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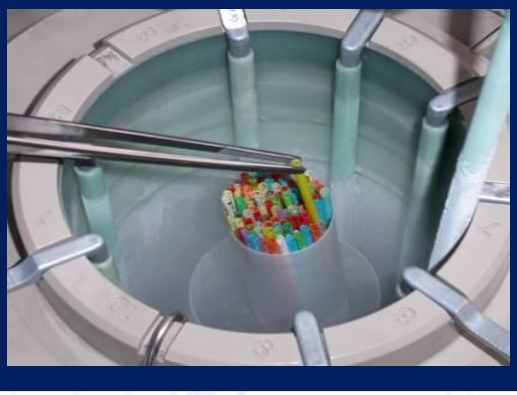
DEVELOPING THE USE OF EPIDIDYMAL SEMEN FOR FARM ANIMAL CRYOBANKS

AND SOME FIELD APPLICATIONS

Tesi di: Dott.ssa Federica Turri

Docente guida: Prof. Gustavo Gandini

Correlatore: Dott.ssa Flavia Pizzi



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DEVELOPING THE USE OF EPIDIDYMAL SEMEN FOR FARM ANIMAL CRYOBANKS

AND SOME FIELD APPLICATIONS

Curriculum: Metodologie e biotecnologie applicate alla genetica animale

D*ocente guida*: Prof. Gustavo Gandini

Correlatore: Dott.ssa Flavia Pizzi

Dott.ssa Federica Turri
Matricola: R08040

Abstract. The aim of this thesis was to increase our knowledge in the use of epididymal semen for the creation of cryobanks for farm animal genetic resources, and more generally to contribute to the area of conservation and sustainable use of farm animal genetic resources (AnGR).Three experiments were conducted in cattle and goat species.
In cattle the effects of two epididymal sperm extraction methods, the float-up and the retrograde flushing technique were compared in terms of quality of epididymal sperm recovered. Results suggest that in this species retrograde flushing technique is the method of choice to obtain viable sperm from the epididymis (Turri et al., 2011. *Reprod Dom Anim*, doi: 10.1111/j.1439-0531.2011.01948.x, *in press).*
In the second experiment, the relationship between body weight, secondary sexual traits and epididymal semen quality in Camosciata della Alpi bucks was evaluated. It was concluded that epididymal semen quality in goat species is influenced by body weight, scrotal circumference, horn diameter and length, and testicular and epididymal weight.
In the third experiment, the effects of time elapsed between animal's death and sperm recovery (0, 24, 48, 72 hours) from the epididymis and the effect of testicles storage temperature (environmental temperature or 5°C) on quality and freezability of epididymal buck spermatozoa were investigated. Sperm recovery was performed by using the retrograde flushing technique. It was concluded that goat epididymal spermatozoa extracted by testicles stored at 5°C until 48 hours post-mortem are able to maintain their quality in terms of total motility, viability and sperm morphologies, also after cryopreservation.
A field application was carried out within the creation of the Lombardia Farm Animal Genetic Resources Cryobank (LABank), when epididymal spermatozoa of the Brianzola sheep were collected instead of conventional semen collection. At present in the LABank genetic material of Varzese cattle, Brianzola sheep and Frisa, Orobica and Verzaschese goats breeds is stored. Information of donors animals and material stored are managed through the software CryoWEB (http://cryoweb.vete_vsa.unimi.it/**).** A coordination system of Italian collections was also developed, through the creation of the "Network of the Italian cryobanks of farm animal genetic resources" CRIONET-IT (http://www.genrescryonet.unimi.it/).
Finally, farmers' motives and values in keeping local cattle breed were investigated trough 371 interviews of farmers of 15 local cattle breeds, in 8 European countries. The work was part of EC EURECA project (Towards self-sustainable EUropean REgional CAttle breeds) aimed to define guidelines for the management of *in situ* and *ex situ* regional conservation programmes, with reference to the cattle species (Gandini et al., 2010. Animal Genetic Resources, 47, 45–58).

Index

Chapter 1

General Introduction

Preface

The Global Plan of Action for Farm Animal Genetic Resources and the Interlaken Declaration of Animal Genetic Resources were adopted by 109 States, the European Community and 42 Organizations, at the invitation of the Food and Agriculture Organization of the United Nations (FAO), through the International Technical Conference on Animal Genetic Resources for Food and Agriculture held in Interlaken, Switzerland, in 2007. This was the first inter-governmental conference focusing exclusively on animal genetic resources (AnGR), that affirms countries' commitment to the implementation of the Global Plan of Action and to establish an effective international framework for the sustainable use, development and conservation of AnGR that are of vital importance to agriculture, food production, rural development and to the environment (FAO, 2007).

The Global Plan of Action for AnGR includes four Strategic Priority Areas: Area 1. Characterization, Inventory and Monitoring of Trends and Associate Risks; Area 2. Sustainable Use and Development; Area 3. Conservation; Area 4. Policies, Institutions and Capacity-building. These four Areas contains a total of 23 Strategic Priorities for Action that propose specific measures aimed to reverse the ongoing trends of erosion of farm AnGR.
Annex 1, provides for each Strategic Priority Area the different Strategic Priorities. Task of each Country is to develop its own Plan of Action with the aim to support the sustainable use, development and conservation of AnGR at regional, national and global levels, based on the priorities listed in Annex 1.

Aim and outline of the thesis

The aim of this thesis is to increase our knowledge in the area of cyoconservation and sustainable use of AnGR. The thesis also provides elements for the development of the Global Plan of Action for animal genetic resources, above introduced. At this respect it can be divided into three parts. First, Chapters 2, 3 and 4, that are in line with Strategic Priority 11 regarding the development of approaches and technical standards for AnGR conservation. Second, Chapter 5, in line with Strategic Priorities 9 and 10, focus on the importance to establish *ex situ* conservation programmes and to implement national and regional long-term conservation strategies. Third, Chapter 6, in line with Strategic Priorities 2 and 6, related to the development of methods for local breeds evaluation and comparison.

Chapters 2, 3 and 4

These three chapters refer to different investigations related to *ex situ* conservation and to animal genebanks development.
With the aim to establish a protocol for the recovery and the cryopreservation of viable sperm from the epididymis of slaughtered animals, as additional opportunity in constructing semen banks (Gandini et al., 2007), research was conducted in cattle and goat species. In Chapter 2, the effects of two epididymal sperm extraction methods, the float-up and the retrograde flushing technique, in combination with two different extenders, with or without egg yolk, are compared in cattle, in order to obtain the more suitable extraction technique in terms of quality of epididymal sperm recovered. In Chapter 3, the quality of epididymal buck semen obtained by using the retrograde flushing technique, given that in cattle species the best results were obtained with this technique (Chapter 2), are analysed in correlation with body weight, scrotal circumference, and horns diameter and length of bucks. In Chapter 4, the effects of time elapsed between animal's death and sperm recovery (0, 24, 48, 72 hours) from the epididymis and the effect of testicles storage temperature (environmental temperature or 5°C) on quality and freezability of epididymal buck spermatozoa are investigated.

Chapter 5

Chapter 5 illustrates the work carried out to create the Lombardia Farm Animal Genetic Resources Cryobank (LABank). This work was developed in the framework of the project "Risorse biologiche e tecnologie innovative per lo sviluppo sostenibile del sistema agro-alimentare", co-funded by Regione Lombardia and the National Research Council, to halt the loss of biodiversity occurring at regional level. LABank was built according to the Guidelines for the Constitution of the National Cryopreservation Programmes for Farm Animals (Hiemstra, 2003) and to the Guidelines for the Cryoconservation of Animal Genetic Resources (FAO, 2011). We have also developed the "Network of the Italian cryobanks of farm animal genetic resources" with the aim to share information on cryopreserved material among Institutions that have storages of genetic material, and to create a network of Italian Institutions collaborating in AnGR cryoconservation.

