Milanino* are composite breeds that could be reproduced. Turkey breeds described by the census are two, *Nero d'Italia* and *Brianzolo* and they are still available. At last, the census reports breeds of other species: *Oca della Lomellina*, endangered, and *Anatra della Bergamasca*, extinct. In 2013, LGBAA listed poultry breeds of various species that are bred in Italy and confirmed such situation.

Chicken and turkey breeds still present in Lombardia region 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 losing 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, 2001; 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. Sustainable and environmentally friendly rearing systems can be identified and spread in large agricultural areas, including sub-urban and marginal areas, improving quality of life.

With remarkable delay compared to other domestic animals, in 2014 the Registro Anagrafico delle Razze Avicole Autoctone (Mipaaf -DM 19536, October 1st, 2014) was established. The Registro follows the effort of the national legislation in adopting international guidelines aimed to preserve AGRs in avian species.

Poultry genetic resources conservation in Italy

When we say "Conservation" we mean a proper management of the genetic resources by men in order to benefit – by a sustainable use – as much as possible both today and in the future. Conservation is a concept that involves the ideas of protection, maintenance, sustainable use, safeguard and valorisation of biological resources (FAO, 2008).

By now it's widely recognized that an effective conservation work devoted to AGR (Animal Genetic Resources) above all needs the identification of specific and integrative goals, both locally and globally. The goals can be summed up as follows (FAO, 2008): a) sharing a definition of the concept of breed; b) historically investigating origins, diffusion, use and features of the breeds; c) making a territorial inventory in order to know numbers and current consistency; d) phenotipically describing and molecularly characterizing living populations, in order to know their genetic diversity, productive abilities (present and future), importance for scientific, economic, ecological, historical and cultural purposes; e) recording population breeding data, surveying their size and periodically reporting their state of risk; f) facilitating the use of the highest number of breeds by means of zootechnical valorisation strategies; g) storing genetic material (particularly semen, oocytes and embryos) that may be used in the future; h) starting education and training programs about the population genetics and the implementation of conservation activities; i) promoting local and international policies to support the farming of local breeds.

Animal biodiversity conservation is generally targeted on maintaining the presence of breeds in their region of origin, in order to allows their natural constant evolution in the natural environment. 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* consists in the utilisation of breeds within their production systems in the original and new diffusion areas. It is the best strategy since it fulfils all the conservation purposes: the use of the breeds in farm animal productions allows its continue evolving by gradually adapting to environmental changes.
- ✓ *Ex situ in vivo* consists in the maintenance of the living animals of the breed outside its production system (herds in protected areas, parks, zoos, experimental and show farms, research centres, hobby breeder farm).
- ✓ *Ex situ in vitro* consists in the storage of genetic material in the form of haploid (semen and oocytes) or diploid cells (embryos) by cryoconservation allowing almost endless storage period.

FAO guidelines (1998) underlines the priority for *in situ* conservation, while the *ex situ* is recognized as a useful complementary strategy providing powerful and safe tools. Therefore, efforts are required in order to build a framework for the effective integration of *in situ* and *ex situ* techniques in conservation programs.

Genetic characterization of animals and populations is an essential activity in the management of small population at risk in order to measure genetic structure and diversity of the breeds, and the rate of inbreeding. It must be remembered that inbreeding reduces diversity and as a consequence adaptability is also reduced, causing a recessive general effect on fitness traits.
Since the 1990s, the characterization of genetic diversity has focused on molecular data and has long been based on the use of neutral genetic markers that provide the following advantages: 1) to overcome the problem of populations without pedigree records, such as poultry breeds; 2) to genotype extensively across the genome in order to estimate precisely the actual proportion of DNA shared by sibs or other relatives. Studies on molecular

diversity relied for many years on genotyping by microsatellite, being markers widely spread in the genome and highly polymorphic. Specific microsatellite panels were developed for 9 animal species, including the chicken - *Gallus gallus* (FAO, 2011).
Until today, some studies on the genetic characteristics of Italian chicken breeds have been performed; in particular, data are available on *Valdarnese* B*ianca*, *Romagnola*, *Modenese, Ancona, Livorno, Pepoi*, *Ermellinata di Rovigo*, *Robusta Maculata, Robusta Lionata* and *Padovana* breeds (De Marchi *et al.*, 2005; Strillacci *et al.*, 2009; Zanetti *et al.*, 2011; Ceccobelli *et al.*, 2013).
A further activity that is essential in a conservation plan is the phenotypic characterization of the breeds (FAO, 2012) aimed to describe and measure the peculiar morphological traits and to study productive and reproductive performance. The valorisation of the breed in alternative productive systems is closely related to the characterization activities of the conservation program; a good knowledge on productive and reproductive features is required in order to study management guidelines specific for the breed; the purpose is the introduction on markets of a product that can be diversified and appreciated by the consumer, well-aware of the environmental impact topics and of animal welfare (Gandini and Oldenbroek, 2007). FAO (2008) has often emphasized that the recovery of the strong link among environment, farming, local breeds and products has been the safest strategy for AGRs conservation in many populations.
Conservation and valorisation projects of Italian poultry breeds have been regionally developing 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, Piemonte and Emilia Romagna, and *Valdarnese Bianca* and *Ancona* breeds also. Few projects have also considered turkey breeds from the Veneto region and the turkey from Parma and Piacenza breed (Cerolini, 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 Tuscany. The breed was included in the "Repertorio Regionale delle Risorse Genetiche Autoctone Animali della Toscana" (LR 50/97) and is the only Italian breed having a "Registro Anagrafico" 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, Spain) several conservation programs of animal genetic resources have also included poultry breeds and semen cryobanks of local breeds and/or specific genetic lines have been created (Woelders *et al*., 2006; Blesbois *et al*. 2007; Santiago-Moreno *et al*., 2011). So far, each laboratory had to study the most feasible cryopreservation procedure in order to identify the reference procedure to be implemented in the cryobank. In fact in birds, semen cryopreservation is not a standardized procedure and its success is still greatly variable and dependent on the species, the genetic types/breeds within the species, and *in vitro* processing. In Italy, the semen cryobank of Italian poultry breeds is not available yet.

Since 2009, a conservation program of Italian poultry breeds from the Lombardia region has been developing at the University of Milan, VESPA Department. In 2012, the CoVAL project was funded and made possible the integration of different research activities all related to conservation (Cozzi et al., 2013).

I have carried out my PhD research activities from 2012 to 2015 within the CoVAL project.

The CoVAL project - Conservation and Valorisation of Italian Avian poultry breeds from Lombardia

The CoVAL project is a three years conservation project funded by the local Institution Regione Lombardia in 2012 (project n. 1723 – call 2010).
The general aim of the project was the conservation of four poultry breeds from Lombardia region: two chicken breeds, *Mericanel della Brianza* (MB) and *Milanino* (MI), and two turkey breeds, *Brianzolo* (BRI) and *Nero d'Italia* (NI).
CoVAL considered both *in situ* priority and *ex situ in vitro* complementary strategies to develop an integrated conservation plan, being innovative at national level.
Different activities have been developed simultaneously in the four breeds in order to build up knowledge on the breeds and plan conservation activities according to FAO international guidelines.
Activities have been planned and run to achieve the following specific goals, divided according to the conservation strategy:

In situ strategy

1. Characterization of the four breeds through the study of genetic markers and phenotypic traits (morphologic, reproductive and productive).
2. Selection of a conservation nucleus of breeders within the chicken breeds corresponding to the desired effective population size considered in FAO Guidelines for management of population at risk.
3. Draft management guidelines for a free range farming system specific for the chicken breeds and assessment of meat production and quality within that system.
4. Dissemination of research results in scientific publications and communication of characteristics and productive potential of poultry breeds to a large audience, from rural productive systems to local Institutions to society.

Ex situ in vitro strategy

1. Identification of a reference procedure for chicken semen cryoconservation according to FAO Guidelines; the reference procedure is the preliminary step required for the creation of a sperm cryobank for Italian poultry breeds.
2. Evaluation of the cryoconservation success of chicken semen collected from the chicken breeds *Milanino* and *Mericanel della Brianza*.

I developed my PhD research plan within the CoVAL project giving my contribution to many activities that will be reported in detail in the following sections of my dissertation.

Brief description of the poultry breeds

The CoVAL breeds from Lombardia region are *Mericanel della Brianza*, the only chicken breed still present, and *Brianzolo* and *Nero d'Italia*, the two turkey breeds traditionally recognized. The composite chicken breed *Milanino* has been re-established since 2009 (project funded by Provincia di Milano) and was also considered (Cerolini et al., 2012).

Mericanel della Brianza

It is a bantam chicken breed very common in Brianza, a hill area in the north-east of Milano. It is still not possible defining the exact period in which this breed appeared, but it is considered likely that it descends from dwarf rural chickens diffused in small rural farms at the beginning of last century.
Mericanel della Brianza is the only Italian bantam chicken breed with a standard officially recognized by the Federazione Italiana delle Associazioni Avicole (FIAV, 2015). Such standard describes chickens with well-balanced and round physical structure and lively and strong temperament. The official standard includes different colour varieties (i.e. plain white, collo oro).

Picture 1: Male of *Mericanel della Brianza* chicken

Milanino

First proofs of the existence of this composite breed were given at the end of 1920 by marquis Trevisani, who mentioned: «A personal initiative born from Isidoro Bianchi's work, accountant who owns a small cottage in Milanino area»; here Bianchi started a breeding farm of this new Italian chicken (Faelli, 1923). The breed is described as rustic and resistant to the harshness of winters, therefore fitting to life in Pianura Padana. Chickens were reared in outdoor fenced pens for meat production and became the peculiarity of the area, being spread in all the villas equipped with poultry houses. However the success of this breed was then eclipsed by the rapid development of intensive poultry farming with the resulting introduction of commercial strains, which caused the extinction of the *Milanino* breed.

Picture 2: Male of *Milanino* chicken

Nero d'Italia

It is a light and very rustic turkey, totally black and iridescent in both sexes. In the past these turkeys were used as brooding hens, thanks to their natural instinct to broodiness and to their light body weight that didn't put the chicks in danger of possible crushing. *Nero d'Italia* breed has an official standard (FIAV, 2015) and has been classified as not very widespread (Zanon and Sabbioni, 2001).

Picture 3: Male of Nero d'Italia turkey

Brianzolo

It is a turkey typical of Lombardia, generally present in rural farming, but at present its number is extremely reduced and it is considered endangered.
Brianzolo is a good grazer, it has early growth and it is resistant to turkey common diseases. The breed includes few colour varieties (black, bronze, criss-cross grey, etc.); grey plumage is considered the prevailing variety in Brianza. *Brianzolo* shows the feature of often having the head and neck with orange instead of red skin and caruncles . This breed has resemblances both in colours and size with *Ronquieres*, a Belgian breed. In 2011, FIAV recognized a breed standard.

Picture 4: Male of *Brianzolo* turkey

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Web Sites

- www.fao.org
- www.unaitalia.com

EXPERIMENTAL TRIALS

During my PhD research period, I have actively participated to many research activities planned within the CoVAL project. In particular, I was involved in studies related to the reproductive characterization of the chicken breeds and their valorisation in meat production system (*in situ* strategy). I was also deeply involved in all the studies related to the cryoconservation of chicken semen (*ex situ* strategy).

The results reported in this thesis only concern the two chicken breeds, in particular *Milanino* breed, that presents high meat-production potentialities.

The management of the small breeding populations has allowed to hatch a high number of birds during the breeding season (January-July), enabling the realization of the valorisation trials and the selection of the breeders for the next season in order to form a conservation breeder nucleus (2014) according to FAO Guidelines for the management of small population at risk. Moreover, it has been possible having a sufficient number of adult chicken males from both breeds to evaluate the specific breed sensitivity to the semen cryopreservation procedure.

Concerning the two turkey breeds, the number of breeders was very limited and the reproductive performance was poor, therefore, it was impossible to hatch many birds in a number that reliable trials could be conducted. The number of turkeys hatched per season has enabled only the selection of a certain amount of breeders, from season to season. In the 3-year period research, fertility and hatchability of turkey breeds have been studied with the purpose of building up knowledge, still completely lacking, on their reproductive performance, that is the basis of all the possible strategies for the conservation of populations over time.

The experimental activities conducted are being fully presented in the following chapters of my thesis. Firstly, the activities concerning the primary *in situ* conservation strategy, and then the ones concerning the *ex situ in vitro* strategy are being discussed.

The results, wherever possible, are presented as paper or, at least, as manuscript submitted to the scientific journals. The results not yet published are being presented in summarized form and concisely discussed in order to bring the reader to realize general conclusion with a strong practical dimension.

In situ strategy

Study on the reproductive performance and selection of a breeder nucleus population.

Aim

The main purpose has been the study of the reproductive performance of the chicken breeds *Milanino* (MI) and *Mericanel della Brianza* (MB), the increase of the population size and the selection of the nucleus population of breeders, according to FAO Guidelines for Management of Animals Genetic Resources.

Materials and Methods

The breeders from both breeds have been housed at the 'Centro Zootecnico Didattico Sperimentale' (CZDS) in the University Campus in Lodi (University of Milan), at the beginning of the reproductive season (December) each year (2012-2015). Birds have been kept in floor pens with litters, single manual nests, pan feeders and automatic nipple drinkers. The environmental conditions were controlled as follows: 15L:10D photoperiod, 18°C ambient temperature, 40-50% UR, forced positive pressured ventilation. Birds have been fed a standard commercial diet for chicken breeders *ad libitum* (16% PG and 2.6% Ca). Breeders have been organized in families, with 1 male and a variable number of females according to the number selected within families each year. Breeders have been selected according to their morphological traits and body weight, and have been organised in families according to their pedigree.
The following sanitary tests have been planned on the breeders before housing and during the reproductive season: microbiological tests on faecal matter in search of *Salmonella spp.* and serological tests in search of *Micoplasma spp.*. Breeders were also tested against avian influenza.

Egg production has been recorded in both breeds from January to July each year. The eggs have been daily collected, labelled with the laying date and the family of origin, and weighed. The eggs have been stored in a room with controlled temperature and RU (14-15°C and 70% respectively) and set every 15 days. The eggs of both breeds have been set in the same incubator from day 1 to 18 and into different hatchers from day 18 to 21 providing standard environmental incubation parameters for the chicken egg. The eggs have been weighed at the end of the conservation period and at the 18th day of incubation in order to calculate the egg weight losses from oviposition to hatching time. At hatch, all chicks have been weighted, labelled with a wing metal tag and vaccinated against Marek's disease.

The numbers of fertile eggs, dead embryos and live chicks were recorded by family and the reproductive parameters fertility, embryo mortality (EM) and hatchability (H) were calculated as proportions on the total eggs set, EM and H were also calculated as proportions on the fertile eggs.

The chicks have been reared throughout the brooding period (1-35 days) at the CZDS following the standard management guidelines for chickens. Birds have been vaccinated against Newcastle disease on day 10 and 30 of age. The mortality has been daily recorded. At the end of the brooding period, the 35 days old chickens have been transferred to a private farm in order to complete the growth in outdoor pens according to the free range rearing system. The chickens have been divided by breed and reared in distinct pens provided with a protected area (small house) and equipped with drinkers and feeders; bird density ranged from 2 to 5 m^2/bird. All the birds have been fed *ad libitum* a standard commercial diet for growing chickens and natural pasture was also available. Each year, a small flock (about 200 chickens) was reared for selection of young breeders. The following bird characteristics have been recorded at 120 and 180 days of age: gender, body weight, shank and skin colour, comb type, colour variety of the plumage. According to the data

collected, a first selection was aimed to reject all the birds with undesired traits and identify the number of birds corresponding to the phenotypic breed standard. A further selection was based upon the pedigree of the birds and the parents' reproductive performance in order to organize the breeding families for the next year.

Statistical Analysis

Reproductive parameters (fertility, embryo mortality, hatchability) have been subjected to statistical analysis, using Chi-square test (SAS, 2009), in order to evaluate the influence of the following categories: setting (the number during the reproductive season is indicative of bird age), family (score), egg weight (g), egg storage (days), egg weight loss during storage and artificial incubation (%).

Results and Discussion

The number and size of the breeding families selected from 2012 to 2014, mortality and mean body weight of male and female breeders in both MI and MB chicken breeds are reported in Table 1.