Chapter 6

For successful programmes of AnGR conservation it is advisable to have the participation of all stakeholders (Hieimstra et al, 2010). Among these, farmers play a dominant role. Chapter 6 deals with the participation to the EC EURECA project (Towards self-sustainable EUropean REgional CAttle breeds) aimed to define guidelines for the management of *in situ* and *ex situ* regional conservation programmes, with reference to the cattle species. In particular Chapter 6 refers about a wide investigation, involving a total of 371 farmers of 15 local cattle breeds in 8 European countries, with the aim to better understand farmers' motives and values in keeping local cattle. My role in this project was to interview face to face 56 farmers of two Italian cattle breeds, the Reggiana and the Modenese Bianca Val Padana, and to participate to the statistical analysis of the data collected in the eight participating European countries.

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Annex 1 :
Strategic Priority Areas and Strategic Priorities for Action

Strategic Priority Area 1 - Characterization, Inventory and Monitoring of Trends and Associated Risks	
Strategic Priority 1	Inventory and characterize animal genetic resources, monitor trends and risks associated with them, and establish country-based early-warning and response systems
Strategic Priority 2	Develop international technical standards and protocols for characterization, inventory, and monitoring of trends and associated risks
Strategic Priority Area 2 - Sustainable Use and Development	
Strategic Priority 3	Establish and strengthen national sustainable use policies
Strategic Priority 4	Establish national species and breed development strategies and programmes
Strategic Priority 5	Promote agro-ecosystems approaches to the management of animal genetic resources
Strategic Priority 6	Support indigenous and local production systems and associated knowledge systems of importance to the maintenance and sustainable use of animal genetic resources
Strategic Priority Area 3 - Conservation	
Strategic Priority 7	Establish national conservation policies
Strategic Priority 8	Establish or strengthen in situ conservation programmes
Strategic Priority 9	Establish or strengthen ex situ conservation programmes
Strategic Priority 10	Develop and implement regional and global long-term conservation strategies
Strategic Priority 11	Develop approaches and technical standards for conservation
Strategic Priority Area 4 - Policies, Institutions and Capacity-building	
Strategic Priority 12	Establish or strengthen national institutions, including national focal points, for planning and implementing animal genetic resources measures, for livestock sector development
Strategic Priority 13	Establish or strengthen national educational and research facilities
Strategic Priority 14	Strengthen national human capacity for characterization, inventory, and monitoring of trends and associated risks, for sustainable use and development, and for conservation
Strategic Priority 15	Establish or strengthen international information sharing, research and education
Strategic Priority 16	Strengthen international cooperation to build capacities in developing countries and countries with economies in transition, for: • characterization, inventory, monitoring of trends and associated risks • sustainable use and development • conservation of animal genetic resources
Strategic Priority 17	Establish regional focal points and strengthen international networks
Strategic Priority 18	Raise national awareness of the roles and values of animal genetic resources
Strategic Priority 19	Raise regional and international awareness of the roles and values of animal genetic resources
Strategic Priority 20	Review and develop national policies and legal frameworks for animal genetic resources
Strategic Priority 21	Review and develop international policies and regulatory frameworks relevant to animal genetic resources
Strategic Priority 22	Coordinate the Commission's efforts on animal genetic resources policy with other international forums
Strategic Priority 23	Strengthen efforts to mobilize resources, including financial resources, for the conservation, sustainable use and development of animal genetic resources

Chapter 2

Influence of recovery methods and extenders on bull epididymal spermatozoa quality

F. Turri[1,2], M. Madeddu[1], T.M. Gliozzi[1], G. Gandini[2], F. Pizzi[1]

[1]*Istituto di Biologia e Biotecnologia Agraria, Unità Organizzativa di Supporto di Lodi, Consiglio Nazionale delle Ricerche, c/o Parco Tecnologico Padano, via Einstein, 26900 Lodi, Italy.*

[2]*Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Università degli Studi di Milano, via Celoria 10, 20134 Milano, Italy.*

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2. Influence of recovery methods and extenders on bull epididymal spermatozoa quality

2.1 Abstract

The aim of this study was to compare the effects of two extraction methods in combination with two different extenders in bull epididymal sperm collection. Testes from 23 sexually mature Limousine bulls were collected at the abattoir. Epididymal sperm recovery was performed using both the float-up and the retrograde flushing technique. Within extraction methods, half testes were processed with a Tris egg yolk extender and half with a Tris egg yolk-free extender. Sperm concentration, motility, viability and morphology were evaluated. Sperm concentration was not significantly different between methods. Flushing technique was significantly better than the float-up method in terms of sperm quality, considering total motility (80.3 ± 2.3 % vs 71.6 ± 2.0 %, $p<0.001$, respectively) and viability (84.5 ± 1.5% vs 77.2 ± 1.3%,$p<0.001$, respectively). Egg yolk influenced positively motility and morphology in the float-up method, whereas decreased viability in flushed samples. Results suggest the use of the retrograde flushing technique to collect cattle epididymal sperm.

2.2 Introduction

The interest in preserving special germplasm has resulted in increased attention to the recovery of functional sperm from the epididymis of dead animals (Foote 2000). Semen collection from epididymis can be the only option we have to preserve male gametes from animals of high value or from endangered species (Leibo and Songsasen 2002; Fickel et al. 2007). Three main methods of post-mortem epididymal sperm recovery have been described in different domestic and wild species. The cutting method implies that numerous cuts are made on the cauda epididymidis with a blade and the spermatic fluid emerging from the tubules is collected (e.g. Kaabi et al. 2003; Soler et al. 2003; Goovaerts et al. 2006; Martinez-Pastor et al. 2006; Martins et al. 2009; Santiago Moreno et al. 2009). The float-up method consists in slicing with a scalpel the cauda epididymidis and the proximal ductus deferens in a Petri dish and suspending in semen extender (e.g. Hishinuma et al. 2003; Cary et al. 2004; Bruemmer 2006). This method has never been reported in cattle. With the retrograde flushing, the lumen of the ductus deferens is cannulated and perfused with semen extenders (e.g. Cary et al. 2004; Martinez-Pastor et al. 2006; Santiago-Moreno et al. 2009).

During cryopreservation, egg yolk is widely used in mammalian sperm since the discovery of its beneficial effect (Phillips 1939; Phillips and Lardy 1940). The positive action on fertility in bull of egg yolk semen extenders have been more recently confirmed by Amirat et al. (2005) and Leite et al. (2010). In particular egg yolk has been shown to prevent pre-term capacitation caused by cold shock (Witte et al. 2009). However there was a lack of information on the use of egg yolk and its effects on fresh semen.

The aim of this work was to compare the effects of float-up and retrograde flushing methods on bull epididymal sperm quality. In addition, we evaluated the effects of egg yolk in the early stages of semen extraction. The investigation was carried out within a programme addressed to optimise semen collection to create a semen bank for endangered cattle breeds.

2.3 Materials and methods

Media were prepared in our laboratory by using chemicals from Sigma Aldrich (Milan, Italy).

2.3.1 Testes collection and preparation

Testes were collected at the abattoir from 23 sexually mature Limousine bulls (mean age 16.1 months, standard error 1.7 months), 40 minutes on average after slaughtering and then transported at room temperature (21.9°C ± 3.34) in a Styrofoam box to the laboratory. Approximately 120 minutes after collection testicles with respective epididymis and ductus deferens attached were isolated from the scrotum and other tissues, and subsequently epididymides were isolated and cleaned of connective tissue. Cauda epididymidis and ductus deferens were then isolated and spermatozoa were collected using the float-up (FL) or the retrograde flushing (RF) methods. To avoid blood contamination, superficial blood vessels were previously punctured with a needle and their contents wiped out. For each bull, it was randomly decided which epididymis to process by FL or by RF technique. Time elapsed between epididymis isolation and sperm collection was recorded. For each extraction method, half of epididymides were processed with a Tris Egg Yolk extender (FL-TEY, RF-TEY) containing Tris 30.28 g/l, citric acid 12.61 g/l, fructose 12.43 g/l, 25% v/v egg yolk and an antibiotic cocktail containing respectively 1g/l of tylosin, gentamicin and lincospectin, with pH=7.5 and osmolarity of 300 mOsm (Cormier et al. 1997). The remaining epididymides were processed with Tris egg yolk-free extender (FL-TEYF, RF-TEYF).