The parameters related to the oviposition performances recorded each reproductive season from 2012 to 2014 in the MI and MB chicken breeds are reported in Table 2.

The reproductive parameters recorded during the artificial incubation of eggs layed in different season from 2012 to 2014 in the MI and MB chicken breeds are reported in Table 3.

Table 1. Annual breeding population size, mortality rate and body weight of male and female breeders in *Milanino* (MI) and *Mericanel della Brianza* (MB) chicken breeds from 2012 to 2014 (M=male; F=female; BW= body weight).

Parameters	MB			MI		
	2012	2013	2014	2012	2013	2014
N. breeders	28	25	58	25	29	59
N. families	6	5	10	5	5	10
N. M	6	5	10	5	5	10
N. F	22	20	48	18	24	49
Mortality M (%)	4.5	0	2	0	0	0
Mortality F (%)	0	0	0	22	12	6
Mean BW- M (g)	953	934	938	2826	3193	3180
Mean BW- F (g)	673	787	754	1897	2352	2399

Table 2. Parameters concerning the oviposition performance recorded from 2012 to 2014 in *Milanino* (MI) and *Mericanel della Brianza* (MB) chicken breeds.

Parameters	MB			MI		
	2012	2013	2014	2012	2013	2014
Oviposition period	2 Jan-30 Jun	7 Jan - 30 Jun	2 Jan - 30 Jun	2 Jan – 9 Jun	7 Jan - 18 Jun	2 Jan - 30 Jun
Oviposition weeks (n)	26	25	26	27	23	26
Mean oviposition (%)	35	34	36	59	54	40
Oviposition range (%)	6-52	9-54	15-62	74-12	74-30	50-24
Total eggs laid (n)	1343	1202	2966	1637	2035	3573
Eggs/female/week (n)	2.47	2.43	2.39	3.57	3.69	2.50
Mean egg weight (g)	33.3	34.2	35.2	59.0	60.7	57.4
Egg weight range (g)	20 – 44	19 – 46	22 – 45	43 – 69	48 – 74	46 – 75

Table 3. Reproductive performance recorded in different seasons from 2012 to 2014 in Milanino (MI) and Mericanel della Brianza (MB) chicken breeds (EM = Embryo Mortality).

Parameters	MB			MI		
	2012	2013	2014	2012	2013	2014
N. incubated eggs	728	454	726	1034	749	902
Fertility (%)	90	89	84	95	96	86
Hatching/incubated eggs (%)	54	57	40	72	69	53
EM/incubated eggs (%)	35	32	44	23	27	33
Hatching/fertile eggs (%)	60	64	48	76	72	62
EM/fertile eggs (%)	40	36	52	24	28	38

The results of the chi-square test showed a significant effect for some categories with differences among breeds.

The setting number is indicative of the hen oviposition week and is therefore an indirect evaluation of the ageing effect within a reproductive cycle. The setting number significantly affected egg fertility and hatchability in both breeds. However, this effect was not constant over the years and, when present, fertility and hatchability did not show a specific trend during the whole reproductive season in agreement with the standard known changes occurring during ageing in breeder commercial lines.

Fertility and hatchability rates were significantly affected by the family category in both MI and MB breeds; however, even this result has not been constant over the years. The maximum and minimum hatchability (%) calculated on family basis in 2012, 2013 and 2014 in both chicken breeds are reported in Table 4.

Table 4. Maximum and minimum hatchability values (% fertile eggs) calculated within family during different reproductive seasons in *Milanino* (MI) and *Mericanel della Brianza* (MB) chicken breeds. The asterisk shows a significant effect (*P*<0.05) of the family category in the chi-square test.

Breeds	Year		
	2012	2013	2014
MB	64-84*	56-75	23-63*
MI	65-83*	69-89*	8-76*

The egg weight did not show a significant effect on fertility and hatchability, with very few exceptions. It is of interest to underline that egg weight has been constant in both breeds throughout the reproductive season. The weight of the egg is correlated to the body weight of the laying hen. In our studies, females always began the reproductive activity after 25-26 weeks of age when the full somatic development was completed and the adult body weight was reached, therefore, no great changes were expected during the oviposition period in body weight and, as a consequence, in egg weight also.

Egg storage before artificial incubation had a great significant effect on hatchability in both breeds. The days of egg storage have been classified in different storage periods: short period (1-4 days), medium period (5-9 days) and long period (10 or more days, with a maximum of 18 days). The increase of the storage period is associated to the decrease in hatchability, and the same clear trend has been found in *Milanino* (Fig. 1) and *Mericanel della Brianza* (Fig. 2) eggs and confirmed in different years. Therefore, the storage of eggs before artificial incubation should not exceed 4 days in order to prevent a decrease in the number of live chicks at hatch.

Figure 1 – Hatchability (% on fertile eggs) of *Milanino* eggs calculated for different egg storage periods before artificial incubation (chi-square test *P* < 0.001).

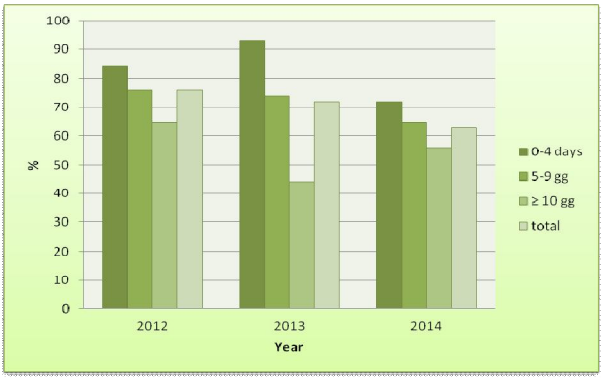
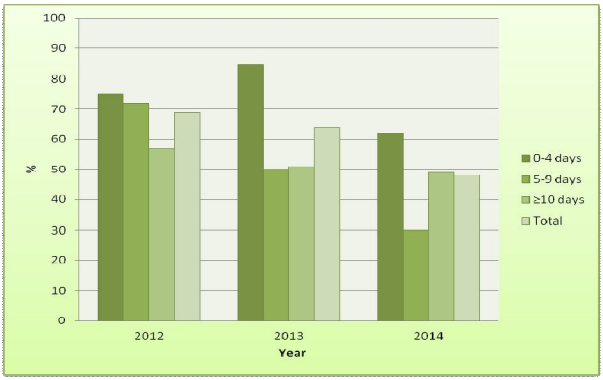
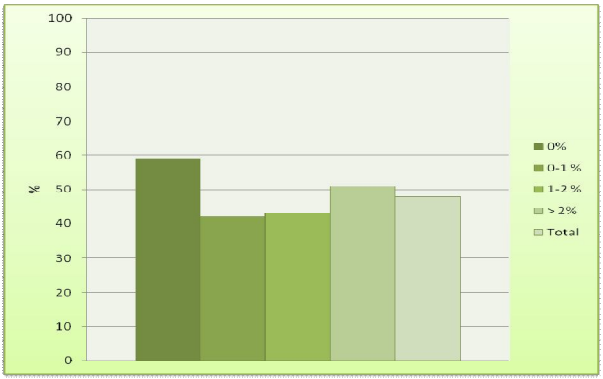


Figure 2 – Hatchability (% on fertile eggs) of *Mericanel della Brianza* eggs calculated for different egg storage periods before artificial incubation (chi-square test *P* < 0.001).



The decrease in egg weight during the storage period is considered an indicative parameter of the correct egg management in such period. In artificial incubation of poultry species, it is well recognised that the egg weight loss before incubation should not exceed 1% of the initial weight, in order not to increase embryo mortality. Egg weight loss measured during storage has been very variable in native breeds, and a negative significant relation was found between hatchability and decrease in egg weight during storage. In 2012, because of a suboptimal control of the temperature in the storage room, the mean egg weight loss from laying to setting was 1,34 and 2,08% in MI and MB breed respectively. In 2013 and 2014, the storage room was equipped for an improved control of T and RU within the recommended values; in MI breed as a consequence, the proportional decrease in egg weight during storage was reduced, then its negative effect on hatchability was prevented. In contrast in MB breed, the rate of egg weight loss was still very high and variable during storage with a resulting significant effect on hatching rates (Figure 3). MB eggs have unique features being very small in size: the shell is very thick and strong, and the amount of albumen is low compared to standard chicken eggs; as a result, it is likely that MB eggs require specific environmental conditions during storage in order to prevent dehydration and then embryo mortality during artificial incubation.

Figure 3 – Hatchability (% on fertile eggs) of *Mericanel della Brianza* eggs calculated for different classes of egg weight loss during storage in the 2014 reproductive season (chi-square test $P < 0.001$).



Conclusions

Original data on the reproductive performance of the Italian chicken breeds *Mericanel della Brianza* and *Milanino* are reported here for the first time.

The present results represent the basic knowledge on reproductive parameters necessary to plan the breeding management within a conservation program in order to improve the reproductive efficiency of the native avian breeds.

Valorisation of chicken breeds in meat production systems

Recent changes in consumers' taste and demand have offered new chances toward the market of local products and, consequently, the valorisation of Italian chicken breeds having potential features for meat production. Today, it can be assumed that the recovery of the ancient relation between animal farming, natural environment, local breeds and local products represents the safest tool for the long term conservation of Animal Genetic Resources in many populations.

Even within the CoVAL project, the valorisation of chicken breeds within productive systems has been considered and developed. The research plan included every year experimental activities aimed to study the rearing management and the quality of products, meat and eggs. The number of chickens required to organize different treatments within trials were planned at the beginning of the reproductive season, hatched and reared during the brooding period at the CZDS. Then, birds were transferred to private farms (partners in CoVAL project) to perform the growing period in outdoor pens.

The general aim was the compiling of management guidelines on outdoor free range farming systems specific for the Italian MI and MB chicken breeds. Bird density and the dietary protein level fed during the growing period are the rearing parameters studied in both breeds in different trials. Each year the same experimental protocol was planned and run at the same time in each breed.

Because of the need to summarize, I am only reporting the results concerning the *Milanino* chicken breed which, over time, has revealed remarkable characteristics suitable for the development of a local meat production system of potential interest for the multi-functional farms within the agricultural sector in the Lombardia region.

Bird density, stress markers and growth performance in the Italian chicken breed Milanino

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Summary

The Milanino is an Italian chicken breed included in a conservation project run by the University of Milan. It is characterized by good fertility, heavy body weights, high adaptation ability to adverse climate conditions, and disease resistance. Because of these characteristics, the Milanino could represent an important genetic resource for alternative production systems. This research was aimed at studying the effect of bird density on growth and slaughter performance, as well as stress response in Milanino chickens kept in outdoor pens. One hundred and sixty Milanino chickens were randomly assigned to 2 experimental groups kept at different densities (2 m^2/bird and 8 m^2/bird) and were slaughtered at 185 days of age. Growth and slaughter performance and stress condition were recorded. The interaction bird density * sex * age significantly affected body weight and an opposite trend was found between females and males: heavier females were found in the high-density group, while heavier males were found in the low-density group. Bird density did not affect carcass weight data. The stress marker (H/L ratio) was significantly higher in birds kept at the higher density (2 m^2/bird). In conclusion, the Milanino provided satisfactory growth performance with different rearing density but the lower density, 8 m^2/bird, should be preferred to minimize welfare problems for male birds.

Keywords: body weight, chickens, breed, stress markers, bird density, slaughter, carcass yield

Description of the problem

According to the State of the World's Animal Genetic Resources for Food and Agriculture, 20% of documented livestock breeds are at risk of extinction: 1500 of the 7600 breeds around the globe may be lost forever in the near future [1]. Such a situation is greatly emphasized in the poultry sector where only selected strains are reared for meat and egg production while local breeds have been excluded from the intensive productive system [2]. In Italy, 90 local avian breeds were described; the majority (61%) were classified as extinct and only 8.9% as still widely spread [3]. The need for conservation of avian genetic resources is well recognized and actions are requested from both public and private institutions [2]. In Italy, efforts to include local avian breeds within conservation programs have been increasing with the support of local institutions, mainly in the regions that have an important poultry tradition. Literature reports provide information on genetic variability, breeding performance, meat and egg quality and behavioral features of some Italian breeds involved in conservation projects in Veneto [4, 5], Emilia Romagna [6] and Lombardia regions [7, 8].

The Milanino is a composite chicken breed (Valdarnese x Orpington) selected mainly for meat production at the beginning of the 20th century in the rural area close to Milan in the North of Italy [9]. The breed is currently included in a conservation research project on poultry autochthonous breeds present in the Lombardia region (CoVAL project n. 1723, funded by Regione Lombardia). The morphological traits characteristic of the breed include white plumage, white skin, white shanks, and a large single comb. The mean egg production recorded in a small population during the breeding season, from January to June, was 58% with a peak of oviposition of 82%. High fertility values were recorded, range 85-91%, during the same whole reproductive season [9]. Because these characteristics, the Milanino represents an important genetic resource for alternative production systems, especially where the demand for organic and free-range meat is

increasing. Studies on biological, productive and behavioral characteristics of the breed are required to develop a peculiar management guide for extensive rearing systems and support the potential use of Milanino birds in sustainable productions, wishing to reach niche markets and marginal areas.
The purpose of this trial was to study the effect of bird density on growth and slaughter performance, and on stress response in Milanino chickens reared in outdoor pens.

Materials and Methods

Birds, Rearing System and Growth Performance

The study was carried out during the 2013 reproductive season, from May to November. One hundred and sixty straight-run Milanino chickens (79M:81F) were hatched at the Poultry Unit, Animal Production Centre, University of Milan (Lodi). The chicks were reared in a controlled environment from 1 to 35 days of age following standard management guidelines for chickens. At hatch, birds were labeled with a metal wing tag, weighed and vaccinated for Marek's and Newcastle diseases. On day 35 of age, birds were transferred to a local private farm and reared until 185 days of age in outdoor pens. The pens were equipped with feeders, drinkers and a suitable shelter to confine chickens at night or during bad weather. Birds were fed *ad libitum* with a starter feed (12.13 MJ/kg of ME, 20% CP) from hatch to 21 day of age and a finisher feed (12.58 MJ/kg of ME, 18% CP) from 22 to 185 day of age. The total amount of feed given to each group was recorded. At transfer on 35 days of age, birds were randomly assigned to two experimental groups (80 birds/group) and kept at different densities in outdoor pens, corresponding to 2 m^2/bird and 8 m^2/bird. Birds were reared straight-run and each experimental group was kept in one pen according to the rural farming system used in the farm. Birds were individually weighed on day 35, 65, 95, 150 and 185 of age. Bird mortality was

recorded daily. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching [10].

Slaughter and Carcass Weight

At 185 days of age, 20 birds per treatment, 10 males + 10 females, were randomly chosen and slaughtered after 12 h feed withdrawal. Chickens were stunned by electrocution (110 V; 350 Hz) before killing. After killing, carcasses were plucked and weighed. Then, non-edible viscera (intestines, proventriculus, gall bladder, spleen, esophagus and full crop) were removed and the weight of the partial eviscerated carcass (PEC) was recorded. Spleen weight was recorded and its proportion on the live body weight was calculated. Then the head, neck, legs, edible viscera (heart, liver, and gizzard) and fat (perivisceral, perineal and abdominal) were removed in order to obtain the ready-to-cook carcass (RCC) [11] and its weight. The proportion of the PEC and RCC on the live body weight was calculated.

Stress Markers

Stress condition was assessed by the measurement of the heterophil/lymphocyte (H/L) ratio and of the spleen weight. In birds, H/L ratio is one of the most accepted indicators of stress [12, 13], and also the relative spleen weight is often used as stress parameter [14].
On the day before slaughter, blood samples were randomly taken from the cutaneous ulnar vein of 40 birds per treatment (20 males + 20 females) to assess the leukocyte formula and then to calculate the H/L ratio. On the day of slaughtering, spleen was dissected from the 20 birds slaughtered as previously described to record its weight and to calculate its proportion on the live body weight.

Statistical Analysis

Analysis of variance on the live body weight data was performed using the MIXED procedure of SAS [15]. The statistical model included fixed (sex, age and bird density) and random (the chicken) effects and the relative interactions (sex * age, sex * bird density, age * bird density, sex * age * bird density). A *T* test was used to compare LS Means.
Analysis of variance on the carcass weight data and on H/L ratio was performed using the GLM procedure of SAS [15]. The statistical model included sex and bird density as sources of variation, and the interaction sex * bird density. A *T* test was used to compare LS Means.