After extraction and extender dilution the following four experimental groups were obtained: FL-TEYF (n=12), FL-TEY (n=13), RF-TEYF (n=11) and RF-TEY (n=10). Experimental groups were unbalanced because two testes designed for RF-TEY group were rejected due to ductus deferens damage. Sperm recovery of these testes was performed by the FL method, in particular the two testes were assigned respectively in FL-TEYF and in FL-TEY groups.

2.3.2 Sperm recovery by float-up method (FL)

Float-up method was performed as described by Cary et al. (2004) with minor modifications. Cauda epididymidis and proximal ductus deferens were incised in a Petri dish with a scalpel, washed with approximately 2.5 ml of warmed extender (37 °C) and then transferred in a second Petri dish and washed again with approximately 2.5 ml of the same extender. Sperm suspension obtained from the two washing steps were filtrated through 200 μm stainless sieve and collected in a glass tube.

2.3.3 Sperm recovery by retrograde-flushing method (RF)

Retrograde flushing method was performed as described by Martinez-Pastor et al. (2006). Cauda epididymidis and ductus deferens were isolated from the rest of the epididymis by making a cut with a scalpel near the junction of the corpus and the proximal cauda. After that, the lumen of the ductus deferens was cannulated with a blunted 22G needle. Sperm cells were then flushed in a retrograde direction from the ductus deferens through the cauda epididymidis with a syringe loaded with approximately 4 ml of warmed (37°C) extender.

2.3.4 Evaluation of epididymal spermatozoa

Sperm quality was analysed immediately after extraction. Sperm concentration, motility, viability and morphology were evaluated.

Sperm concentration was estimated with a Corning Colorimeter 253 (Corning Limited Halstead, Essex England; wavelength: 540 nm, dilution of semen aliquots 40:1000 vol/vol in NaCl 7%; two replicates per sample) by converting transmittance in sperm concentration using a pre-established conversion table.

Sperm motility parameters were assessed using a CASA (Computer Assisted Semen Analysis) system (The Hobson sperm tracker 7V2B: Hobson Tracking Systems Ltd, UK). Each sample was diluted ($30x10^6$ cells/mL) in NaCl 0.9% and incubated for 20 min in a 37°C water bath. Then, a pre-warmed (37°C) Makler counting chamber (10 μm depth) was loaded with 10 μL of sample. At least two or three fields per sample were acquired, making a total of 50 motile sperms. The following parameters were recorded: total motility (%), active cells (% of sperm

with VAP >25μm/s and STR >75% , hyperactive cells (% of sperm with VCL >70 μm/s, ALH >5 μm and LIN <30%), average path velocity (VAP, μm/s; the average velocity of the smoothed cell path), curvilinear velocity (VCL, μm/s; the average velocity measured over the actual point to point track followed by the cell), straight-line velocity (VSL, μm/s; the average velocity measured in a straight line from the beginning to the end of the track), linearity index (LIN, %; the average value of the ratio VSL/VCL), straightness index (STR, %; the average value of the ratio VSL/VAP), amplitude of lateral head displacement (ALH, μm; the mean width of the head oscillation as the sperm swim), and beat cross-frequency (BCF, Hz; the frequency of sperm head crossing the average path in either direction).

Viability was evaluated by eosin-nigrosin stain (Bakst and Cecil, 1997). Ten microliters of semen were mixed with 500 μl of eosin-nigrosin for two minutes before preparing each smear that was allowed to air dry. Then slides were permanently sealed with with Eukitt mounting medium (O. Kindler GmbH, Germany) and topped with a 22x40 mm coverslip. Two separate smears were prepared for each epididymis. Two hundred cells for smear were assessed.

Sperm cell morphology was assessed by examination of eosin-nigrosin stain fixed samples. A Leica microscope (model DMLB 30 FLUO; Leica Microsystems Imaging Solutions, Cambridge, UK) with a 100x oil immersion objective was used. The following morphological categories were used: abnormal heads (small, giant, pyriform, narrow at the base head), midpiece (filiform, deformed, double) and tail (coiled around the head, single bent, angle tail implantation, absent). For each replicate smear, the number of normal and abnormal sperm was expressed as a percentage of the total number of live sperms. The mean of the two independent smears per experimental group was used for statistical analysis. Frequency of cytoplasmatic droplets (proximal and distal) was recorded on normal spermatozoa. Due to outlier values three bulls were excluded from the morphological analysis.

2.3.5 Statistical analysis

Statistical analyses were carried out using the SAS™ package v 9.1 (SAS Institute Inc., Cary, NC). Cell concentration, viability, CASA motility parameters and sperm morphology data were analyzed using the GLM (General Linear Model) procedure. Fixed effects included the extraction method, in combination with the egg yolk addition in the extender, and the age of bull and time for extraction as covariates. Results were expressed as adjusted least squares means ± standard error means (LSM ± SEM). The Shapiro-Wilk test for normality was performed to check data distribution. The test indicated that normal distribution can be

assumed for most analysed variables, even for some of the binomial (percentage) quantities. For some of the morphological parameters, however, the assumption of normal distribution can lead to a somewhat conservative statistical comparison. The majority of the analyzed variables show a normal distribution, for this reason untransformed data were analysed.

2.4 Results

Age of bulls affected negatively total motility (covariate -2.05 months, $p<0.05$), hyperactive cells (covariate -4.32 months, $p<0.05$) and viability (covariate -2.01 months, $p<0.001$) parameters.

2.4.1 Extraction technique effects

Time of extraction of semen from the epididymides was significantly ($p<0.001$) higher of approximately five minutes in the RF method (19.44 ± 0.87 min) than in the FL method (14.16 ± 0.78 min). Sperm concentration was not significantly different between techniques, 294.41 (± 27.29) $x10^6$ cells/ml in the FL method and 257.07 (± 31.25)$x10^6$ cells/ml with the RF technique.
The results of the influence of extraction technique on sperm kinetics and viability parameters are shown in Table 1 and 2.

Table 1. Sperm kinetics and viability parameters (LSM ± SEM) in fresh epididymal sperm samples, by extraction technique

Parameters	Experimental Group[1]	
	FL (n=25)	RF (n=21)
Total motility (%)	71.6 ± 2.0[a]	80.3 ± 2.3[b]
Active cells (%)	9.0 ± 1.6	6.8 ±1.8
Hyperactive cells (%)	55.0 ± 4.2	52.6 ± 4.8
Average path velocity (μm/s)	53.4 ± 1.8	49.4 ± 2.1
Curvilinear velocity (μm/s)	112.8 ± 3.5	109.6 ± 4.0
Straight-line velocity (μm/s)	13.7 ± 0.9	12.4 ± 1.1
Amplitude of lateral head displacement (μm)	6.3 ± 0.4	6.6 ± 0.4
Beat cross-frequency (Hz)	9.5 ± 0.7	8.3 ± 0.8
Linearity index (%)	12.1 ± 0.9	11.2 ± 1.1
Straightness index (%)	30.4 ± 1.9	28.8 ± 2.2
Viability (%)	77.2 ± 1.3[a]	84.5 ± 1.5[b]

[1]Extraction technique: FL= Floated samples; RF= Flushed samples.
[2]a,b: values within each row with different letters are significantly different ($p<0.05$).

The extraction technique had significant effects on total motility ($p<0.05$) and on viability ($p<0.01$). In particular, samples extracted with RF technique showed

higher motility and viability than those obtained with FL method, by 12% and 9% respectively (total motility: RF= 80.32 ± 2.32% vs FL= 71.58 ± 2.03%; viability: RF= 84. 48 ± 1.48% vs FL= 77.16 ± 1.30%). No significant differences were observed in the other nine kinetics parameters.

The extraction method did not influence significantly sperm morphology (Table 2); however in RF technique total normal sperm it appeared to be higher, but the difference was not significant, than in FL technique and total sperm abnormalities were 62% lower. On average, the majority (98%) of defects in both the extraction techniques were in the tail. No sperm midpiece defects were observed.