Results and Discussion

Growth Performance

The fixed effects (sex, age and bird density) and some of the interactions (sex * age, bird density * age and sex * bird density * age) considered in the analysis of variance significantly affected body weight during the growing period. ($P < 0.05$). Body weights were significantly different between the two sexes (males = 1,551.27 g ± 14.07, females = 1,280.08 g ± 14.36, $P < 0.001$) but not before day 65 and the difference increased with the age of the birds (Table 1). Males were significantly heavier than females from 65 to 185 days of age. The present result shows a clear sexual dimorphism in the Milanino breed. Mean body weight was different between the two sexes already on hatch and this sexual dimorphism became markedly and constantly evident from 65 days of age onwards. According to their growth performance, both males and females could be slaughtered at different ages to produce different chicken meat products characteristic of the Italian poultry market [16]. Ready-to-cook carcasses could be produced with males and females slaughtered on 80 and 110 days

of age respectively, and meat cuts with males and females slaughtered on 150 and 185 days of age respectively.

Table 1. Body weight (g) of males and females recorded at different ages during the growing period in the Italian chicken breed Milanino

Age (days)	Males	s.e.	Females	s.e.
1	34.04	21.83	39.99	21.83
35	540.04	21.34	473.62	21.34
65	1,344.96^{A}	21.16	1,079.82^{B}	21.87
95	2,100.55^{A}	21.15	1,554.53^{B}	21.68
150	2,423.86^{A}	21.96	2,128.51^{B}	22.19
185	2,864.16^{A}	22.27	2,404.03^{B}	22.97

$^{A, B}$ Values within a row with different superscripts differ significantly at P<0.001

The body weight of Milanino chickens on 185 days of age ranged between 2886 g and 2381 g and these values are higher than those reported for other Italian chicken breeds, such as the Padovana breed reared outdoor and slaughtered on a similar age [17] and the Modenese and Romagnola breeds slaughtered on 210 days of age [6].

Bird density significantly affected body weight (2 m^2/bird = 1,394.88 g ± 14.44; 8 m^2/bird = 1,436.47 g ± 13.72, $P < 0.05$); however, the effect was age dependent and present only on 150 days of age (Table 2). On that age, the chickens kept at the lower density were significantly heavier ($P < 0.001$). Moreover, also the interaction among age, sex and bird density significantly affected body weight (Table 3). In females, bird density showed a significant effect on body weight only on 185 days of age ($P < 0.05$), and heavier females were found in the group kept at the higher density (2 m^2/bird). In males, bird density showed a significant effect on body weight on 150 and 185 days of age ($P < 0.001$), and heavier males were found in the group kept at the

lower density (8 m^2/bird). Therefore, bird density significantly affected the growth and the effect was age dependent and different between males and females. In males, the best growth performance was recorded in birds kept at the lower density of 8 m^2/bird and such positive effect was present from 150 days of age onwards. In contrast, in females, the highest mean body weight was recorded in birds kept at the higher density, corresponding to 2 m^2/bird, and such an effect was present only in adult birds on 185 days of age. The effect of bird density on male growth performance may be related to male sexual behavior. Fights among males are frequent at the onset of sexual maturity in concomitance with the increase of testosterone blood level and the frequency of fights is positively related to rearing densities [18, 19]. A higher frequency of fights can occur among males kept at the higher density of 2 m^2/birds, and as a consequence male growth performance could have been negatively affected. The same situation may also explain the highest weight of adult females reared at the same high density: if males spend more time to fight than to mate, females can feed undisturbed longer and gain more weight. In domestic fowls, the hierarchical system is rigorously established by aggressive behaviors, but once the hierarchy has been organized, the aggressiveness decreases and is substituted for demonstrations of dominance and submission [20].

Table 2. Body weight (g) recorded at different ages in Milanino chickens reared in outdoor pens at different bird density

Age (days)	Density 2 (m^2/bird)	s.e.	Density 8 (m^2/bird)	s.e.
1	33.19	22.13	40.84	22.13
35	495.06	21.94	518.60	21.94
65	1,199.95	21.94	1,224.83	20.92
95	1,823.96	22.09	1,831.22	20.56
150	2,195.93^{A}	22.59	2,356.44^{B}	21.37
185	2,621.32	22.84	2,646.87	22.23

$^{A, B}$ Values within a row with different superscripts differ significantly at $P<0.001$

According to the present results, a critical rearing period is from 95 to 150 days of age in male birds, in concomitance with the onset of sexual maturity, and from 150 to 185 days of age in females, in concomitance with the onset of mating behavior. The separate rearing of males from females after 10 weeks of age may be suggested and studied in order to better control sexual behavior. A way to overcome the aggressiveness of males due to the development of the reproductive function during the long rearing period of Milanino birds could be to use males to produce capons, a traditional niche market product on Christmas period in Italy [21]. It is known that castration is followed by a deficiency of testosterone and males soon show changes in appearance and behavior, becoming more docile and unwilling to mate [22]. Caponization is usually performed from 4 weeks to 8 weeks of age [23]; therefore, this practice will prevent the hormonal and behavioral changes associated with the onset of the reproductive function.

The overall mean feed consumption measured in the growing period, from 35 to 185 days of age, was very similar in both treatments and corresponding to 94 g/bird/day. Daily feed

consumption progressively increased from 53 to 129 g/bird in the same period. The cumulative feed consumption recorded was 14.1 kg/bird/150 days rearing period. According to the present results on growth performance, further studies are suggested to collect more detailed data on variations in feed consumption according to the age and the sex of birds.

Mortality recorded during the brooding period (1-35 days of age) was 2.4%. Mortality recorded during the growing period (36-185 days of age) was 1.2 and 2.4% in the low and high density group respectively.

Table 3. Body weight (g) recorded at different ages in male and female Milanino chickens reared in outdoor pens at different bird density

Age (days)	Sex[1]	Density 2 (m^2/bird)	s.e.	Density 8 (m^2/bird)	s.e.
1	**F**	33.68	31.63	46.31	31.63
	M	32.71	31.63	35.37	31.63
35	**F**	460.11	31.63	487.13	31.63
	M	530.01	31.63	550.06	31.63
65	**F**	1,056.58	31.63	1,103.05	29.73
	M	1,343.31	30.42	1,346.61	29.43
95	**F**	1,552.00	32.30	1,557.05	28.45
	M	2,095.71	30.14	2,105.39	29.69
150	**F**	2,094.77	32.30	2,162.25	29.94
	M	2,297.10^{A}	31.59	2,550.63^{B}	30.51
185	**F**	2,450.46^{a}	33.00	2,357.60^{b}	31.48
	M	2,792.17^{A}	31.59	2,936.15^{B}	31.40

$^{A, B}$ Values within a row with different superscripts differ significantly at P<0.001

$^{a, b}$ Values within a row with different superscripts differ significantly at P <0.05

[1]M = males; F = females

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Slaughter Performance

The sex was the only source of variation significantly affecting the carcass weight data. Bird density and the interaction between density and sex did not affect carcass weight data ($P > 0.05$). Significant results related to the slaughter performance are reported in Table 4. The live body weight (BW) measured before slaughter was higher in males ($P < 0.05$), compared to the BW of females, and carcass weight showed the same difference between sexes ($P < 0.05$). The weight of the PEC and RCC was heavier in males ($P < 0.001$) compared to the same weight recorded in females (Table 4). The PEC expressed as percentage of the BW had higher values in males ($P < 0.05$), while the RCC expressed as percentage of the BW presented no significant difference between males and females ($P > 0.05$).

Table 4. Carcass weight data recorded in male and female Milanino chickens slaughtered on 185 days of age.

Carcass weight data[1]	Females	Males	s.e.
LW (g)	2,398.80^{A}	2,661.75^{B}	59.42
CW (g)	2,191.00^{a}	2,369.95^{b}	52.50
PEC (g)	1,893.75^{A}	2,264.50^{B}	59.25
RCC (g)	1,583.00^{A}	1,785.90^{B}	38.50
PEC (% LW)	79.20^{A}	84.93^{B}	1.36
RCC (% LW)	66.16	67.13	0.77

[1]LW = live weight; CW = carcass weight; PEC = partial eviscerated carcass; RCC = ready-to-cook carcass

$^{A, B}$ Values within a row with different superscripts differ significantly at P<0.001

$^{a, b}$ Values within a row with different superscripts differ significantly at P<0.05

The Milanino, like other local breeds reared in free-range systems, should be able to provide satisfactory productive performance. The recommendations of the Network for Animal Health and Welfare in Organic Agriculture [24] suggest that the use of commercial strains should be avoided to reduce welfare problems and, in contrast, chickens with a slow growing rate should be preferred for extensive production systems because they show more "natural" behavioral patterns. On the other hand, strains or breeds with slow growth rate require a very long rearing time [25, 26], resulting in high production costs. With this background, the position of local breeds as an organic and free-range product could be strengthened if their true ability to have good performance in very different environmental conditions will be confirmed. In the present study, bird density and the interaction between density and sex did not affect carcass weight data. Therefore, the Milanino can be considered a high adaptable breed, able to have good productive performance under both organic and free range systems. The slaughter performance of the Milanino breed of chicken was good in both sexes and a high proportion of carcass yield was measured, corresponding to 66% and 67% RCC in female and male birds respectively. Lower proportions of RCC were reported in other Italian chicken breeds, 63% and 62% in the Modenese and Romagnola breeds respectively, both slaughtered at 210 days of age [6]. A lower RCC proportion (64%) has been also reported in Delaware breed (64%), slaughtered at 105 days of age and today promoted for small-scale poultry production in U.S. [27]. The proportion of semi- and full eviscerated carcasses measured in the male Milanino chickens are in agreement with the results reported in the Baicheng-You indigenous breed, one of the native breeds commonly raised for high-quality meat production in China [28]. However, it should be considered that the comparison of results from different reports is difficult due to differences in bird management, age and weight at slaughter.

Stress Parameters

The most important parameter involved in stress response, the H/L ratio, was significantly affected by sex ($P < 0.05$) and bird density ($P < 0.05$). Circulating H/L ratio was higher in female (0.75) than in male (0.41) chickens (Table 5), and in birds kept at the higher density (0.74 *vs* 0.42) (Table 6). In contrast, the proportion of the weight of the spleen on the body weight was not different ($P > 0.05$) between the two sexes and the two bird densities. The interaction between sex and bird density considered in the statistical model did not show a significant effect ($P > 0.05$) on stress parameters.

Table 5. Stress parameters recorded in male and female Milanino chickens raised in outdoor pens.

	Females	**Males**	**s.e.**
H/L ratio[1]	0.75[a]	0.41[b]	0.09
Relative spleen (%)	0.11	0.13	0.007

[1]H/L ratio = circulating heterophil/lymphocyte ratio

[a, b] Values within a row with different superscripts differ significantly at P<0.05

Table 6. Stress parameters recorded in Milanino chickens reared in outdoor pens at different bird density

	Density 2 (m^2/bird)	Density 8 (m^2/bird)	s.e.
H/L ratio[1]	0.74[a]	0.42[b]	0.09
Relative spleen (%)	0.13	0.12	0.007

[1]H/L ratio = circulating heterophil/lymphocyte ratio

[a, b] Values within a row with different superscripts differ significantly at $P<0.05$

A number of environmental stressors affects H/L ratio [12, 14], which makes this measurement one of the most accepted indicator of the chronic stress condition in birds. The rearing system and management might have great influence on stress condition in poultry. There are many stress-inducing factors in domestic bird rearing, such as feed restriction, high stocking densities or inability to perform specific behaviors like dust or sand bathing [19]. An increased circulating H/L ratio indicates a stress state [12, 29] and a high H/L ratio is negatively correlated with body weight [14, 30]. To evaluate the best density at which to rear Milanino chickens during the growing period it is important to understand if bird density affects the stress of the birds and, finally, growth performance. In the present study, the birds kept at the higher density presented a higher H/L ratio and it affected male, not female, body weight at the end of the rearing period. In fact, as reported by Queiroz and Cromberg [19], environmental stress is a possible triggering factor of aggressive behavior and this could contribute to a higher frequency of fights among males kept at 2 m^2/bird close to puberty. H/L ratio was higher in females suggesting different stress effects between males and females, in the rearing conditions utilized for this study. Aggressiveness and subordination are complex behavioral expressions that involve genetic differences between breeds, strains and individuals [31]. Milman and Duncan [20] reported mating problems related to an increased aggressiveness in broiler

breeders during the reproductive phase. Males showed extreme aggressiveness during mating, causing serious injuries to females or even causing death. It would be of interest to investigate the mating behavior in the Milanino breed to understand if male aggressiveness may be related to the higher stress condition found in females.

Overall results provided in this report on the free-range rearing system for the Milanino chicken breed are the first available. The present trial was performed in field conditions, therefore each experimental group was reared in one pen. Even in this situation, which could have created highly variable results, significant effects of bird density on growth performance and stress parameters were found. The present results suggest further studies are required to improve the management of the birds during the rearing period in free-range system.

Conclusions and Applications

1. The Milanino is a heavy breed with sexual dimorphism in relation to adult body weight: 2864 g in males and 2404 g in females at 185 days of age.
2. Flock density affects body weight for this breed. The major effect was recorded in males, who gained more weight at the low density.
3. Slaughter performance was recorded at 66-67% carcass yield for the breed. Carcass yield was not affected by flock density.
4. Flock density did affect bird stress levels with high bird density associated with an increase in H/L measurements. A rearing density of 8 m^2/bird is recommended in outdoor pens to prevent welfare problems in both male and female chickens.

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Growth performance, carcass characteristics and meat composition of Milanino chickens fed different protein levels

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Abstract

Milanino is a heavy Italian chicken breed included in a conservation project of the University of Milan and is an important genetic resource for alternative production systems. This research was aimed to study the effect of the dietary protein level on growth and slaughter performance, and meat composition in free-range reared Milanino chickens.
One hundred and twenty Milanino chickens were fed different protein level (HP=20% CP and LP=16% CP), reared according a free range system and slaughtered at 150 and 180 days of age. Growth and slaughter performance, and meat (breast and thigh) composition were recorded.
The protein level of diet did not affect the overall Milanino mean body weight recorded in the straight-run group in the whole rearing period. However, the growth rate within sex was significantly different between the dietary treatments: heavier females were found in the HP group from 125 days onwards, while no differences were recorded in male body weights. The protein level of diet did not affect both carcass weight data and meat composition.
The present results suggest the use of a low protein diet for rearing straight-run Milanino chickens for long rearing period. However in females, a high protein diet is recommended from 125 days of age onwards.

Introduction

Widespread societal concerns with animal welfare and environmental issues caused by intensive farming are primary factors contributing to an emerging interest in the diversification of poultry industry toward more extensive and sustainable systems of production such as free-range or organic (Sundrum, 2001). Although consumers are accustomed to paying low prices for poultry meat, they are increasingly interested in products that

they perceive as naturally produced or environmentally friendly and provide good welfare to the birds (Fanatico *et al.,* 2007). The recommendations of the Network for Animal Health and Welfare in Organic Agriculture (2002) suggest that the use of commercial strains should be avoided to reduce welfare problems and, in contrast, chickens with a slow growing rate should be preferred for extensive production systems because they show more "natural" behavioural patterns. Moreover, fast-growing meat strains of chicken are characterised by a very low degree of adaptation and resistance to natural environment (Reiter and Bessei, 1999), while the slow-growing strains can fully benefit from extensive rearing system (pasture availability, older age). Despite a higher retail price than conventional poultry products, these types of chickens have aroused lively interest in national markets. In France, the "Label Rouge" program, developed almost 40 years ago, provides the 30% of the poultry meat sales despite its price is twice the conventional price. The "Label Rouge" program is nowadays a model to develop pasture-raised chicken production systems in other countries, such as U.S. (Westgren, 1999; Fanatico and Born, 2002). In Italy, among those slow-growing genotypes there are several local chicken breeds involved in conservation projects in Veneto (De Merchi *et al.,* 2005; Zanetti *et al.,* 2010) and Emilia Romagna (Sabbioni *et al.,* 2006) regions. Carcass characteristics or peculiar qualitative meat traits have been identified in those Italian chicken breeds supporting their potential use in innovative small scale farming systems.