Table 2. Percentage (LSM ± SEM) of normal and abnormal sperm as a function of extraction technique.

Parameters		Experimental Group[1]	
		FL (n=21)	RF (n=19)
Normal sperm	WCD[2] (%)	59.2 ± 4.3	62.9 ± 4.7
	PD[2] (%)	2.6 ± 0.5	2.0 ± 0.5
	DD[2] (%)	11.0 ± 3.7	18.1 ± 4.1
	Total (%)	72.8 ± 4.7	83.0 ± 5.1
Sperm abnormalities	Head (%)	0.5 ± 0.2	0.3 ± 0.2
	Tail (%)	26.6 ± 4.7	16.7 ± 5.0
	Total (%)	27.1 ± 4.7	17.0 ± 5.1

[1]Extraction technique: FL= Floated samples; RF= Flushed samples.
[2]WCD=without cytoplasmatic droplets; PD=proximal droplets; DD=distal droplets.

2.4.2 Egg Yolk addition effects

The effects of extenders within extraction technique are shown in Tables 3 and 4. In FL technique, egg yolk addition induced a significant increase ($p < 0.001$) of total motility (+32%), VAP (+32%) and BCF (+48%). Consequently differences in total motility between FL and RP methods, above analysed independently by the extender (Table 1), were reduced and differences in VAP and BCF kinetic parameters were increased. Within RF method, egg yolk addition reduced significantly ($p<0.001$) viability, by 9%.

Regarding sperm morphology (Table 4), samples extracted with the egg yolk addition in the extender showed a higher number of total normal sperm and consequently a lower number of total sperm abnormalities in both extraction methods. In particular, within FL method, with egg yolk addition there was a significant increase ($p<0.05$) of total normal sperm (+35%), due mainly to the increase ($p<0.05$) of sperm without cytoplasmatic droplets (+35%). In this group, egg yolk extender led also to a significant ($p<0.05$) decrease of tail

abnormalities and consequently of total sperm abnormalities. Similar positive trends on morphological parameters were detected in the RF method, but in this case differences between extenders were not statistically significant.

Table 3. Sperm kinetics and viability parameters (LSM ± SEM) in fresh epididymal sperm samples, by extraction technique and extender.

Parameters	Experimental Group[1]			
	FL – TEYF (n=13)	FL – TEY (n=12)	RF – TEYF (n=10)	RF – TEY (n=11)
Total motility (%)	61.8 ± 2.6[a]	81.4 ± 2.7[b]	78.4 ± 3.1[b]	83.3 ± 2.7[b]
Active cells (%)	7.6 ± 2.2	10.4 ± 2.3	6.3 ± 2.6	7.5 ± 2.3
Hyperactive cells (%)	52.6 ± 5.6	57.6 ± 5.8	61.5 ± 6.7	45.2 ± 6.0
Average path velocity (μm/s)	46.0 ± 2.3[a]	60.9 ± 2.4[b]	49.7 ± 2.7[ac]	50.1 ± 2.4[a]
Curvilinear velocity (μm/s)	107.3 ± 4.5[a]	118.5 ± 4.7[ac]	116.5 ± 5.4[a]	104.4 ± 4.8[ab]
Straight-line velocity (μm/s)	12.6 ± 1.3	14.8 ± 1.3	13.3 ± 1.5	11.8 ± 1.4
Amplitude of lateral head (μm)	6.6 ± 0.5	5.9 ± 0.5	7.2 ± 0.6	6.0 ± 0.5
Beat cross-frequency (Hz)	7.7 ± 0.8[a]	11.4 ± 0.9[b]	9.2 ± 1.0[ab]	7.7 ± 0.9[a]
Linearity index (%)	11.7 ± 1.3	12.6 ± 1.3	11.4 ± 1.5	11.0 ± 1.4
Straightness index (%)	32.8 ± 2.7	28.0 ± 2.8	30.85 ± 3.2	26.8 ± 2.8
Viability (%)	77.6 ± 1.7[a]	76.8 ± 1.8[a]	88.7 ± 2.0[b]	80.8 ± 1.8[a]

[1]FL – TEYF= Floated samples; Tris Egg Yolk-Free extender; FL – TEY= Floated samples; Tris Egg Yolk extender, RF – TEYF= Flushed samples; Tris Egg Yolk-Free extender; RF – TEY= Flushed samples; Tris Egg Yolk extender.
[2]a,b,c: values within each row with different letters are significantly different ($p<0.05$).

Table 4. Percentage (LSM ± SEM) of normal and abnormal sperm as a function of extraction technique and extender.

Parameters		Experimental Group[1]			
		FL – TEYF (n=11)	FL – TEY (n=10)	RF – TEYF (n=10)	RF – TEY (n=9)
Normal sperm	WCD[2] (%)	50.5 ± 5.9[a]	68.1 ± 6.2[b]	58.2 ± 6.6[ab]	67.7 ± 6.4[ab]
	PD[2] (%)	1.9 ± 0.6	3.3 ± 0.7	2.0 ± 0.7	1.9 ± 0.7
	DD[2] (%)	9.6 ± 5.2	12.5 ± 5.4	18.7 ± 5.8	17.6 ± 5.6
	Total (%)	61.9 ± 6.4[a]	83.9 ± 6.7[b]	78.9 ± 7.1[ab]	87.3 ± 6.9[b]
Sperm abnormalities	Head (%)	0.5 ± 0.3	0.6 ± 0.3	0.2 ± 0.3	0.4 ± 0.3
	Tail (%)	37.5 ± 6.4[a]	15.5 ± 6.8[b]	20.8 ± 7.2[ab]	12.3 ± 7.0[b]
	Total (%)	38.0 ± 6.4[a]	16.0 ± 6.7[b]	21.1 ± 7.1[ab]	12.7 ± 6.9[b]

[1]FL – TEYF= Floated samples; Tris Egg Yolk-Free extender; FL – TEY= Floated samples; Tris Egg Yolk extender; RF – TEYF= Flushed samples; Tris Egg Yolk-Free extender; RF – TEY= Flushed samples; Tris Egg Yolk extender.
[2]WCD=without cytoplasmatic droplets; PD=, proximal droplets; DD=distal droplets.
[3]a,b,: values within each row with different letters are significantly different ($p<0.05$).

2.5 Discussion and conclusion

Recovery and cryopreservation of viable sperm from the epididymides of slaughtered animals can be an alternative tool to collect male gametes, especially in wild species and in situations where traditional collection is difficult for lack of expertise and/or facilities.

Several authors compared different extraction methods to optimize epididymal spermatozoa extraction techniques and did not find significant differences between methods: retrograde flushing and float-up methods were tested in stallions (Cary et al. 2004) and retrograde flushing and cutting methods in red deer and ibex (Martinez-Pastor et al. 2006; Santiago-Moreno et al. 2009). Nevertheless, Martinez-Pastor et al. (2006) and Santiago-Moreno et al. (2009) concluded that flushing would be a more recommendable method for post-mortem salvaging of sperm from the cauda epididymidis.

Overall, our results show that when bull epididymal spermatozoa are extracted from the epididymis with the retrograde flushing technique, sperm quality is better than using the float-up method. Sperm recovery time from epididymis was approximately 5 minutes longer in the retrograde flushing method, mainly due to time needed to cannulate the ductus deferens, with a difference of some importance respect the FL technique when working on rather large numbers.
The extraction method did not influence concentration of the sperm recovered, as observed by Cary et al. in the horse (2004) and by Martinez-Pastor et al. in the red deer (2006). With float-up method, an average sperm concentration of 294.41×10^6 cells/ml was collected in our experiment, but no reports are available in the literature for comparison. Sperm concentration with RF method was 257.07×10^6 cells/ml, and almost equal to that obtained by Alapati et al. (2009) who recovered from the cattle epididymis the same number of sperm per ml.
Total motility of RF samples, significantly higher than FL samples, ranged from 61% to 94.5%, with a mean of 80.32%, similar to what observed by Alapati et al. (2009). Percentage of viable sperm was also significantly higher in flushed than floated samples. It has been suggested (Martinez-Pastor et al. 2006) that in FL technique blood and interstitial fluid may alter composition, pH and osmolarity of the spermatic fluid, therefore exposing spermatozoa to deleterious conditions. Following these lines of reasoning, the higher motility and viability of our RF samples could perhaps be explained by the fact that with this technique spermatozoa, due to the few cuts performed, are less exposed to contact with blood and other fluids.

In this study percentages of normal and abnormal sperm did not significantly differ between the two extraction methods, even if the RF technique showed a lower (-38% with respect to FL method) number of total sperm abnormalities.

The importance of sperm morphology in bull fertility has been documented (Salisbury et al. 1978; Barth and Oko 1989; Freneau et al. 2000, Holroyd et al. 2002). The presence of yolk in the extender (TEY) decreased significantly total sperm abnormalities in both extraction methods. The total number of sperm abnormalities in the RF-TEY (12.71 ± 6.92%) was 30% lower than observed by Alapati et al. (2009). Different hypotheses have been proposed to explain the protective mechanism of egg yolk. Pace and Graham (1974) first suggested that the low-density lipoproteins (LDLs) present in egg yolk offer protection to the cell membrane, an hypothesis supported by many subsequent studies (Watson 1976, 1981; Foulkes 1977, Moussa et al. 2002; Amirat et al. 2004). However, the mechanism of how LDL protects sperm remains elusive. Several studies hypothesized that LDL, especially its phospholipids, could bind or adhere to sperm membranes (direct association) and provide protection by forming a protective film (Quinn et and Chow 1980) or by replacing lost phospholipids (Foulkes et al. 1980; Graham and Foote 1987; Trimeche et al. 1996) at the surface of sperm membranes (Dong et al. 2009). The use of Tris egg yolk extender within the FL group was associated to significant increases of the total motility, BCF and VAP. Total motility FL values, in association with the use of the EY, were comparable to those registered by Cary et al. (2004) in the horse (74%) and by Lone et al. (2011) in the sheep (78%). Our observations on the positive effects of egg yolk on sperm motility confirm the findings of Fernàndez-Santos et al. (2006) in the red deer, who revealed that sperm motility was better preserved when egg yolk was present in the extender in the early stages of sperm extraction and dilution. Conversely, EY influence negatively viability in RF samples. However this effect could depends on the higher coefficient of variation (CV) of the RF-TEY group (RF-TEY CV 10.75% vs RF-TEYF CV 4.62%). However this effect could depends by the presence of three outlaying samples of the RF-TEY group, with values ranging from 63% to 74% versus the other 8 samples ranging from 80% to 92%, with a mean of 87%.

In conclusion, for post-mortem salvaging of sperm from the cauda epididymidis in the cattle species flushing would be a more recommendable method than the float-up method. Flushed samples had higher quality probably due to less contamination by blood. Moreover the addition of egg yolk has been found to have a significant beneficial effect on protecting epididymal sperm collected in float-up method, whereas in flushing its action wasn't substantial. Then our results suggest that the use of a medium with egg yolk addition can have a

positive effect already in the early stage, during extraction and recovery phase, before cooling and freezing process.
Further studies should be addressed to verify our results obtained in fresh semen with cryopreservation and fertility tests and to lay the basis for the routine application of these methods for the creation of cattle semen cryobanks, as proposed by Gandini et al. (2007).

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Chapter 3

Relationship between body weight, secondary sexual traits and epididymal semen quality in Camosciata della Alpi bucks

3. Relationship between body weight, secondary sexual traits and epididymal semen quality in Camosciata della Alpi bucks

3.1 Abstract

The aim of this study was to evaluate the relationship between body weight, secondary sexual traits and epididymal semen quality in Camosciata della Alpi young bucks. Body weight (BW), scrotal circumference (SC), horns diameter (HD) and horns length (HL) were recorded monthly on 55 young bucks from the second to the thirty-fourth week of life. After slaughter post-mortem evaluation of carcass weight, testicles weight (TW), epididymal weight (EW) and of epididymal sperm quality were done. Epididymal sperm recovery was performed using the retrograde flushing technique. Sperm concentration, motility, viability and morphology were evaluated on the collected samples. A linear positive relation between BW and SC, HL, TW occurred ($p<0.05$). Moreover some parameters of epididymal semen quality in Camosciata delle Alpi goat breed were influenced by secondary sexual trait as BW on normal sperm, ($p<0.05$), SC on the reduction of proximal droplets, ($p<0.01$), HD on the reduction of abnormal sperm, ($p<0.05$), HL on sperm concentration, ($p<0.01$). Gonads measurements (TW, EW) were significantly correlated with some parameters of epididymal sperm production (semen volume, epididymal yield, concentration and total number of spermatozoa).

3.2 Introduction

In dairy goats artificial insemination (AI) is increasingly used for reproductive management and, in conjunction with progeny testing, as a tool for genetic improvement (Furstoss et al., 2009). Associated to the use of AI, there is a growing interest for better knowledge on the reproductive characteristics of these animals. The ideal method for evaluating fertility in males, other than its ability to produce offspring, is by evaluation of its semen (Hafez, 1993). Optimal semen production is, therefore, a prerequisite for the selection of bucks (Mekasha et al., 2007). Sperm output has been shown to be positively associated with body weight in bucks (Mekasha et al. 2007) and body size is closely associated with testicular mass, a phenomenon which explains why testicular growth occurs in parallel to increases in body size (Coulter and Foote 1977; Ahmad and Noakes 1996; Mekasha et al. 2007, 2008). Delayed growth in body

size and testicular mass obviously leads to reproductive wastage and economic loss. Increase of testicular size and body weight in small-ruminants is influenced by several factors, including breed, age, nutrition and photoperiodic changes (Ortavant 1977; Laubser et al. 1982; Bielli et al. 2000; Karagiannidis et al. 2000b; Mekasha et al. 2007). Testicular size has been shown to be a reliable measurement of the reproductive growth status, spermatogenic capacity and seminal characteristics in goats (Daudu 1984). As sperm production depends on testicular size, both direct testicular measurements, as volume, length and width, and determination of scrotal circumference (SC, as a relevant indirect measurement of testicular growth), have been used to predict sperm production and semen quality (Elmore et al., 1976; Ritar et al., 1992). Horns are epidermal and bony appendages used by males of many ungulate species for intra-sexual fights and competition during the rut (Santiago-Moreno et al., 2007). In addition to their function in combat, these secondary sexual characteristics probably serve as signals of male vigor that females may use to select mates (Geist, 1966a, b). Therefore, dominant males with most developed horns are naturally selected for reproduction (Santiago-Moreno et al., 2007). Horn growth appears to be modulated by testosterone and prolactin hormones (Santiago-Moreno et al., 2005). In addition, these hormones also act directly to maintain secretory activity of male accessory sex glands (Ravautl et al., 1977; Tripathi and Mukhopadhyay, 1988), to control the spermatogenesis (Regisford and Katz, 1993; Lincoln et al., 1996) together with LH and FSH (Courot and Ortavant, 1981) and semen production. Since horn size is correlated with male dominance and fighting ability, and consequently lifetime reproductive success (Clutton-Brock et al., 1988), we can hypothesize that semen parameters may be related to horn development both in wild and domestic species. It is well known that interactions between body, horn and testis growth and sperm production are complex since early pre-pubertal ages and, in many aspects, such events can be influenced by both the genetic background of animals and the environment (Yarney and Sanford 1989, 1993; Langford et al. 1998; Belibasaki and Kouimtzis 2000). To our knowledge, scant information exists on these aspects in the Camosciata delle Alpi goat, a major dairy goat breed.

Parallel to the demand and to the necessity for more knowledge about the reproductive characteristics of breeding male, the interest in preserving special germplasm has resulted in increased attention to the recovery of functional sperm from the epididymis of dead animals (Foote 2000). Moreover, semen collection from epididymis can be the only option we have to preserve male gametes from animals of high genetic value or from endangered species (Leibo and Songsasen 2002; Fickel et al. 2007). Three main methods of post-mortem epididymal sperm recovery have been described in different domestic and wild

species (Turri et al. 2011); the retrograde flushing method has never been reported in goat species.