The interest in local breeds has increased noticeably in the last decade, mostly because biodiversity conservation has become an important issue for the international scientific community (FAO, 2009). The Milanino is a composite chicken breed (Valdarnese x Orpington) selected mainly for meat production at the beginning of the 20th century in the rural area close to Milan in the North of Italy (Cerolini *et al.,* 2012). The breed is currently included in a conservation research project on poultry autochthonous breeds

present in the Lombardia region (CoVAL project n. 1723, funded by Regione Lombardia). The Milanino could represent an important genetic resource for alternative production systems, especially where the demand for organic and free-range meat is increasing. Productive performance analysis is highly relevant to the inclusion of local breeds in conservation programmes (Ruane, 1999). In fact, local breeds with slow growth rate require a very long rearing time (Castellini *et al.,* 2006), resulting in high production costs in particular related to feed consumption. However, high-priced conventional diets typically meet requirements for fast-growing broilers in indoor production. EU legislation for free-range poultry meat specifies maximum stocking densities, age at slaughter, as well as a diet that is at least 70% cereals at finishing, ensuring a low-protein diet for slow growth (European Union, 2008). A low-protein diet to support a slower rate of growth and improve meat quality is used in the well-known Label Rouge French program (Sundrum, 2006). Studies on the rearing and productive characteristics of the Milanino are required to develop an extensive farming program for the Milanino meat production. In previous trials, the age of transfer to outdoor pens (unpublished results) and bird density during the growing period (Mosca et al., 2015) were studied. The purpose of this trial was to study the growth and slaughter performance and the meat composition in Milanino chickens fed two different diets during the growing period: a diet typically used in commercial strains with high level of protein and a poorer and less expensive diet with low level of protein.

Materials and Methods

Birds, Rearing System and Growth Performance

The study was carried out during the 2014 reproductive season, from May to November. One hundred and twenty Milanino chickens (61M:59F) were hatched at the Poultry Unit, Animal

Production Centre, University of Milan (Lodi). The chicks were reared in a controlled environment from 1 to 35 days of age following standard management guidelines for chickens. At hatch, birds were labelled with a metal wing tag, weighed and vaccinated for Marek's and Newcastle diseases. On day 21 of age birds were weighed and provided with a second vaccination for Newcastle disease. Birds were fed *ad libitum* with a starter feed (12.13 MJ/kg of ME, 22% CP) from hatch to 35 day of age. On day 35 of age, birds were transferred to a local private farm and reared until 180 days of age in outdoor pens at 8 m^2/bird density. The pens were equipped with feeders, drinkers and a suitable shelter to confine chickens at night or during bad weather. At transfer on 35 days of age, birds were randomly assigned to two experimental groups (60 birds/group) and fed *ad libitum* two different grower diets corresponding to high level of protein (HP = 20% crude protein) and low level of protein (LP = 16% crude protein) until the age of slaughter. Both diets were a crumbled vegetable diet that consisted of 4% lipids, 4% fibre, 6% ash, and 12.58 MJ/ME/kg. The total amount of feed given to each group was recorded. Birds were reared straight-run and each experimental group was kept in one pen (corresponding to one protein level, HP and LP) according to the rural farming system used in the farm. Birds were individually weighed on day 35, 65, 95, 125, 150 and 180 of age. Bird mortality was recorded daily. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Slaughter and Carcass Weight

At 150 and 180 days of age, 20 birds per treatment (10 males + 10 females) were randomly chosen and slaughtered after 8 h feed withdrawal. Chickens were stunned by electrocution (110 V; 350 Hz) before killing. After killing, carcasses were plucked and weighed. Then, non-edible viscera (intestines, proventriculus, gall

bladder, spleen, oesophagus and full crop) were removed and the weight of the partial eviscerated carcass (PEC) was recorded. Then the head, neck, legs, edible viscera (heart, liver, and gizzard) and fat (perivisceral, perineal and abdominal) were removed in order to obtain the ready-to-cook carcass (RCC) (Romboli *et al*, 1996) and its weight. The proportion of the PEC and RCC on the live body weight was calculated. The weight of main viscera (intestines, spleen, heart, liver and gizzard) was recorded. Fat weight was not recorded because a negligible amount of fat was present in these birds.

After processing, RCC were cooled in a cold tunnel and refrigerated at 4°C for 24 h. On the day after slaughter, the right breast and thigh without skin were removed and stored at -20°C up to 20 days for further meat analyses.

Analytical Determination

Breast and tight meat samples were homogenized (Ultra Turrax T25, IKA) for 2 min. Moisture (950.46), total protein (981.10), ether extract (991.36) and ash (920.153) contents of the meat homogenates were analysed in duplicate according to the AOAC methods (2000).

Statistical Analysis

Analysis of variance on the live body weight data was performed using the MIXED procedure of SAS (SAS Institute, 1999). The statistical model included fixed (sex, age and protein level) and random (the chicken) effects and the relative interactions (sex * age, sex * protein level, age * protein level, sex * age * protein level). *T* test was used to compare LS Means.

Analysis of variance on the carcass weight data and on meat composition was performed using the GLM procedure of SAS (SAS Institute, 1999). The statistical model included sex, slaughter age and protein level as sources of variation, and the relative

interactions (sex * slaughter age, sex * protein level, slaughter age * protein level, sex * slaughter age * protein level). *T* test was used to compare LS Means.

Results

Growth Performance

According to the results of the analysis of variance, the fixed effects sex and age, and the interactions sex*age and protein level*sex*age significantly affected body weight during the growing period ($P < 0.05$).

Body weights were significantly different between the two sexes (males = 1,482.15 g ± 14.66, females = 1,209.31 g ± 16.87, $P < 0.001$) and the difference increased with the age of the birds. Males were significantly heavier than females from 35 to 180 days of age (Table 1).

The protein level of the diet did not affected body weight considering the whole group in the whole rearing period (HP = 1,355.63 g ± 16.02; LP = 1,335.83 g ± 15.58, $P > 0.05$). However, the growth rate within sex was significantly different between the dietary treatments. Heavier females were found in the group fed the higher protein level (20% *vs* 16%) from 125 days of age onwards (Table 2). In contrast, male body weight was very similar in both dietary treatments during the whole growing period (Table 2).

The overall mean feed consumption measured in the growing period, from 35 to 180 days of age, was very similar in both treatments (HP and LP) and corresponding to 95 g/bird/day. Daily feed consumption progressively increased from 50 to 130 g/bird in the same period. The cumulative feed consumption recorded was 13.7 kg/bird/145 days rearing period. Mortality recorded during the brooding period (1-35 days of age) was 2.5%. Mortality recorded during the growing period (36-180 days of age) was 3.3 and 1.7% in the HP and LP group respectively.

Table 1. Body weight (g) of males and females recorded at different ages during the growing period in the Italian chicken breed Milanino

Age (days)	Males	s.e.	Females	s.e.
1	34.14	21.83	39.95	21.83
21	252.03	21.77	235.35	21.77
35	538.28[a]	21.86	467.62[b]	21.86
65	1,459.15[A]	21.21	1,137.59[B]	23.95
95	1,911.91[A]	21.07	1,504.74[B]	24.07
125	2,264.62[A]	21.20	1,833.00[B]	24.20
150	2,604.05[A]	21.12	2,157.44[B]	24.66
180	2,775.15[A]	22.49	2,306.40[B]	28.68

[A, B] Values within a row with different superscripts differ significantly at P<0.001
[a, b] Values within a row with different superscripts differ significantly at P<0.05

Table 2. Body weight (g) recorded at different ages in male and female Milanino chickens fed two different protein levels.

Age (days)	Sex[1]	HP[2]	s.e.	LP[2]	s.e.
1	**F**	34.64	30.01	45.01	30.01
	M	32.51	30.01	35.22	30.01
21	**F**	220.98	31.60	249.71	31.60
	M	243.86	31.60	260.20	31.60
35	**F**	471.46	32.36	464.32	32.36
	M	537.78	32.36	538.78	32.36
65	**F**	1,156.86	35.42	1,118.32	32.26
	M	1,454.07	28.73	1,464.23	31.21
95	**F**	1,534.35	35.37	1,475.12	32.66
	M	1,891.49	28.73	1,932.33	30.82
125	**F**	1,896.83[a]	35.37	1,769.17[b]	33.09
	M	2,234.27	28.73	2,294.97	31.18
150	**F**	2,219.65[a]	36.57	2,095.22[b]	33.08
	M	2,601.30	29.01	2,606.80	30.70
180	**F**	2,406.21[a]	44.96	2,206.60[b]	35.63
	M	2,747.77	30.29	2,802.53	33.25

[a, b] Values within a row with different superscripts differ significantly at P <0.01

[1] M = males; F = females.

[2] HP= High protein level (20% crude protein); LP=Low protein level (16%crude protein)

Slaughter Performance

According to the results of the analysis of variance, the sex and the age of slaughter were the only sources of variation significantly affecting the carcass weight data. All significant results related to the slaughter performance are reported in Table 3.

As expected, the live body weight (BW) measured before slaughter was higher in males compared to females ($P < 0.001$) and in 180 days old birds compared to 150 days old birds ($P < 0.05$). The weight of the PEC and RCC showed the same differences between sexes ($P < 0.001$) and ages ($P < 0.001$). The proportion of RCC was significantly higher in males than in females ($P < 0.05$), and in contrast the proportion of PEC was very similar in males and females ($P > 0.05$). The proportions of PEC and RCC significantly increased when the slaughter age increased from 150 ($P < 0.05$) to 180 ($P < 0.001$) days. The weight of all viscera, except the intestines, was higher in males compared to females ($P < 0.001$). The weight of intestines ($P < 0.05$) and heart ($P < 0.001$) was higher in 180 days old birds compared to younger birds .

Table 3. Least square means of carcass weight data by age and sex of the Milanino breed of chicken

Carcass weight data[1]	Sex		Age (days)		s.e.
	Females	Males	150	180	
BW (g)	2,043.40^{A}	2,614.10^{B}	2,214.50^{c}	2,443.00^{d}	46.03
PEC (g)	1,760.50^{A}	2,215.00^{B}	1,862.50^{C}	2,113.00^{D}	38.22
RCC (g)	1,329.50^{A}	1,744.12^{B}	1,418.87^{C}	1,654.75^{D}	28.25
PEC (% BW)	86.04	84.80	84.26^{c}	86.58^{d}	0.58
RCC (% BW)	64.96^{a}	67.05^{b}	64.30^{C}	67.71^{D}	0.44
Intestines (g)	91.84	84.55	81.08^{c}	95.39^{d}	2.67
Cecum (g)	16.30^{A}	20.63^{B}	17.73	19.20	0.55
Gizzard (g)	52.44^{A}	68.96^{B}	60.16	61.24	3.31
Spleen (g)	2.30^{A}	3.46^{B}	2.87	2.89	0.16
Heart (g)	8.63^{A}	13.82^{B}	9.50^{C}	12.95^{D}	0.48
Liver (g)	34.99^{A}	40.37^{B}	36.79	38.59	0.77

[1]BW = live body weight; PEC = partial eviscerated carcass; RCC = ready-to-cook carcass

$^{A, B}$ Values within a row with different superscripts differ significantly at P<0.001 between the sexes

$^{a, b}$ Values within a row with different superscripts differ significantly at P<0.05 between the sexes

$^{C, D}$ Values within a row with different superscripts differ significantly at P<0.001 between the two ages of slaughter

$^{c, d}$ Values within a row with different superscripts differ significantly at P<0.05 between the two ages of slaughter

Meat composition

According to the results of the analysis of variance, the sex and the age of slaughter were the only sources of variation significantly affecting the meat composition characteristics. All significant results related to the chemical characteristics of meat are reported in Table 4.

Dry matter and protein contents (%) of the breast meat were higher in females compared to males ($P < 0.05$) and in birds slaughtered at 180 days of age compared to 150 days ($P < 0.05$) (Table 4). In contrast, ether extract and ash contents (%) were very similar in the breast meat of both sexes and of birds slaughtered at 180 and 150 days of age. In the thigh meat, dry matter and ether extract contents (%) were higher in females compared to males ($P < 0.001$), and the protein content (%) increased in birds slaughtered at the older age ($P < 0.05$) (Table 4).

Table 4. Least square means of chemical meat composition by age and sex of the Milanino breed of chicken

	Sex		Age (days)		
	Females	**Males**	**150**	**180**	**s.e.**
Breast muscle					
Dry matter (%)	28.74[a]	27.19[b]	27.32[c]	28.21[d]	0.23
Total proteins (%)	25.77[a]	24.97[b]	24.74[C]	26.00[D]	0.15
Total lipids (%)	0.55	0.15	0.54	0.15	0.17
Ash (%)	1.17	1.11	1.11	1.17	0.02
Thigh muscle					
Dry matter (%)	27.18[A]	24.64[B]	25.53	26.30	0.28
Total proteins (%)	21.12	20.60	20.46[c]	21.26[d]	0.22
Total lipids (%)	2.93[A]	1.09[B]	2.32	1.71	0.27
Ash (%)	1.06	1.07	1.05	1.08	0.02

[A, B] Values within a row with different superscripts differ significantly at P<0.001 between the sexes

[a, b] Values within a row with different superscripts differ significantly at P<0.05 between the sexes

[C, D] Values within a row with different superscripts differ significantly at P<0.001 between the two ages of slaughter

[c, d] Values within a row with different superscripts differ significantly at P<0.05 between the two ages of slaughter

Discussion

Milanino is confirmed to be a heavy breed with a characteristic sexual dimorphism in relation to adult body weight (Mosca *et al.,* 2015), being 2,775 g in males and 2,306 in females on 180 days of age. This sexual dimorphism became markedly and constantly evident from 35 days of age onwards. The body weight of Milanino chickens on 180 days of age ranged between 2,798 and 2,278 g and these values are higher than those reported for other Italian chicken breeds reared outdoor and slaughtered on similar or older age, such as the Padovana (De Marchi *et al.,*2005; Zanetti *et al.,* 2010), the Modenese and Romagnola (Sabbioni *et al.,* 2006) and the Bionda Piemontese and Bianca di Saluzzo breeds (Schiavone *et al.,* 2015). Few other Italian chicken breeds, such as the Ermellinata di Rovigo (Zanetti *et al.,* 2010) and the Robusta Maculata (Rizzi and Chiericato, 2010), presented a body weight similar to the Milanino breed at 180 days of age. At 150 days of age, Milanino birds are heavier (males = 2,604 g; females= 2,157 g) than the Bresse birds. Bresse is the only French chicken breed with an AOC (=Appellation d'Origine Controllée) brand and a production of 1.4 million/chickens/year. Specific rules for rearing the Bresse are prescribed by law and the age of slaughter must be at least 112 and up to 150 days of age (Verrier *et al.,* 2005).

In poultry production, the level of dietary protein for broiler chickens was widely studied (Olomu and Offiong, 1980; Ojewola and Longe, 1999), while data on optimal nutrient density for slow growing genotypes, especially for indigenous chickens, is limited. Some studies report that commercial broilers generally are fed a relatively high nutrient diet to achieve a fast growth rate, and a low nutrient diet is used to secure the slow growth rate of indigenous breeds (Komprda *et al.,* 2000; Sundrum, 2006). The well-known French Label Rouge program requires slow growing genotypes and a low nutrient diet (Fanatico and Born, 2002). Wang *et al.* (2013) found a higher body weight in both fast and

slow growing genotypes given a high nutrient diet at 63 days; however, the opposite relationship occurred at 105 days of age. In this study the protein level of the diet did not affect the overall Milanino mean body weight recorded in the straight-run group in the whole rearing period. A similar result was found in a Chinese native breed, the Wuding, whose body weight was not affected by the dietary protein level during all rearing period from 1 to 60 days (Li *et al.,* 2013). The present results suggest that the low protein level was adequate to satisfy the protein requirements for the full growth of the birds. Therefore, the use of a low protein diet for rearing straight-run Milanino chickens for long rearing period is suggested. However in females, the best growth performance was recorded in birds fed the higher protein level from 125 days (18 weeks) of age onwards. This effect could be due to a relative higher protein requirement related to the major development of the female reproductive tract occurring few weeks before the onset of sexual maturity. It is generally known that laying hens starts to lay eggs at 18-20 weeks of age (Cerolini and Zaniboni, 2008). In layer type strains, protein requirement is 15% between 12 and 17 weeks of age and increases up to 17% at 18 weeks of age (Schiavone, 2008). At the onset of sexual maturity, a higher protein requirement for the development of ovary and oviduct occurs, and, as a consequence, Milanino females fed a higher protein diet gained more body weight. According to the practice of separate-sex feeding, a high protein feed is recommended to rear Milanino females from 125 days of age onwards.