The aim of this work was to evaluate the body development of Camosciata delle Alpi young bucks from weaning to the early pre-pubertal ages, and to determine if inter-individual differences in body weight, testicles and horns size are related to differences in sperm quality, by the assay of epididymal spermatozoa collected post-mortem with the retrograde flushing method. The investigation was carried out within a programme addressed to optimize semen collection to create a semen bank for endangered breeds.

3.3 Material and Methods

3.3.1 In vivo evaluations

Fifty-five Camosciata delle Alpi male goats were bred from birth to approximately 34 weeks of age in two farms located in Northern Italy. During the experimental period, from February to December 2010, animals were kept on farm under natural light and were fed with the same diet, grass hay and commercial mixture with 18% of crude protein. Each animal was identified using a tag number to be identifiable both for ante than post mortem evaluation. Body weight (BW), scrotal circumference (SC), horns diameter (HD) and horns length (HL) were measured monthly, approximately from the second to the thirty-fourth week of life. The live BW was recorded in kg with an electronic portable scale, SC was measured in cm on the greatest scrotal diameter with a flexible tape, HD and HL were also measured in cm with a flexible tape. At the end of the biometrical study, all bucks were slaughtered in groups of 8-10 animals in 6 different days.

3.3.2 Post-mortem evaluations

Post mortem body weight (PMBW) (mean age at slaughter 35.3 weeks, standard error 1.27 weeks) was measured weighing the carcass, without head and legs, with a mechanical balance at the abattoir. Testicles of each buck were collected at the abattoir on average 90 minutes on average after slaughtering. Testicles of 28 animals were transported to the laboratory at environmental temperature (E) instead testicles of 27 animals during transportation to the laboratory were refrigerated at 5°C (R). For the investigation of the relationship between body weight, secondary sexual traits and epididymal semen quality one testicle per pair was considered. The analysis of the pair of testicles, addressed to evaluate the effects on spermatozoa quality of time elapsed between animal's death and

sperm recovery and of temperature during testicles preservation, is reported elsewhere in Chapter 4.
At the arrival in the laboratory each testicle, with the respective epididymis and vas deferens attached, was isolated from scrotum and other tissues. Testicle weight (TW) was measured with a digital scale. Then, cauda epididymis with ductus deferens was carefully removed from each testis, cleaned of connective tissue, isolated from the rest of the epididymis by making a cut with a scalpel near the junction of the corpus and the proximal cauda. Epididymal weight (EW) was measured with the same modality.

3.3.3 Sperm recovery and epididymal semen quality evaluation

To avoid blood contamination, superficial blood vessels were previously punctured with a needle and their contents wiped out. Spermatozoa were collected using the retrograde flushing method with a Tris egg yolk extender consisting of 20% v/v egg yolk, 7% wt/vol glycerol, 2.42% wt/vol Tris buffer, 1.0% wt/vol fructose, 1.38% wt/vol citric acid, 5.5 mg tylosin, 27.5 mg gentamycin, 16.5 mg lincospectin, and 33.0 mg spectinomycin per 100 mL (Blash et al., 2000). Retrograde flushing method was performed as described by Turri et al. (2011). The sample of each epididymis was collected in a plastic tube and immediately after evaluated for semen volume (ml), epididymal yield (ml), sperm concentration (10^9/ml), total number of spermatozoa (10^9), motility and kinetics parameters, viability and sperm morphology, as described in Chapter 2.

3.3.4 Statistical analysis

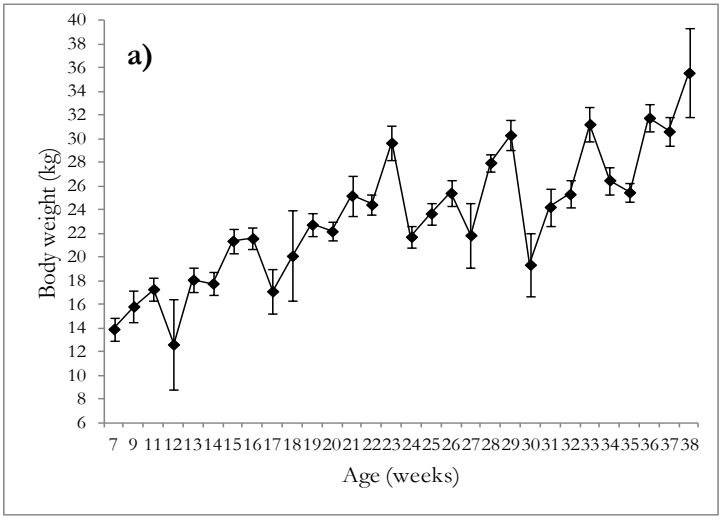
Statistical analyses were carried out using the SAS™ package v 9.2 (SAS Institute Inc., Cary, NC). The Nonlinear Model (PROC NLIN) procedure was used to fit the logistic model to bucks BW, SC, HL and HD data, to describe the evolution of these variables against time, in order to estimate the growth curve until slaughter time (mean age at slaughter 35.3 weeks, standard error 1.27 weeks), obtaining a more accurate association between these 4 biometrical variables with epididymal semen output. The general form of the equation for the logistic growth curve is: $y = k/1+((k-n_o)/n_o)*\exp^{(-r*t)}$; where n_o is the expected value of y at time t=0, k is the height of the horizontal asymptote and r is a measure of the growth rate. The three parameters (n_o, k and r) were estimated independently for each of the four variables.
The General Linear Model (PROC GLM) procedure was employed to analyse the relationship between BW, SC, HD, HL, TW, EW, PMBW and semen quality parameters. In the model the fixed effect of farm was included. Results are given as adjusted least squares means ± standard error means (LSM ± SEM). The Spearman coefficient of correlation was used to evaluate linear association between all the variables.

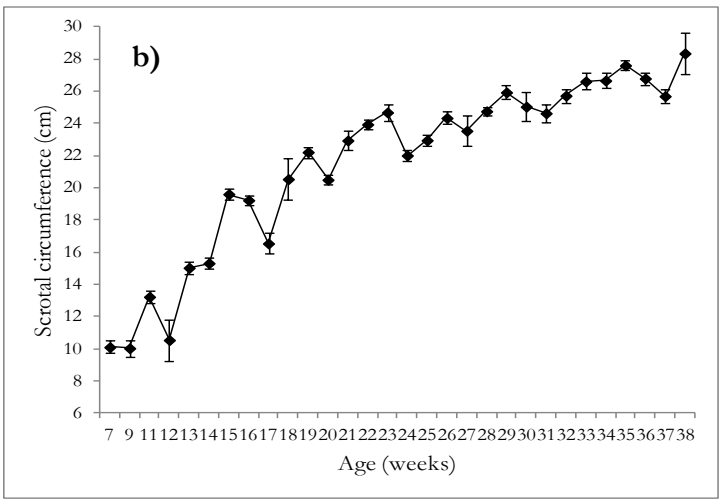
3.4 Results

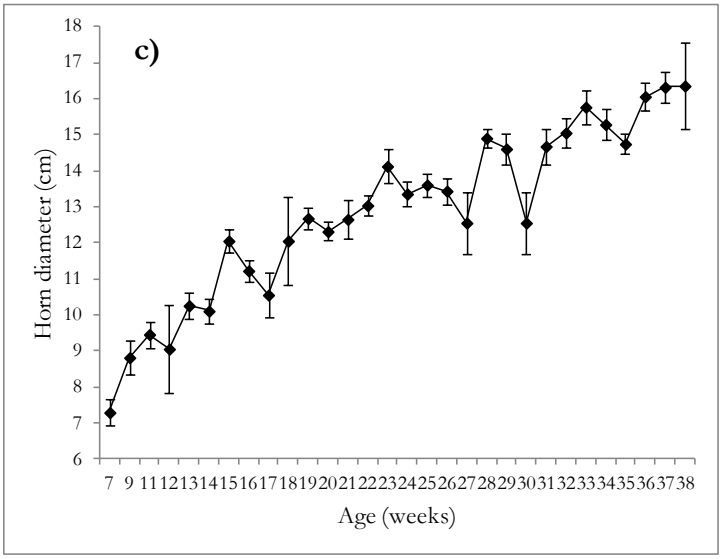
3.4.1 Body weight (BW), scrotal circumference (SC) and horn parameters (HD, HL)

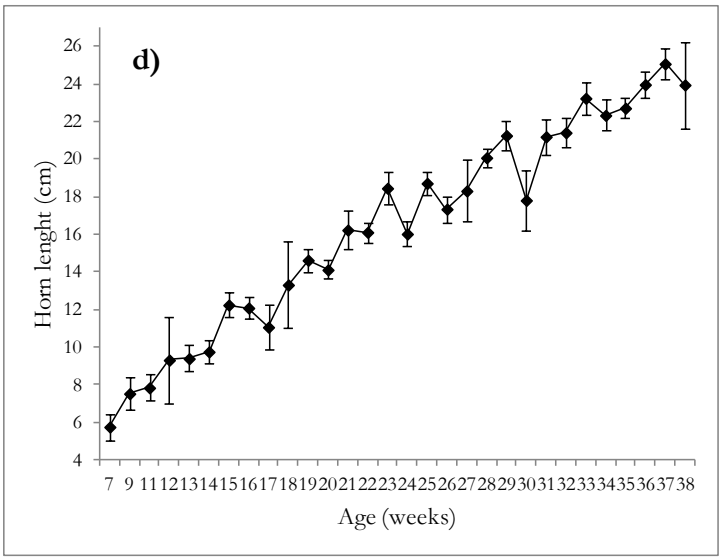
Body weight of bucks increased from 13.92 ± 1.00 kg at 7 weeks to 25.49 ± 0.85 kg at 35 weeks of age (Fig 1a). Transients decreases in body weight were observed during the body development. Scrotal circumference increased from 10.09 ± 0.70 cm at 7 week to 27.60 ± 0.60 cm at 35 week of age (Fig 1b). Horn diameter increased from 7.28 ± 0.39 cm at 7 week to 14.74 ± 0.30 cm at 35. week of age (Fig 1c). Horn length varied from 5.76 ± 0.69 cm at 7 week to 22.72 ± 0.52 cm at 35 week of age (Fig 1d). All the 4 variables were influenced by age ($p<0.001$).

Figure 1 – Age related changes (weeks) in body weight(a), scrotal circumference (b), horn diameter (c) and length (d) (LSM ± SEM).









No significant difference occurred between farm on EBW, EHD and EHL, but all the variables showed higher values in Farm B. A positive relation was present between EBW before slaughter and EHL (covariate 0.36 g, $p<0.05$) and PMBW (covariate 1.27 g, $p<0.001$). Farm had significant effect on ESC measurement (Farm A= 24.92 ± 0.61 cm, Farm B= 28.74 ± 0.71 cm; $p<0.01$). Values of TW and EW varied from the two farms. In farm A, both TW (122.51 ± 3.90 g) and EW (7.30 ± 0.31 g) were significantly higher ($p<0.001$, $p<0.05$ respectively) than in farm B (96.01 ± 4.54 g and 6.17 ± 0.36). Moreover TW was affected by EBW (covariate 1.75 g, $p<0.05$) and ESC variable (covariate 1.99 g, $p<0.05$). No positive relation between EW and other variables occurred.

3.4.2 Semen quality evaluation

Epididymal sperm was successfully collected from 54 of the 55 bucks. One testicle was rejected due to the absence of sperm in the collected fluid . The LSM ± SEM for the sperm parameters assessed in the 54 samples are outlined in Table 1 and Table 2. Farm had significant effect on semen volume ($p<0.001$), epididymal yield ($p<0.001$), number of spermatozoa ($p<0.05$) and total motility ($p<0.01$). In particular samples of farm A showed higher volume, epididymal yield, number of spermatozoa and total motility. No significant differences were observed in the other kinetics parameters and semen viability. Moreover, concentration and number of spermatozoa were affected positively by the age at slaughter (covariate 156.43 number of sperm/ml, $p<0.05$; covariate 532.88 total number of sperm, $p<0.01$ respectively).

Table 1. Results (LSM ± SEM) in terms of volume, concentration, number of spermatozoa, kinetics parameters and viability of fresh epididymal sperm samples collected from bucks, by farm

Parameters	Farm	
	A (n=27)	B (n=28)
Volume (ml)	2.71 ± 0.12[a]	2.04 ± 0.12[b]
Epididymal yield (ml)	0.45 ± 0.05[a]	0.19 ± 0.05[b]
Concentration (10^9/ml)	1.08 ± 0.10	1.17 ± 0.10
Number of spermatozoa (10^9)	3.19 ± 0.31[a]	2.27 ± 0.31[b]
Total motility (%)	80.61 ± 2.30[a]	69.94 ± 2.30[b]
Active cells (%)	37.14 ± 2.45	38.46 ± 2.45
Hyperactive cells (%)	35.71 ± 2.45	34.06 ± 2.45
Average path velocity (μm/s)	54.48 ± 2.48	56.55 ± 2.48
Curvilinear velocity (μm/s)	119.89 ± 2.60	121.01 ± 2.60
Straight-line velocity (μm/s)	31.02 ± 1.72	33.86 ± 1.72
Amplitude of lateral head displacement (μm)	6.51 ± 0.64	7 .28 ± 0.64
Beat cross-frequency (Hz)	10.04 ± 0.70	10.75 ± 0.60
Linearity index (%)	25.01 ± 1.01	27.00 ± 1.01
Straightness index (%)	59.52 ± 1.69	61.78 ± 1.69
Viability (%)	71.21 ± 1.75	73.84 ± 1.75

[1]a, b: values within each row with different letters are significantly different ($p < 0.05$).

Concerning sperm morphologies, as shown in Table 2., significant differences were present between the two farms on the total number of normal sperm ($p<0.05$) and then on sperm abnormalities ($p<0.05$). Samples of farm A showed a total number of normal sperm significantly higher than farm B due mainly to the major percentage of sperm without cytoplasmatic droplet (+14.7%) and the minor percentage of distal droplet (-32%). Almost all the majority of sperm defects in both farms were in tails (farm A=92%; farm B=95%), in farm A this kind of defect was significantly lower than farm B ($p<0.05$). Due to the

significantly decrease of defects in tail (-175%) then the total number of sperm abnormalities was significantly lower ($p<0.05$) in farm A than farm B. No sperm midpiece defects were observed.
Biometrical variables showed effects on some sperm morphological traits: ESC affected negatively the percentage of proximal droplets (covariate -0.38 cm, $p<0.05$) and EHD affected negatively the percentage of tail defects(covariate -0.45 cm, $p<0.05$). Also age of animals affected negatively the percentage of proximal droplets (covariate -1.55 weeks, $p<0.01$) and the percentage of head defects (covariate -0.09 weeks, $p<0.05$).

Table 2. Percentage (LSM ± SEM) of normal and abnormal sperm of fresh epididymal sperm samples collected from bucks, by farm

Parameters		Farm	
		A (n=27)	B (n=28)
Normal sperm	WCD[1](%)	75.80 ± 3.66	66.10 ± 4.20
	PD[1] (%)	1.10 ± 0.78	1.70 ± 0.89
	DD[1] (%)	21.80 ± 3.50	28.76 ± 4.02
	Total (%)	98.70 ± 0.57[a]	96.57 ± 0.66[b]
Sperm abnormalities	Head (%)	0.10 ± 0.06	0.16 ± 0.07
	Tail (%)	1.18 ± 0.55[a]	3.25 ± 0.63[b]
	Total (%)	1.28 ± 0.57[a]	3.41 ± 0.66[b]

[1]WCD, without cytoplasmatic droplets; PD, proximal droplets; DD, distal droplets.
[2]a,b:values within each row with different letters are significantly different ($p < 0.05$).