In the present study, only the overall feed consumption has been measured in the growing period, from 35 to 180 days of age. According to the feed consumption data and the growing performance, the estimate feed efficiency value may be higher than 5. Few data on feed consumption and feed conversion ratio of local chicken breeds are available. Tixier-Boichard *et al.*(2006) provided the feed efficiency of seven French local chicken breeds that ranged between 4 and 6.5. Among these chicken breeds, the

best known Bresse presented a feed efficiency of 4.59. Compared with the standard broiler, the local chicken breeds are characterized by slow growth rates, requiring a very long rearing time (Culioli *et al.,* 1990), and low feed efficiency values, resulting in high production costs. The supplementation of poor nutrient, such as protein, density diets to slow growing birds is a key factor to reduce the production cost. Further studies are required to customize the feeding program to the peculiar nutrient requirements of the chicken breed in order to support the potential use of Milanino birds in sustainable productions, wishing to reach niche markets and marginal areas.

The carcass weight data confirmed the clear sexual dimorphism related to adult body weight characterizing the Milanino chicken breed. The weight of the PEC of Milanino chickens slaughtered between 150 and 180 days of age ranged between 1,722 and 2,253 g and these values are higher than those reported for other Italian chicken breeds reared outdoor and slaughtered on similar or higher age, such as the Padovana (De Marchi *et al.,* 2005), the Ermellinata di Rovigo (Zanetti *et al.,* 2010), the Modenese and Romagnola (Sabbioni *et al.,* 2006) and the Bionda Piemontese and Bianca di Saluzzo breeds (Schiavone *et al.,* 2015). The PEC of Milanino is heavier than the carcass of the Bresse chickens, whose minimum weight is fixed by law: 1200 g and 1800 g for males and females respectively (Verrier *et al.,* 2005).

According to their BW and to the meat products peculiar of the Italian poultry market (Cerolini, 2008), Milanino birds slaughtered on both 150 and 180 days of age could be used to produce meat cuts. The standard RCC is usually obtained with lighter birds weighting 1700-1800 g at slaughter; therefore, Milanino chickens should be slaughtered at younger ages to obtain a similar product.

A good slaughter performance was recorded in the Milanino breed and the general proportion of carcass yield (RCC) was 66%. Lower proportion of RCC were reported in other Italian chicken breeds slaughtered at 150 days of age or more: 63% and 62% in the Modenese and Romagnola breeds respectively

(Sabbioni *et al.,* 2006), 63% in the Padovana breed (Zanetti *et al.,* 2010), 59% and 58% in females of Bianca di Saluzzo and Bionda Piemontese breeds respectively (Schiavone *et al.,* 2015). In Milanino chickens, males showed the best performance (67%) compared to females (65%). This result can be related to the full development of the reproductive organs, having bigger size and weight in females, in birds slaughtered on adult ages. Moreover, the proportion of the RCC increased until the end of the growing period reaching the 68% at 180 days of age.

The chemical composition of Milanino meat is characterised by high protein and low fat contents compared to the standard broiler meat (USDA, 2015). The chemical composition of breast and thigh meat showed variations according to the sex and the age at slaughter. In females, meat contains higher proportions of dry matter (breast and thigh), protein (breast) and fat (thigh). The protein content of the meat increased in birds slaughtered at the older age, therefore the protein deposition in muscle tissue occurs until 180 days of age.

De Marchi *et al.* (2005) reported similar effects of the sex and the age on the chemical composition of the breast meat in the Padovana chicken breed. The dry matter and protein contents of Milanino chicken meat were similar to those reported in organic chickens (Castellini *et al.,* 2002) and in other Italian breeds, such as the Modenese and Romagnola (Sabbioni *et al.,* 2006). In contrast, Zanetti *et al.* (2010) reported lower dry matter and protein contents in the breast meat of the Padovana chickens. In females, the dry matter and protein contents found in the Milanino meat were higher compared to the same contents found in Bianca di Saluzzo and Bionda Piemontese meat (Schiavone *et al.,* 2015). The lipid and ash contents of breast meat in the Milanino breed was very similar in both sexes and in birds slaughtered at 150 and 180 days of age, in agreement with results measured in other Italian breeds (Sabbioni *et al.,* 2006; Zanetti *et al.,* 2010; Schiavone *et al.,* 2015). Souza *et al.* (2011) reported a lower protein and a similar lipid content of breast meat in two

free-range broiler strains (Super Pesadao and Paraiso Pedres) slaughtered at 85 days of age compared to Milanino chickens.
Thigh meat has improved nutritive value, corresponding to higher protein and lower lipid content, in the Milanino breed compared to free-range broiler strains (Souza *et al.,* 2011). In contrast, the lipid content of Milanino meat was higher than the lipid content of meat obtained from the Thai indigenous breed, one of the native breeds commonly raised with low production costs under family farming systems in Thailand (Wattanachant *et al.,* 2004). However, the comparison between thigh meat composition of different local breeds is difficult due to the few data available on thigh meat composition.

Conclusions

Milanino is confirmed to be a heavy breed with a characteristic sexual dimorphism in relation to adult body weight. Growth performance is not compromised feeding low protein level to birds reared according to the straight-run free range system. High general proportion of carcass yield (RCC=66%) was recorded. Furthermore, Milanino meat was characterised by high protein and low fat contents compared to the standard broiler meat. The present results support the potential of Milanino chicken meat production for niche markets.

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Ex situ in vitro strategy

Semen cryopreservation

The general aim has been the identification of a reference procedure for the cryopreservation of chicken semen according to the "FAO guidelines - Cryoconservation of animal genetic resources" (2012). The reference procedure will be implemented for the constitution of a sperm cryobank of Italian poultry breeds, today not available at national level.
The following specific aims have been considered:

- the improvement of the cryoconservation success of chicken semen packaged into straws;
- the application of the reference procedure to the semen collected from the MI and MB males in order to assess the breed sensitivity to semen cryoconservation.

Commercial breeder lines have been used in the trials planned to study the reference cryoconservation procedure to take advantage of the following conditions related to birds: low cost, large numbers available all the year long, easy housing in cages and handling for semen collection, large volume and good sperm concentration of the ejaculates. Results are presented in form of manuscripts submitted for publication.

Effect of cooling rate on the survival of cryopreserved chicken spermatozoa: comparison of different heights on liquid nitrogen vapor.

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Introduction

Biodiversity and gene conservation are of great interest around the world. Although focused more on endangered species, these issues are also important for domestic livestock, especially in intensive poultry breeding which caused a rapid decline of genetic diversity in local breeds. (Váradi et al., 2013). According to the DAD-IS (Domestic Animal Diversity Information System), more than 50% of poultry species are in the endangered category (Hoffmann, 2005). Consequently, there was an urgent need to create gene banks. In addition to *in vivo* management, *in vitro* conservation is a strategic instrument to secure genetic diversity, taking into account the risk of epidemic diseases (Blesbois et al., 2007). Currently, local poultry genetic materials are stored in vitro in only four national gene banks (France, Netherlands, North-America and Japan) (Blackburn HD, 2006; Woelders et al., 2006; Blesbois et al., 2007; Blesbois, 2011). Although the cryopreservation is a valuable instrument for poultry industry the freezing of poultry semen is not yet a procedure commonly used in practice (Bellagamba et al.,1993; Fulton, 2006; Long, 2006; Blesbois, 2007). The percentages of fertility obtained by artificial insemination with frozen semen are not very high and this is due to some physiological and biological characteristics of chicken sperm that make it more sensitive to freezing (Donoghue and Wishart, 2000; Blesbois, 2007; Blanco et al., 2008). During the years have been studied many freezing methods, with different cryoprotectants such as glycerol, dimethyl acetamide (DMA), DMSO and recently N-methylacetamide (Tselutin et al., 1999; Sasaki et al., 2010), different sperm packaging such as pellets, vials and straws and with slow and rapid freezing procedures (Seigneurin and Blesbois., 1995; Tselutin et al., 1999; Blesbois et al., 2007; Blanco et al., 2012). In spite of these several studies, today the average fertility in chickens with frozen semen is equal to 60% (Blesbois, 2011), ranging from 0 to 90%. New studies are needed to increase the success of freezing methods. Although

glycerol is usually observed as a suitable cryoprotectant for poultry semen (Seigneurin and Blesbois, 1995), it has biological limitations as its contraceptive effect (Hammerstedt and Graham, 1992). It must therefore be removed before the use of semen in artificial insemination, which is an important deficiency. In addition, the required post thawing centrifugation of glycerol-treated spermatozoa leads to their detriment. Dimethylacetamide (DMA) may offer an alternative because semen samples can be thawed without additional processing, and high levels of fertility have been obtained with this cryoprotectant (Tselutin et al., 1999, Chalah et al., 1999). The best fertility rates are realized with DMA when the spermatozoa are frozen in pellets (Blesbois et al., 2007), but this method does not permit the proper identification of semen samples and also can occur cross-contamination (Wishart, 2009). All these problems can be avoided by the use of straws for semen packaging as required by the FAO guidelines (FAO, 2011). Previous reports of different freezing rates have usually utilized a programmable freezing machine (Santiago-Moreno., 2011; Blesbois et al., 2007; Blanco et al., 2012), which is not always available particularly in field condition. The straws can be frozen on a piece of styrofoam floating on nitrogen liquid (e.g. Dong *et al.,* 2009). It is difficult to compare different non-programmable freezing systems because they are influenced by many factors, as temperature inside and outside the straw, volume surface ratio of the straw and the ventilation. (FAO, 2011). Therefore, experimentation is needed to determine which conditions are optimal. The aim of the present study was to improve the success of cryopreservation in chicken semen packaged in straws and frozen in nitrogen vapor. The effect on sperm quality of different cooling rates obtained using different heights between straws and the liquid nitrogen have been studied. The final goal was to identify a reference procedure to implement in a semen cryobank for conservation of Italian chicken breeds.

Materials and Methods

Twenty-seven adult Lohmann male fowl (*Gallus domesticus*) were housed at 22 weeks of age in individual cages and kept at 20° C and 14L:10D photoperiod, at the Poultry Unit, Animal Production Centre, University of Milan (Lodi, Italy). Birds were given *ad libitum* access to a standard commercial chicken breeder diet (2800 kcal ME/kg, 15% crude protein, 3% ether extract, 10.5% ash, 3.10% calcium) and drinking water. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). After 2-week semen collection training period, all males were routinely collected twice a week from March to July. Semen was collected according to the technique initially described by Burrows and Quinn (1935). Each day of collection, ejaculates were randomly pooled (9 ejaculates/pool) into 3 semen samples and pools obtained in different days were always formed with different ejaculates to reduce the effect of the bird. The ejaculates were pooled into graduated tubes, semen volume was recorded and sperm concentration was measured using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm. Then, pooled semen samples were diluted in modified Lake prefreezing extender (8 g D-fructose, 5 g potassiumacetate,19.2 g sodium glutamate, 3 g polyvinylpyrrolidone, 0.7 g magnesium acetate, 3.75g glycine, adjusted to1L with distilled water; pH7.0, and osmolality 340 mOsmol/kg) to a concentration of 1.5×10^9 sperm/mL, cooled at 4° C for 20/30 minutes and transferred to the laboratory for further quality assessment, including sperm viability and motility, and freezing processing. Sperm viability was measured using the SYBR14/PI dual staining procedure (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano (2011) with minor modifications. In brief, the incubations were done at room temperature and the Lake's diluent (6 g glucose, 1.28 g potassium citrate,15.2 g sodium glutamate, 0.8 g magnesium acetate, 30.5 g

BES, 58 ml NaOH adjusted to1L with distilled water; pH7.05, and osmolality 411 mOsmol/kg) was used. Assessment of 200 spermatozoa was made in duplicate aliquots for every sample and evaluated microscopically at 100X total magnification using a Zeiss (Axioskop 40- AxioCamICc 1) microscope and FITC filter fluorescence. SYBR-14, a membrane-permeant DNA stain, stains only living spermatozoa in green. PI stains the nuclei of membrane-damaged cells red, so spermatozoa that exhibit green fluorescence are considered live, those that exhibit red fluorescence are considered dead. Sperm motility was assayed using a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Fresh pooled semen samples were further diluted in refrigerated 0.9 % NaCl to a concentration of 1.0 x 10^8 sperm/mL and incubated for 20 minutes at room temperature; then, 10 μL semen were placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and evaluated under the microscope at room temperature. The motion parameters recorded were: motile spermatozoa (%), progressive motile spermatozoa (%), curvilinear velocity [VCL, (μm/s)], straight-line velocity [VSL, (μm/s)], average path velocity [VAP, (μm/s)], amplitude of lateral head displacement [ALH, (μm)], beat cross frequency [BCF, (Hz)], linearity [LIN, (%)], straightness [STR, (%)] and wobble [WOB, (%)]. A minimum of three fields and 500 sperm tracks were analyzed at 10X magnification for each sample. After the assessment of sperm viability and motility, semen samples were further diluted to 1 x 10^9 sperm/mL with Lake prefreezing extender containing 18% DMA, leaving to 6% final DMA concentration (Zaniboni et al., 2014), equilibrated at 5°C for 1 min and loaded into 0.25-mL French straws (IMV Technologies, France). Each pooled semen sample was divided into three aliquots loaded into differently colored straws corresponding to different treatments during freezing in nitrogen vapor. The treatments considered were

different heights between the straws and the liquid nitrogen bath, providing different freezing rates. Two consecutive experimental protocols were run, the first in May and the second in July. In experiment 1 were compared the heights: 1, 3 and 5 cm. According to the results of experiment 1, in experiment 2 were compared the heights: 3, 7 and 10 cm. Freezing was performed using floating racks consisted of a wire netting sustained by a styrofoam frame of different heights in order to support the straws above the liquid nitrogen bath at the required treatment heights (1, 3, 5, 7, 10 cm). Three styrofoam boxes were loaded to a depth of about 4 cm with liquid nitrogen and the cover closed to allow the vapor to stabilize. The cover of the boxes was then opened and the racks with straws were placed to float on the liquid nitrogen. After 10 min, straws were plunged into liquid nitrogen and stored in cryotank. A total of 9 (n. of replicates) pooled semen samples were processed per experiment and a total of 18 straws were stored per treatment. The temperatures inside straws (n=3) exposed to each of the treatments were measured simultaneously with a frequency of one per 20 seconds during freezing in both experiments. The cooling velocity of the frozen semen was measured using thermocouple thermometer (80PK-1 K, Fluke-51/RS, Fluke Corporation, USA). The straws were thawed in water bath at 38°C for 30 sec and sperm quality was assessed in thawed semen. Sperm viability and motility were measured as previously described in fresh semen samples, with the exception of using 0.9% NaCl at room temperature for sample dilution before sperm motility analysis.

Statistical analysis

Analysis of variance on sperm quality parameters recorded in fresh and frozen/thawed semen samples was performed using the MIXED procedure of SAS (SAS, 1999). Treatments (different heights on the liquid nitrogen vapor), time (fresh and thawed semen), and the relative interactions were considered as fixed

effects and the pooled semen sample was considered as random effect. The *t* test was used to compare LSMeans. The recovery rates (%) of sperm viability, motility and progressive motility after cryopreservation were calculated as follows: [(mean on thawed semen*100)/mean on fresh semen]. Analysis of variance on the recovery variables was performed using the GLM procedure of SAS (SAS, 1999), and the treatment was the only source of variation included in the model. The *t* test was used to compare LSMeans. Data measured as proportions were transformed into arsin values before statistical analysis

Results

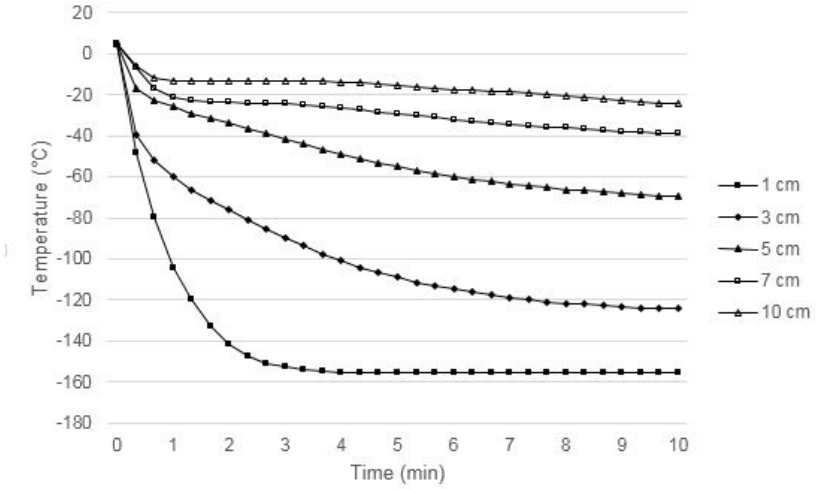
Fresh semen

The mean volume and sperm concentration recorded in fresh ejaculates were 0.2 ± 0.08 mL and 3.55 ± 0.84 x 10^9 sperm/mL respectively.

Measurement of freezing rates

As expected, the height of the straws above liquid nitrogen had a large effect on cooling rate as shown in Figure 1.

Fig. 1. Change in temperature of semen in straws while being suspended in 1 cm, 3 cm, 5 cm, 7 cm and 10 cm above liquid nitrogen during cryopreservation in nitrogen vapors.



Experiment 1: Effect of freezing semen packaged in straws and kept at 1, 3 and 5 cm in nitrogen vapor on sperm quality after thawing.

According to the results of the analysis of variance, sperm quality parameters were significantly affected by the freezing–thawing process (time effect $P < 0.001$) and not affected by the freezing rate (treatment 1, 3, 5 cm in nitrogen vapor $P > 0.05$) and the interaction time*treatment ($P > 0.05$). High proportion of motile sperm was recorded in all treatments (Table 1) and the recovery rate of motile sperm was also high, being between 36 and 41% (Table 2). Lower proportions of progressive motility were recorded, in particular after freezing/thawing (Table 1); therefore, lower recovery rates were found and only 20-24% of progressive motile sperm survived cryopreservation (Table 2). This result shows an increased sensitivity of the cells with progressive motility to the freezing process. All kinetic parameters

significantly decreased after freezing/thawing also and the same trend was recorded in all treatments (Table 1). The mean values of sperm viability recorded before and after cryopreservation were 74 and 41% respectively (Table 1). High proportions of viable sperm were recovered after freezing/thawing and no differences were found between treatments (Table 2).

Table 1. Sperm motility parameters measured in fresh semen and in semen frozen with three different heights over nitrogen vapors: 1 cm, 3 cm and 5 cm. (mean ± SE)

Sperm parameters	Fresh	Heights over nitrogen vapors		
		1 cm	3 cm	5 cm
Viability (%)	74.4 ± 1.9[a]	42.1 ± 2.2[b]	41.6 ± 2.2[b]	40.4± 2.2[b]
Motility (%)	78.1 ± 1.6[a]	29.7 ± 2.3[b]	31.1 ± 2.3[b]	27.3 ± 2.3[b]
Progressive motility (%)	10.8 ± 0.8[a]	1.4 ± 1[b]	1.3 ± 1[b]	1.2 ± 1[b]
VCL (µm/s)	47.2 ± 1.3[a]	27.8 ± 1.7[b]	27.6 ± 1.7[b]	27.2 ± 1.7[b]
VSL (µm/s)	15.2 ± 0.5[a]	7.3 ± 0.7[b]	7.2 ± 0.7[b]	7.1 ± 0.7[b]
VAP (µm/s)	26.9 ± 0.8[a]	13.8 ± 1[b]	13.7 ± 1[b]	13.6 ± 1[b]
LIN (%)	32 ± 0.4[a]	26 ± 0.6[b]	25.8 ± 0.6[b]	26 ± 0.6[b]
STR (%)	56.2 ± 0.5[a]	52.4 ± 0.8[b]	52.3 ± 0.8[b]	52.3 ± 0.8[b]
WOB (%)	56.9 ± 0.4[a]	49.4 ± 0.5[b]	49.3 ± 0.5[b]	49.6 ± 0.5[b]
ALH (µm)	3.2 ± 0.1[a]	2.9 ± 0.1[b]	2.8 ± 0.1[b]	2.5 ± 0.1[b]
BCF (Hz)	6.9 ± 0.2[a]	5.2 ± 0.4[b]	5.9 ± 0.4[b]	5.1 ± 0.4[b]

Motility, the percentage of motile spermatozoa; progressive motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble. [a,b] Values within each row with different superscript letters are significantly different ($p < 0.001$).

Table 2. Recovery rates of sperm quality recorded in semen frozen with three different heights over nitrogen vapors: 1 cm, 3 cm and 5 cm. (mean ± SE)

Sperm parameters	Recovery (%)		
	Heights over nitrogen vapors		
	1 cm	3 cm	5 cm
Viability (%)	56.6 ± 2.3	55.8 ± 2.3	54.7 ± 2.3
Motility (%)	39 ± 3.8	40.8 ± 3.8	35.8 ± 3.8
Progressive motility (%)	24.2 ± 6.6	20.5 ± 6.6	20.4 ± 6.6

Experiment 2: Effect of freezing semen packaged in straws and kept at 3, 7 and 10 cm in nitrogen vapor on sperm quality after thawing.

In experiment 2, two new heights, 7 and 10 cm, were compared to 3 cm as representative of the range 1-5 cm studied in experiment 1. According to the results of the analysis of variance, sperm quality parameters were significantly affected by the freezing–thawing process (time effect $P < 0.001$). The freezing rate (treatment 3, 7, 10 cm in nitrogen vapor) and the interaction time*treatment significantly affected sperm motility ($P < 0.001$) and viability ($P < 0.001$), and three kinetic parameters (STR, ALH and BCF; $P < 0.05$). The highest percentage of viable and motile spermatozoa in cryopreserved semen was observed in the treatment 3 cm (46% and 22%, respectively) (Table 3). The recovery rate of viable sperm in the treatment 3 cm was 58% and it was significantly higher than those measured in the treatments 7 cm (47%) and 10 cm (44%) (Table 4). This result shows that sperm integrity is better preserved during cryopreservation freezing the semen in nitrogen vapor at a height of 3 cm. Also the recovery rate of motile cells was significantly higher in treatment 3 cm compared to the other treatments (26% *vs.* 16.6%). Progressive motility was greatly reduced after freezing/thawing and less than 1% of progressive motile sperm were recorded after

thawing in all treatments (Table 3); however, the recovery rate of progressive motile sperm was significantly higher in the treatment 3 cm compared to the treatments 7 and 10 cm (Table 4). The freezing/thawing process significantly reduced all the kinetic parameters in all the treatments (Table 3), with the only exception of ALH value that did not differ between fresh and thawed semen in the treatment 3 cm. After freezing/thawing, STR, ALH and BCF mean values measured in the treatment 3 cm were significantly higher compared to the mean values measured in treatments 7 and 10 cm (Table 3).

Table 3. Sperm motility parameters measured in fresh semen and in semen frozen with three different heights over nitrogen vapors: 3 cm, 7 cm and 10 cm. (mean ± SE)

Sperm parameters	Fresh	Heights over nitrogen vapors		
		3 cm	7 cm	10 cm
Viability (%)	78.3 ± 2.1^{a}	46.1 ± 2.3^{b}	36.4 ± 2.3^{c}	34.8 ± 2.3^{c}
Motility (%)	86 ± 1^{a}	22 ± 1.3^{b}	14.1 ± 1.3^{c}	14.3 ± 1.3^{c}
Progressive motility (%)	16.2 ± 0.4^{a}	0.6 ± 0.4^{b}	0.3 ± 0.4^{b}	0.3 ± 0.4^{b}
VCL (µm/s)	53.3 ± 1.5^{a}	28.5 ± 1.7^{b}	27.3 ± 1.7^{b}	27.5 ± 1.7^{b}
VSL (µm/s)	18.1 ± 0.5^{a}	7 ± 0.6^{b}	6.2 ± 0.6^{b}	6.4 ± 0.6^{b}
VAP (µm/s)	30.9 ± 1^{a}	13.8 ± 1^{b}	12.9 ± 1^{b}	13.2 ± 1^{b}
LIN (%)	34.1 ± 0.5^{a}	24.4 ± 0.6^{b}	22.7 ± 0.6^{b}	23.2 ± 0.6^{b}
STR (%)	58.9 ± 0.5^{A}	50.4 ± 0.6^{B}	48.3 ± 0.6^{C}	48.3 ± 0.6^{C}
WOB (%)	57.9 ± 0.6^{a}	48.3 ± 0.7^{b}	46.9 ± 0.7^{b}	47.9 ± 0.7^{b}
ALH (µm)	3.2 ± 0.1$^{\alpha}$	2.8 ± 0.2$^{\alpha}$	1.9 ± 0.2$^{\beta}$	2 ± 0.2$^{\beta}$
BCF (Hz)	7.8 ± 0.3^{a}	4.4 ± 0.4^{b}	2.7 ± 0.4^{c}	2.1 ± 0.4^{c}

Motility, the percentage of motile spermatozoa; progressive motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble. $^{a-c}$ Values within each row with different superscript letters are significantly different ($p<0.001$). $^{A-C}$ Values within each row with different superscript letters are significantly different ($p<0.05$). $^{\alpha-\beta}$ Values within each row with different superscript letters are significantly different ($p<0.01$).

Table 4. Recovery rates of sperm quality parameters recorded in semen frozen with three different heights over nitrogen vapors: 3 cm, 7 cm and 10 cm. (mean ± SE)

Sperm parameters	Recovery (%)		
	Heights over nitrogen vapors		
	3 cm	7 cm	10 cm
Viability (%)	58.7 ± 1.16[a]	46.6 ± 1.16[b]	44.1 ± 1.16[b]
Motility (%)	25.7 ± 2.3[a]	16.5 ± 2.3[b]	16.6 ± 2.3[b]
Progressive motility (%)	3.9 ± 0.5[a]	1.9 ± 0.5[b]	1.6 ± 0.5[b]

[a,b] Values within each row with different superscript letters are significantly different ($p<0.001$).

Discussion

Freezing semen packaged in straws over nitrogen vapor is a simple, quick and inexpensive method, widely used for cryopreservation of mammalian semen, even in commercial AI Centers. This method has the great advantage to allow the adoption of temperature gradients suitable for freezing semen in liquid nitrogen without the need of expensive dedicated equipments. In fact, the distance between the straw and the liquid nitrogen bath indirectly determines the thermal gradient during the real freezing phase when the change from the liquid to the solid state occurs. The present results provide the range of heights suitable to freeze chicken semen packaged into straws over liquid nitrogen vapor. The optimal range is identified from 1 to 5 cm over the liquid nitrogen bath and it allows to recover the best proportion of viable and motile sperm after freezing/thawing. The range of 1-5 cm provided fast cooling rates associated with the recovery of 56% viable sperm and 36% mobile sperm in experiment 1, and of 59% viable sperm and 26% mobile sperm in experiment 2. The heights of 7 and 10 cm provided slower cooling rates associated with higher cell damage and loss in sperm viability and mobility, therefore they are not

suitable to freeze chicken semen. Santiago-Moreno et al. (2011) obtained the best results in the percentage of sperm motility after freezing with medium freezing rate, compared to a slow and a rapid freezing rate. Their best values (15%) were lower than ours (ranged from 22% to 31%). Also about the sperm viability our data were higher (40-46 %) than their data (13-24%). The sperm of certain avian species, in particular imperial eagles and chickens, may accomplish rapid cooling by maintaining reasonable percentages of viability after thawing (Blanco et al. 2000). Very low proportions of progressive motile sperm (<1.4%) have been measured in chicken semen after freezing/thawing even with the best fast freezing rates, confirming other values already reported in the literature. Purdy et al. (2009) reported 15% motility and 1.8% progressive motility in chicken semen after freezing over nitrogen vapour at the height of 1 cm for 7 minutes and thawing. Even Santiago-Moreno et al. (2012) presented similar values of percentage progressive motility (<5%) and sperm motility (25%), while the values of viability (10-30%) are significantly lower than ours. Each height corresponds to a different freezing curve. The temperature inside the straws kept at 1 and 3 cm above liquid nitrogen dropped below -40°C within the first min, the same temperature into straws kept at 5 cm was reached after the third min, and the temperature never dropped below -24°C and -39°C into straws kept at 7 and 10 cm respectively even after 10 min. In Morris et al. (1999), the best human sperm survival was obtained with freezing curve in which the temperature of -40 °C was reached earlier compared to the other curves. During the process of freezing, cells undergo several changes and the cooling rate is critical for their survival, in particular to avoid the intracellular ice formation. (Mazur, 1977; Viveiros et al., 2001; Woelders and Chaveiro, 2004). The present results confirm that fast cooling rates are required to reduce injures in chicken sperm during the freezing process. The critical temperature of -25°C must be reached within 30 s and -40°C within 3 min. Motility is compromised as a result of poultry semen cryopreservation and

30 to 60% reductions occur after freeze/thaw cycle (Long 2006) and so also all the kinetic parameters. Froman and Feltmann (2000) reported VSL to be the most accurate estimate of sperm cell velocity. In Froman (2007), VSL must be >30 μm/s for a sperm from an overlaid sperm suspension to penetrate an Accudenz solution. In our study, VSL values were lower than 30 μm/s, but were in agreement with the values obtained from Santiago-Moreno et al (2012). Regarding the other kinetic parameters, our values are similar or just lower than those described by Santiago-Moreno et al (2012). This could be because their chicken were native Spanish breeds unlike our birds were Lohmann. Our values of ALH (amplitude of lateral head displacement) in treatment of 3 cm were not different from fresh semen; it has been documented that the ALH value is a good predictor for successful in vitro fertilization in human (Chan et al., 1990). In conclusion this study showed that the freezing of rooster semen can be done over nitrogen vapor using a wide range of very small heights (from 1 to 5 cm), which can be associated with a gradient of very rapid freezing, avoiding the use of expensive programmable freezer. These freezing method preserved poultry semen, maintaining adequate percentage of viable and motile sperm and some kinetic parameters involved in the progressive motility and made it suitable for inseminations, next step to confirm the results obtained *in vitro*.

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Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm

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Abstract

The aim of this study was to assess the combined effect of dimethylacetamide (DMA) and two non-permeating cryoprotective agents, trehalose and sucrose, on the quality of post-thaw chicken semen. Ejaculates were obtained from twenty-seven Lohmann male fowl (*Gallus domesticus*). Each day of collection, all ejaculates were pooled in three semen samples, each one splitted into four aliquots, processed according to the following treatment: Lake pre-freezing extender + 6% DMA (LPF, control treatment), LPF + 0.1 M trehalose (LPF-T treatment), LPF + 0.1 M sucrose (LPF-S treatment) and LPF + 0.1 M trehalose + 0.1 M sucrose (LPF-TS treatment). All semen samples were loaded into straws and frozen in nitrogen vapors. After a week, the straws were thawed in water bath at 38°C for 30 s and sperm quality (viability, mobility and kinetic parameters) was assessed immediately after thawing (T0) and at 5 (T5), 10 (T10) and 15 minutes (T15) thereafter. The majority of the sperm quality parameters, except VCL and BCF, showed a major significant decrease between fresh and cryopreserved semen (T0) and a further progressive significant decrease after 5, 10 and 15 min after thawing. The curvilinear velocity (VCL) did not show significant changes during the whole *in vitro* processing, and the beat cross frequency (BCF) showed a significant decrease only 10 min after thawing. The cryoprotectants did not affected sperm viability, and sperm motility and progressive motility. In contrast, the different cryodiluents combinations significantly affected the kinetic parameters, with the only exception of VCL. The presence of trehalose, alone or with sucrose, combined to DMA improved the quality of motion in cryopreserved sperm in comparison to DMA alone (LPF) and DMA with sucrose (LPF-S). In particular, the highest values in linearity (LIN) and wobble (WOB) were measured in the treatment LPF-T. The treatments significantly affected the recovery rate of progressive motile sperm that presented the best value soon after thawing in the LPF-T

treatment; moreover, the presence of trehalose, alone (LPF-T) or with sucrose (LPF-TS), significantly improved the recovery rate of progressive motile sperm also at T5 and T10 compared to LPF and LPF-S. The present results show a positive synergic action of DMA and trehalose on motile function of thawed chicken sperm.

Introduction

The most feasible method for *ex situ* management of genetic resources in birds is semen cryopreservation [Gee, 1995; Hammerstedt, 1995; Blesbois et al., 2008], which has firstly been studied in the chicken and then in other domesticated birds, such as turkey, duck and goose [Lake, 1986; Surai and Wishart, 1996; Blesbois, 2007]. Despite years of research, the cryopreservation of poultry sperm still cannot be carried out efficiently [Long, 2006; Blesbois, 2007]. The low quality of frozen-thawed poultry semen as well as the poor fertilization rates, obtained in avian as opposed to mammalian species, are attributable to the unique morphological characteristics of avian sperm, which make them more susceptible to freezing damage [Donoghue and Wishart, 2000; Long, 2006]. A variety of semen cryopreservation protocols involving different cryoprotective agents (CPAs), packaging methods and freezing and thawing rates have been developed in different poultry species [Blesbois 2007; Blesbois 2011; Iaffaldano 2015]. The choice of the CPA is certainly among the most important factors for an effective poultry semen freezing protocol. Despite decades of research on the use of permeant CPAs (P-CPAs) [Sexton, 1975; Tselutin et al, 1999; Blanco et al, 2011], quality of avian sperm after freezing/thawing procedures and relative fertilization rates remain highly variable. In chickens, the average fertility after artificial insemination of frozen/thawed semen is equal to 60%, ranging from 0 to 90% [Blesbois, 2011]. In various mammalian species, sperm cryosurvival has been improved by combining P-CPAs with non-permeant CPAs (N-CPAs) [Woelders et al., 1997; Aboagla and Terada, 2003;

Guttierrez-Perez et al., 2009]. The combination of P-CPAs and N-CPAs provides different mechanisms to protect spermatozoa during freezing/thawing procedures. P-CPAs 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 mechanisms of sperm death [Holt, 2000; Swain and Smith, 2010]. However, P-CPA themselves 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]. In contrast, N-CPAs are generally large, nontoxic, hydrophilic molecules (sugars, proteins or aminoacids) playing a different protective effect: because of the inability to diffuse across the plasma membrane, these substances create an osmotic pressure that lowers the freezing temperature of the medium and decrease extra-cellular ice formation [Aisen et al., 2002]. The use of N-CPAs, that act mainly as osmoprotectants, could reduce the amount of P-CPAs needed in sperm cryopreservation. Among the disaccharides, sucrose and trehalose are N-CPAs widely studied in different mammalian species: bulls [Woelders et al., 1997], goats [Aboagla and Terada, 2003] boars [Guttierrez-Perez et al., 2009] and rabbits [Rosato and Iaffaldano, 2013]. In contrast, the effect of trehalose and sucrose on the post-thaw quality of poultry sperm was poorly studied and few reports are available. Recently, Blanco et al [2011] tested trehalose and/or sucrose in combination with the P-CPA dimethylacetamide (DMA) and reported an improved post-thawing motility of turkey semen, which was dependent upon DMA concentration. Although sucrose and trehalose have received some attention as osmoprotectants for chicken sperm in the past [Sexton, 1975; Terada et al., 1989], there is a lack of current original studies on the effect of N-CPAs in this species.

The aim of this study was to assess the combined effect of DMA and the N-CPA trehalose and sucrose on the quality of post-thaw chicken semen.

Materials and Methods

Twenty-seven adult Lohmann male fowl (*Gallus gallus domesticus*) were housed at 28 weeks of age in individual cages and kept at 20° C and 14L:10D photoperiod at the Poultry Unit, Animal Production Centre, University of Milan (Lodi, Italy). Birds were fed *ad libitum* a standard commercial chicken breeder diet (2800 kcal ME/kg, 15% CP)) and drinking water. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching [FASS, 2010]. After 2-week semen collection training period, all males were routinely collected twice a week from May to June. Semen was collected according to the technique initially described by Burrows and Quinn [1935]. Each day of collection, males were divided in three different groups (nine birds/group) and all ejaculates collected within one group were pooled into one semen sample. Pools obtained in different days were always formed with different ejaculates to reduce the effect of the bird. The ejaculates were pooled into graduated tubes, semen volume was recorded and 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 JP, McDaniel GR. 1985]. Then, each pooled semen sample was splitted into four aliquots, each one assigned to one treatment. Semen aliquots were diluted to a concentration of 1.5 x 10^9 sperm/ml using 4 different cryodiluents: Lake pre-freezing modified extender (LPF, control treatment; 8 g D-fructose, 5 g potassiumacetate,19.2 g sodium glutamate, 3 g polyvinylpyrrolidone, 0.7 g magnesium acetate, 3.75 g glycine, adjusted to 1L with distilled water; pH 7.0, osmolality 340 mOsmol/kg), LPF added with 0.1 M trehalose (LPF-T treatment), LPF added with 0.1 M sucrose (LPF-S

treatment) and LPF added with 0.1 M trehalose + 0.1 M sucrose (LPF-TS treatment). The diluted semen was immediately cooled and kept at 4° C for 30 minutes. During this incubation, semen samples were transferred to the laboratory for further quality assessment and freezing processing. Sperm quality assessment included viability and motility. Sperm viability was measured using the dual fluorescent staining SYBR14/propidium iodide (PI) procedure (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano [2011] with minor modifications. In brief, the incubations were done at room temperature and the 7.1 diluent [Lake and Ravie, 1981] was used. Assessment of 200 spermatozoa was made in duplicate aliquots for every sample and evaluated microscopically at 1000X total magnification using a Zeiss (Axioskop 40- AxioCamICc 1) microscope and FITC filter fluorescence. Sperm motility was assayed using a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Fresh pooled semen samples were further diluted in refrigerated 0.9% NaCl to a sperm concentration of 100 x 10^6/ml and incubated for 20 minutes at room temperature. Then, 10 µl semen were placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and evaluated under the microscope at room temperature. The motion parameters recorded were: motile spermatozoa (%), progressive motile spermatozoa (%), curvilinear velocity [VCL, (µm/s)], straight-line velocity [VSL, (µm/s)], average path velocity [VAP, (µm/s)], amplitude of lateral head displacement [ALH, (µm)], beat cross frequency [BCF, (Hz)], linearity [LIN, (%)], straightness [STR, (%)] and wobble [WOB, (%)]. A minimum of 3 fields and 500 sperm tracks were analyzed at 100X total magnification for each sample. After the assessment of sperm quality, semen aliquots were further diluted to 1 x 10^9 sperm/ml with the corresponding extender (LPF, LPF-T, LPF-S and LPF-TS) containing 18% dimethylacetamide (DMA) to 6%

final DMA concentration [Zaniboni et al., 2014], equilibrated at 5°C for 1 min and loaded into 0.25-ml French straws (IMV Technologies, France). Four different straw colors were used according to the 4 different treatments. Straws were transferred on racks (made of wire netting supported by a Styrofoam frame) floating over a nitrogen bath at 3 cm of height (unpublished results), frozen for 10 min and then plunged into liquid nitrogen. Straws were stored in cryotank for at least 7 days. Semen collection was repeated on four days to process 12 pooled semen samples (12 replicates per treatment) and a total of 24 straws were stored per treatment. The straws were thawed in water bath at 38°C for 30 s and sperm quality was assessed in thawed semen. Sperm viability was recorded immediately after thawing (T0), and after 10 min; sperm motility was recorded immediately after thawing (T0), and after 5 (T5), 10 (T10) and 15 minutes (T15). Sperm viability and motility were measured as previously described, with the exception of using 0.9% NaCl at room temperature for sample dilution before sperm motility analysis.

Statistical analysis

Analysis of variance on sperm quality parameters recorded in fresh and frozen/thawed semen samples was performed using the MIXED procedure of SAS [SAS, 1999]. Treatment (DMA; DMA+trehalose; DMA+sucrose; DMA+trehalose+sucrose), time (fresh semen; 0, 5, 10 and 15 min after thawing), and the relative interaction (treatment * time) were considered as fixed effects and the pooled semen sample was considered as random effect. The *t* test was used to compare LSMeans.
The recovery rates (%) of sperm viability at different time (T0, T10) after cryopreservation were calculated as follows: [(mean on thawed semen*100)/mean on fresh semen]. The same formula was used to calculate the recovery rates (%) of sperm motility and progressive motility at different time (T0, T5, T10, T15) after cryopreservation. Analysis of variance on the recovery variables

was performed using the GLM procedure of SAS [SAS, 1999], and the treatment was the only source of variation included in the model. The *t* test was used to compare LSMeans.

Results

The mean volume and sperm concentration recorded in fresh ejaculates were 0.2 ± 0.06 ml and 3.70 ± 0.78 x 10^9 sperm/ml respectively.

The results of the analysis of variance showed that the effect of time ($P < 0.001$) and of the treatment ($P < 0.05$) significantly affected the majority of the semen quality parameters; in contrast, the interaction time*treatment did not affected semen quality measured before and after cryopreservation.

The majority of the sperm quality parameters were significantly affected by the freezing-thawing process, and the further *in vitro* incubation after thawing, as reported in Table 1. A major decrease in the mean values was recorded between fresh and frozen-thawed semen samples (T0) and a further progressive significant decrease was recorded during 15 min interval after thawing. The mean proportion of viable sperm recorded in fresh semen was 72% and a significant progressive decrease to 36% and 28% was recorded after freezing/thawing at T0 and T10 respectively. The mean proportion of motile sperm showed a major significant decrease between fresh and cryopreserved semen (T0) and a further progressive significant decrease at 5, 10 and 15 min after thawing (Table 1). A similar trend was also observed for all kinetic parameters, with the exception of VCL and BCF. VCL mean values recorded during the whole *in vitro* processing did not show significant changes, and BCF mean values recorded before cryopreservation, at T0 and T5 were similar and a significant decrease occurred at T10 and T15 (Table 1).

Table 1. Sperm quality parameters (LSMeans ± S.E.) measured in fresh semen and in immediately thawed semen (T0) and after five (T5), ten (T10) and fifteen (T15) minutes.

Sperm parameters[1]	Fresh	T0	T5	T10	T15	S.E.
Viability (%)	71.8 ^{A}	35.9^{B}		28.4 ^{C}		1.3
Motility (%)	86.8 ^{A}	33.3 ^{B}	25.8 ^{C}	19.9 ^{D}	15.9 ^{E}	1.5
Progr. motility (%)	18.6 ^{A}	3.1 ^{B}	1.9 ^{C}	1.2 ^{CD}	0.8 ^{D}	0.5
VCL (µm/s)	57.2	37.5	33.9	31.5	29.9	1.2
VSL (µm/s)	20.6 ^{A}	11.0 ^{B}	9.6 ^{C}	8.5 ^{D}	7.8 ^{D}	0.4
VAP (µm/s)	35.1 ^{A}	19.7 ^{B}	17.5 ^{C}	15.9 ^{D}	14.7 ^{D}	0.7
LIN (%)	36.9 ^{A}	29.0 ^{B}	27.5 ^{C}	26.8 ^{CD}	25.8 ^{D}	0.8
STR (%)	59.4 ^{A}	55.1 ^{B}	54.1 ^{BC}	52.8 ^{CD}	52.2 ^{D}	0.8
WOB (%)	62.0 ^{A}	52.4 ^{B}	51.1 ^{BC}	50.2 ^{CD}	48.9 ^{D}	0.8
ALH (µm)	3.3 ^{A}	2.9 ^{B}	2.8 ^{BC}	2.6 ^{CD}	2.5 ^{D}	0.1
BCF (Hz)	5.9 ^{A}	6.4 ^{A}	6.2 ^{A}	5.5 ^{B}	4.9 ^{B}	0.3

[1]Viability, the percentage of viable spermatozoa; Motility, the percentage of motile spermatozoa; Progr (Progressive) motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble.

^{A,B,C,D,E} Values within a row with different superscripts differ significantly at $P<0.001$

The cryoprotectants did not significantly affected sperm viability, motility and progressive motility, and similar mean values were recorded in all treatments (Table 2). In contrast, the different DMA and N-CPAs, trehalose and sucrose, combinations significantly affected the kinetic parameters, with the only exception of VCL. The presence of trehalose, alone or with sucrose, combined with DMA improved the quality of motion in cryopreserved sperm in comparison to DMA alone (LPF) and DMA with sucrose (LPF-S). In particular, the highest values in LIN and WOB were measured in the treatment LPF-T (Table 2).
According to the analysis of variance, the treatment significantly affected the recovery rate of progressive motile sperm only. High proportions of viable sperm were recovered immediately after thawing (range 47-53%) and after ten minutes incubation (35-44%) and no differences were found between treatments. The same result was found for the recovery rate of motile sperm and the range value recorded at T0, T5, T10 and T15 was 33-42%, 27-34%, 20-27% and 16-21% respectively. Low recovery rates of progressive motile sperm were found in all treatments (Table 3). The best recovery rate of progressive motile sperm, 24%, was recorded soon after thawing in the LPF-T treatment; moreover, the presence of trehalose, in LPF-T and LPF-TS, significantly improved the recovery rate of progressive motile sperm also at T5 and T10 compared to LPF and LPF-S. The recovery of progressive motile sperm progressively decreased after thawing and very low similar values were found at T15 in all treatments, included those with trehalose (Table 3).

Table 2. Influence of cryodiluent composition on post-thaw sperm quality parameters (LSMeans ± S.E.) of chicken semen.

Sperm parameters[1]	LPF[2]	LPF-S[2]	LPF-ST[2]	LPF-T[2]	S.E.
Viability (%)	46.9	44.7	43.4	46.4	1.3
Motility (%)	35.8	35.3	35.9	38.4	1.5
Progr. motility (%)	4.8	4.9	5.2	5.6	0.5
VCL (µm/s)	36.8	37.6	38.1	39.5	1.2
VSL (µm/s)	11.1 ^{A}	10.8 ^{A}	11.7 ^{B}	12.4 ^{B}	0.4
VAP (µm/s)	20.0 ^{A}	19.8 ^{A}	20.7 ^{AB}	21.9 ^{B}	0.7
LIN (%)	29.2 ^{A}	27.5 ^{B}	29.7 ^{A}	30.8 ^{C}	0.8
STR (%)	54.4 ^{A}	52.8 ^{B}	55.3 ^{AC}	56.2 ^{C}	0.8
WOB (%)	53.0 ^{A}	51.3 ^{B}	52.9 ^{A}	54.5 ^{C}	0.8
ALH (µm)	2.8 ^{a}	2.8 ^{a}	2.9 ^{ab}	3.1 ^{B}	0.1
BCF (Hz)	5.4 ^{A}	5.2 ^{A}	6.1 ^{B}	6.4 ^{B}	0.3

[1]Viability, the percentage of viable spermatozoa; Motility, the percentage of motile spermatozoa; Progr. (Progressive) motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble.

[2]LPF, Lake pre-freezing diluent containing DMA alone; LPF-S, LPF containing DMA and sucrose; LPF-ST, LPF containing DMA, sucrose and trehalose; LPF-T, LPF containing DMA and trehalose.

[A,B,C] Values within a row with different superscripts differ significantly at P<0.001

[a,b] Values within a row with different superscripts differ significantly at P<0.05

Table 3. Influence of cryodiluent composition on recovery rates of progressive motile sperm (LSMeans ± S.E.) of chicken semen.

Time after thawing (min)	LPF[1]	LPF-S[1]	LPF-ST[1]	LPF-T[1]	S.E.
T0	15.1 [a]	13.7 [a]	13.6	23.6 [b]	2.1
T5	7.1 [a]	6.6 [a]	13.1 [b]	14.5 [b]	1.4
T10	3.9 [a]	4.1 [a]	9.1 [b]	8.7 [b]	1.1
T15	3.1	3.0	5.5	4.8	0.8

[1]LPF, Lake pre-freezing diluent containing DMA alone; LPF-S, LPF containing DMA and sucrose; LPF-ST, LPF containing DMA, sucrose and trehalose; LPF-T, LPF containing DMA and trehalose.
[a,b] Values within a row with different superscripts differ significantly at $P<0.05$

Discussion

Currently, the need for *ex situ* conservation of avian genetic resources is widely recognized [Fulton, 2006], but the storage of semen collected from rare breeds and/or pure lines in sperm cryobanks has been so far considered in few national programs for conservation of animal genetic resources, i.e. in France and The Netherland in Europe and in North America [Blackburn 2006; Woelders et al., 2006; Blesbois et al., 2007; Blesbois 2011]. In 2012, FAO guidelines for cryopreservation of animal genetic resources describe two procedures for chicken semen characterized by different diluents, cryoprotectants and temperature gradient [FAO, 2012]. A further cryoprotectant, methylacetamide, was successfully used to develop a procedure for freezing chicken sperm in Japanese [Sasaki et al., 2010] and Korean breeds [Lee et al., 2012] designated as 'Natural Monument'. Recent reviews confirmed the difficulty of obtaining cryopreserved avian semen without significant loss of fertilizing potential [Blesbois, 2012] and the need that future studies should be conducted with the aim of improving relative sperm fertility,

dependent by sperm quality, and fertility duration after freezing [Tajima, 2013]. Therefore, the standardization of cryopreservation procedures for chicken semen is still a matter of research.

In the present study, non-permeant cryoprotectants were tested in combination with DMA to improve chicken sperm survival and function, corresponding to the motion ability, after cryopreservation in semen packaged in straws, according to the FAO cryopreservation guidelines [FAO, 2012]. The effect of the concomitant presence of permeant- and non permeant cryoprotectants was assessed soon after thawing and after a short *in vitro* storage period in order to study the survival of sperm to the cryopreservation process and the potential cell lifespan after thawing. The final goal was to identify a reference procedure to be implemented in a sperm cryobank of Italian chicken breeds.

The control cryopreservation procedure used in the present study was adapted from a pellet procedure previously set up in our laboratory (Zaniboni et al, 2014). In order to improve the post-thaw quality of sperm packaged into straws, freezing was performed in nitrogen vapour 3 cm above the liquid nitrogen bath and thawing was performed at 38°C for 30 s (unpublished results). The sperm viability, motility and progressive motility recorded in chicken semen samples frozen/thawed according to the control procedure were 35, 33 and 3% respectively. Using a similar cryopreservation method, lower viability (24%) and motility (15%) values were reported in frozen/thawed semen from Spanish chicken breeds [Santiago-Moreno et al., 2011]. Purdy et al. [2009] also reported lower motility (15%) and progressive motility (1.8%) in chicken semen added with DMA and frozen over nitrogen vapour.

The proportions of viable, motile and progressive motile sperm recovered soon after thawing undergo a fast and progressive decrease within a very short interval. The loss is much more severe for sperm motility compared to viability; in fact, the proportion of motile and progressive motile sperm decrease by

52% and 74% respectively within 15 min after thawing, whereas the decrease of viable sperm was almost 20% within 10 min. The kinetic parameters indicative of the sperm quality motion follow a similar trend after thawing, even if the rate of decrease is less consistent.

In various mammalian species, sperm cryosurvival was improved by combining P-CPAs with N-CPAs [Woelders et al., 1997; Aboagla and Terada, 2003; Guttierrez-Perez et al., 2009] and, among N-CPAs, trehalose and sucrose were widely studied [Woelders et al., 1997; Aboagla and Terada, 2003; Guttierrez-Perez et al., 2009; Rosato and Iaffaldano, 2013]. A significant positive synergic action between glycerol and trehalose was reported in 1989 on motility of thawed chicken semen also [Terada et al., 1989]. However, since then no exhaustive reports were published on the effect of sucrose and trehalose on the success of cryopreservation in chicken sperm. The present study aimed to investigate the potential positive synergic action of DMA and N-CPAs, trehalose and sucrose, on the quality of cryopreserved chicken sperm. The results show that trehalose, but not sucrose, play a positive protective action during the cryopreservation of chicken sperm. In particular, even if sperm viability, motility and progressive motility were not affected, many kinetic parameters indicative of the quality of sperm movement were improved in frozen/thawed semen samples processed in presence of trehalose or trehalose and sucrose.

The lack of effect of trehalose combined with P-CPAs, DMA and glycerol, on the proportion of viable sperm after cryopreservation was previously reported in the *Gallus gallus* and *Alectoris Barbara* species [Madeddu et al., 2010]. Trehalose and/or sucrose combined to DMA did not improve post-thaw sperm motility also in the *Gru canadiensis* and, in contrast, a positive effect was found in the *Meleagris gallopavo* species [Blanco et al., 2011].

The presence of trehalose, alone or combined to sucrose, into the freezing diluent had a positive effect on VSL, LIN, WOB and BCF of post-thaw chicken semen. VSL was reported as the most

accurate estimate of sperm cell velocity [Froman and Feltmann, 2000] and it was also identified as an important quantitative trait related to fertility [Froman et al, 1999]. In fresh semen, VSL must be > 30 µm/s for a sperm to be able to penetrate a dense Accudenz solution [Froman, 2007]. Lower VSL values were found in frozen/thawed chicken sperm, corresponding to 11-12 µm/s, in agreement with the results reported in a previous scientific study where only P-CPAs were used [Santiago-Moreno et al, 2012]. The improvement of VSL in semen samples protected with trehalose and DMA during freezing and thawing might suggest, as a consequence, the potential improvement of the fertilizing ability of cryopreserved sperm. The kinetic parameters LIN and WOB, indicative of progressiveness [Santiago-Moreno et al., 2015], were also improved in semen samples processed in presence of trehalose. LIN has been largely investigated in mammalian species [Vazques et al.,2015], where a sperm subpopulation with high values of VAP and LIN could make the difference between high or low fertility. BCF has been associated to the ability of mammalian sperm of penetration into the *zona pellucida* of the oocyte. In humans in particular, VCL and BCF were significantly higher in sperm able to perform penetration in *in vitro* assay compared to those failing [Fetterolf and Rogers, 1990]. Finally, the recovery of progressive motile sperm soon after thawing and 5 and 10 min thereafter increased in semen samples provided with trehalose before freezing, thus the sensitivity of the progressive motile sperm present in the fresh sample to the freezing/thawing process was decreased.

Conclusions

Cryopreservation of living germplasm for the purpose of storing genetic resources is one of the most complex challenges in *ex situ* animal conservation programs. In avian species, despite many years of research on the use of permeating agents to cryoprotect semen, survival of sperm after freezing/thawing procedures

remains highly variable. According to the experience in mammals, the investigation on the interaction between permeating cryoprotectants, like dimethylacetamide, and natural osmoprotectants, such as sucrose or trehalose, can be a suitable strategy to improve the success of sperm cryopreservation in birds also.

Our study found a positive synergic action of trehalose and DMA on motile function of frozen/thawed chicken sperm; in contrast, sucrose combined with DMA did not show a similar positive effect. The positive cryoprotective action of trehalose was on the quality of sperm motion, not on the proportion of viable and motile sperm, and on the recovery of progressive motile sperm after cryopreservation. In particular, trehalose improves some key parameters of sperm motion positively related to the fertilizing ability of male gametes. Further studies are required to build on fundamental knowledge about the mechanism of the cryoprotective action of trehalose and to study its full potential as cryoprotectant alone or combined to DMA in chicken semen. It will be of interest to deeper investigate the quantitative DMA/trehalose ratio with the goal to study if a consistent decrease in DMA is possible in presence of trehalose to fully prevent the toxic effect directly related to its concentration.

Authors' Contributions

FM was involved in planning the research activities, performed experimental, laboratory and statistical analyses and drafted the manuscript. MM was involved in planning the research activities and performed the laboratory analyses. AAS was involved in semen collection and laboratory analyses. LZ was involved in planning the research activities, performed the statistical analysis and organized the results. SC was responsible for the research project, conceived the study and was involved in planning the research activities, and revised the manuscript critically for

important intellectual content. All authors read and approved the final manuscript.

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Sensitivity to semen cryopreservation in Milanino and Mericanel della Brianza chicken breeds

Introduction

The results obtained in the previous experimental protocols have made possible the development of a cryoconservation reference procedure for the *Gallus gallus* species. A further experimental protocol has been planned and carried out in order to apply the reference procedure for the cryoconservation of semen collected from *Mericanel della Brianza* and *Milanino* males and to assess semen quality before and after freezing/thawing.

Materials and Methods

Mericanel della Brianza (n=27) and *Milanino* (n=19) cockerels, hatched at the CZDS in 2014, have been kept at the partner farm *Il Roncone* (Figino Serenza, CO) in outdoor floor pens. The birds have been trained to semen collection and selected for semen production, as formerly described. The donor birds have been regularly milked twice a week. The ejaculates have been diluted to $1.5x10^9$/ml in prefreezing diluent, refrigerated soon after collection and then moved to the VESPA laboratory (Milano) for further processing. Cryopreservation was performed according to the results of the previous trials. In brief, diluted semen samples were further diluted to $1x10^9$/ml in prefreezing diluent added with DMA (6% final dilution) and trehalose (0.1 M). After 1 min incubation at 5 °C, semen was loaded into 0.25-mL French straws and frozen in nitrogen vapours 3 cm above the liquid nitrogen bath. Thawing was performed in water bath at 38°C for 30 sec. Semen quality was assessed before and after cryopreservation:

sperm viability and motility were measured according to the techniques described in the previous trials.

Results and Discussion

Fowls used in this study have been kept in outdoor pens as an alternative to individual cages, cause of extreme stress and poor semen production. Indeed, good semen production was obtained in *Milanino* cockerels and in total 12 pools of semen have been acquired. On the contrary, low semen production was obtained in *Mericanel della Brianza* cockerels, both in terms of ejaculates collected per day and of total volume, as a consequence only 3 pools of semen have been acquired. The low number of samples has only made possible the acquisition of preliminary results, which have to be confirmed in future studies.

The average values in all qualitative parameters measured in fresh semen and after freezing/thawing are indicated in Table 1 for both breeds. The quality of fresh semen is higher in *Milanino* than *Mericanel della Brianza* chickens, and the same condition occurs after cryoconservation. As expected, the cryopreservation procedure causes an important cellular damage in both breeds, with a significant decrease of quality: the percentage of sperm viability and motility is reduced to 29 and 15% respectively in *Mericanel della Brianza* and to 42 and 33% in *Milanino*. The quality of cryopreserved semen assessed in the Italian chicken breeds is comparable, or even higher, to the quality of semen collected from other local breeds and processed in similar conditions, as previously reported in scientific reports (Siudzinska and Lukaszewicz, 2008; Santiago-Moreno *et al*., 2012). In *Mericanel della Brianza* breed, the mean recovery rate of viable, motile and progressive motile sperm after cryopreservation has been 38, 33 and 51% respectively. In *Milanino* breed, the recovery rates of viable, motile and progressive motile sperm after cryopreservation are indicated in Table 2. It's interesting underline the large variation of the results (minimum and maximum values), with motility recovery values higher than 50%

in 6 semen samples, and progressive motility values higher than 40% in 5 semen samples. The functional and biochemical characterization of the ejaculates with reduced sensitivity to cryopreservation is of undeniable interest; the characterization is aimed to identify biochemical markers related to the sperm ability to prevent the occurrence of cellular damages during the cryopreservation process and to develop *in vitro* and/or *in vivo* strategies to preserve sperm fertility.

Table 1 – Mean values of quality and kinetic parameters measured in fresh (FR) and cryopreserved semen (CR) in *Mericanel della Brianza* (MB) and *Milanino* (MI) breeds.

Parameters	Breed			
	MB		MI	
	FR	CR	FR	CR
Viability (%)	77.4	29.3	91.4	42.2
Motility (%)	51.2	15.3	83.6	32.8
Progressive motility (%)	7.90	1.8	20.3	3.9
VCL (µm/s)	37.7	32.9	55.6	36.6
VSL (µm/s)	15.3	11.1	20.6	11.7
VAP (µm/s)	23.6	18.2	34.2	19.9
LIN (%)	39.7	31.8	36.8	31.6
STR (%)	63.6	58.1	60.2	58.2
WOB (%)	62.1	53.7	61.0	54.1
ALH (µm)	2.86	1.96	3.1	2.7
BCF (Hz)	7.76	5.47	7.2	7.3

Table 2 – Mean, minimum and maximum recovery values calculated for viability, motility and progressive motility of cryopreserved semen in Milanino chicken breed.

Parameter	Recovery		
	Mean	Minimum	Maximum
Viability (%)	46	24	64
Motility (%)	37	14	74
Progressive motility (%)	18	1	74

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GENERAL DISCUSSION

The native poultry breeds in Lombardia region are only reared in fancy farms with the goal of exhibiting the best birds (morphologically speaking) in sector fairs. The population size of each breed is usually very limited and, as a result, the biodiversity survival is at risk.

The inclusion of those breeds in a conservation program is essential for the safeguard of the genetic resources and to ensure their in situ conservation through the valorisation of the breeds in farming production systems. Implementing conservation activities – following international and national guidelines – ensures the knowledge, the development and the natural diffusion of the breeds on the territory, with the resulting creation of important opportunities for the innovation of the productive system. In fact, valorisation of local poultry breeds can coincide with the development of niche markets for high quality products, based on a sustainable and environmentally friendly farming system, suitable also for the marginal rural areas. Furthermore, these small populations – particularly fit for extreme environments - are often a supplementary source of income for rural communities, with the consequent maintenance of farming areas and gastronomic traditions.

At last, these genetic resources are sources of genetic variation of fundamental relevance to ensure future genetic improvement. Native breeds show specific traits such as the ability to environmental adaptation, the hardiness, the resistance to diseases, and the quality of the products; such features are potential parameters for future selection strategies, but today they are not considered in commercial strains, mainly selected for high production performance.

CoVAL project has been developing many activities, creating the conditions necessary for the development of a conservation program for poultry breeds, based on the primary *in situ* and the complementary *ex situ in vitro* strategies.

The project has produced many results: from the genetic and phenotypic characterization of the breeds to the communication and marketing strategies to facilitate their widespread diffusion on the territory; an economic analysis was also included for the supply chain sustainability evaluation.
As extensively described throughout this dissertation, my research activity has been mainly focused on the valorisation of poultry breeds in free range system for meat production and on semen cryopreservation. The most significant results concerning such aspects and their possible future developments are summarized in the following list, with the intention of making the comprehension of the conclusions more immediate.

1. The project activities have generated a high number of data on the productive and reproductive traits of the two chicken breeds. The results can be used for the upgrading of international databases concerning animal genetic resources (ex. DAD-IS) still inadequate for the Italian poultry breeds.
2. A nucleus population in both chicken breeds have been constituted, following FAO guidelines, in accordance with the criteria that allow a correct conservation over time, useful to limit the genetic drift and the risks originated from inbreeding.
3. The reproductive function of chicken breeds is characterized by a good fertility with a high embryo mortality. Some critical points in the management of breeders and eggs have been identified and consistent changes in the management of the hatching eggs have been made to limit embryo mortality (ex. conservation time of eggs). Other prospective critical points have come to light during the project, as the nutritional requirements of the breeders and the need for specific microclimatic conditions during egg conservation and incubation according to the breed. An in-depth analysis of these factors is highly recommended in future experimental protocols.

4. CoVAL has produced a management guideline for chicken extensive free range rearing. The guideline suggest the animal density of 8 m^2/bird in outdoor pens and the *ad libitum* supplementation of a standard commercial diet for growing chickens with 16% CP from 35 to 180 days of age.
5. In *Milanino* breed, the very long growing period has pointed out animal managing problems concerning behaviours related to the onset of sexual maturity. Further investigations are required for the identification of management guidelines specific to the growing and ethologic features of each sex within breed.
6. The growth curve of *Milanino* breed supports its commercial use for standard meat products according to the Italian poultry market (ready-to-cook carcass, meat cuts) since 90 days of age. Further studies to discover the best slaughtering body weights and ages for meat production are necessary.
7. The *Milanino* chicken meat is of high quality and elevated nutritional value compared to standard poultry meat produced in the intensive sector. A further characterisation of qualitative features in such product is very important for the development of regional filieres.
8. CoVAL has identified a reference procedure for the semen cryopreservation in local poultry breeds. The procedure involves not-permeant and permeant cryoprotectants in a innovative combination. The availability of this reference procedure should allow the constitution of the sperm cryobank for *ex situ in vitro* conservation of Italian poultry genetic resources, today absent in Italy, but already existing in other European countries (France, The Netherlands, Hungary, Spain).

APPENDIX

Scientific production

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