3.4.3 Correlation between in-vivo and post-mortem morphological evaluations

Correlation coefficient between in-vivo and post-mortem morphological evaluations are shown in Table 3. Estimated BW was significantly correlated with EHL ($p<0.001$, $r=0.51$), PMBW ($p<0.001$, $r=0.71$), TW ($p<0.001$, $r=0.53$), and EW ($p<0.001$, $r=0.50$). Also among TW and EW a highly linear association was evident ($p<0.001$, $r=0.63$). Both the two variables showed a moderate correlation coefficient with PMBW. A significant correlation was detected also between EHL with EW ($p<0.05$, $r=0.33$) and PMBW ($p<0.01$, $r=0.43$), and between EHD with PMBW ($p<0.01$, $r=0.42$).

3.4.4 Correlation between in-vivo and post-mortem morphological evaluations with semen quality

A positive linear correlation occurred between EBW, EHD, PMBW and the percentage of normal sperm $p<0.01$, $r=0.37$; $p<0.05$, $r=0.29$; $p<0.01$, $r=0.36$, respectively as showed in Table 4. Also EHL and concentration ($p<0.01$, $r=0.44$) showed a positive association. TW and EW variables were positive correlated with almost all the seminal parameters presented in Table 4. TW had a positive linear association with epididymal volume ($p<0.01$, $r=0.38$), epididymal yield

($p<0.05$, $r=0.32$), concentration ($p<0.05$, $r=0.27$) and consequently with the number of spermatozoa collected from each epididymis. TW had a positive association also with normal sperm ($p<0.01$, $r=0.35$) and with normal sperm without cytoplasmatic droplets ($p<0.01$, $r=0.38$). Regarding EW, a positive correlation between epididymal volume ($p<0.01$, $r=0.31$) concentration ($p<0.05$, $r=0.31$), number of spermatozoa collected from each epididymis ($p<0.01$, $r=0.45$), normal sperm ($p<0.01$, $r=0.35$) and with normal sperm without cytoplsmatic droplets ($p<0.05$, $r=0.32$) occurred. No linear associations were detected between all the in-vivo and post-mortem morphological evaluations with kinetics and viability variables and between SC and semen quality parameters shown in Table 4.

Table 3. Spearman correlation and p values between in-vivo and post-mortem morphological evaluations.

Parameters	EBW	ESC	EHL	EHD	TW	EW	PMBW
EBW	1	0.11(NS)	0.51(***)	0.24(NS)	0.53(***)	0.50(***)	0.71(***)
ESC		1	0.16(NS)	0.03(NS)	0.08(NS)	0.04(NS)	0.11(NS)
EHL			1	0.12(NS)	0.16(NS)	0.33(*)	0.43(**)
EHD				1	0.26(NS)	0.23(NS)	0.42(**)
TW					1	0.63(***)	0.35(**)
EW						1	0.40(**)
PMBW							1

***= $p<0.001$; ** = the $p<0.01$; * = $p<0.05$; NS=not significant.

Table 4. Spearman correlation and p values between in-vivo and post-mortem morphological evaluations with semen quality.

Parameters	Volume (ml)	Epididymal yield (ml)	Concentration (10^9/ml)	Number of spermatozoa (10^9)	Normal sperm (%)	WCD[1] (%)
EBW	0.14(NS)	0.12(NS)	0.11(NS)	0.13(NS)	0.37(**)	0.20(NS)
EHL	0.16(NS)	-0.10(NS)	0.44(**)	0.24(NS)	0.16(NS)	0.13(NS)
EHD	0.00(NS)	0.06(NS)	0.03(NS)	-0.01(NS)	0.29(*)	0.01(NS)
TW	0.38(**)	0.32(*)	0.27(*)	0.45(***)	0.35(**)	0.38(**)
EW	0.31(**)	0.24(NS)	0.31(*)	0.38(***)	0.35(**)	0.32(*)
PMBW	0.01(NS)	0.01((NS)	0.19(NS)	0.10(NS)	0.36(**)	0.20(NS)

***= $p<0.001$; ** = the $p<0.01$; * = $p<0.05$; NS=not significant.

EBW=estimated body weight; EHL=estimated horn length; EHD estimated horn diameter; TW=testicular weight; EW=epididymal weight; PMBW=post-mortem body weight.

[1] Normal sperm without cytoplsmatic droplets.

3.5 Discussion and conclusion

3.5.1 In-vivo and post-mortem morphological evaluations

In this study EBW, EHL and EHD were not significantly different between farm, but higher values occurred in farm B. While farm had a significant effect on SC, with higher values again in farm B. Considering the relationships between the 4 biometrical variables estimated at slaughter age performed in this study, we can said that EBW of Camosciata delle Alpi bucks was positively associate to EHL. This means that also HL development, as other secondary sexual traits (scrotal circumference and testicular weight) indicated by several authors (Coulter and Foote 1977; Bongso et al. 1982; Ahmad and Noakes 1996; Mekasha et al. 2007, 2008), occurs in parallel to the increase of live body weight. Scrotal circumference and testicular measurements are important components in breeding evaluation. In our study also a linear positive relation between EBW and TW and SC occurred, confirming that the growth of the genital organ is manifested in parallel to the increase of body weight as demonstrated in previous reports (Mekasha et al. 2007, 2008; Agga et al. 2011). Our findings showed that a positive correlation between in-vivo and post-mortem morphological parameters is present, to confirm of our previous evaluations. The EBW was moderately correlated with EHL, PMBW, and genital measurement after slaughter (TW and EW) as obtained by Agga et al. (2011) in rams and Mekasha et al. (2007, 2008) in bucks. Also PMBW variable showed positive correlation with several parameters as EHD and EHL, TW and EW, suggesting that at higher slaughter weight correspond better conditions of the secondary sexual traits. A positive and relatively high correlation ($r=0.63$) among TW and EW was present as obtained again by Agga et al. (2011) in rams and Ritar et al. (1992) in bucks.

3.5.2 Semen quality evaluation

Semen volume, epididymal yield, number of spermatozoa, total motility and normal sperm parameters were significantly different between farms. In particular samples of farm A were significantly better than samples of farm B. Considering that the retrograde flushing method has never been reported in goat species, no reports are available in the literature to compare this kind of data. Just Blash et al. (2000) have reported an example of epididymal sperm collection in goat but with a different epididymal sperm extraction method, the cutting method.

This study showed that a positive linear association between in-vivo and post-mortem morphological evaluations with some epididymal semen quality parameters exists. In particular SC was negatively ($p<0.01$) associated with the occurrence of proximal droplets as demonstrated by Vásquez et al. (2003) in bull

and HD affected negatively the percentage of sperm defects in tail ($p<0.05$). Age at slaughter affected some secondary morphological sperm abnormalities indices of immature sperm, decreasing the incidence of proximal droplets ($p<0.01$), as obtained again by Vásquez et al. (2003), and head defects ($p<0.05$). Moreover age at slaughter, in our case, have influenced positively bucks sperm production in term of concentration and total number of sperm recovered from the epididymides. A positive correlation between in-vivo and post-mortem morphological evaluations with some epididymal semen quality parameters of Camosciata delle Alpi bucks were present. The EBW and EHD were moderately correlated ($p<0.05$) with the number of normal sperm. Consequently a positive correlation ($p<0.01$) occurred also between PMBW and the number of normal sperm. Moreover our findings showed that a positive and significant correlation of TW and EW with some parameters of epididymal sperm production, as semen volume, epididymal yield, concentration and number of spermatozoa was present, and EHL with concentration again, suggesting that with an increasing of genital organs weight and of horn length also the capacity of Camosciata bucks to produce sperm can improve. For this reasons bucks of farm A, showing a significant higher testicular and epididymal weight, were able to produce higher semen volume, epididymal yield, concentration and a total number of epididymal spermatozoa. Findings of this study strongly agrees with Ritar et al. (1992), Mekasha et al. (2007) and Kabiraj et al. (2011) who reported that sperm production in bucks, in terms of total number of spermatozoa and concentration respectively, was positively correlated with TW.

In conclusion this study reveals that in Camosciata delle Alpi bucks scrotal circumference, horn length and testicular development occurs in parallel to the increase of live body weight. A good quality of goat epididymal sperm can be obtained by using the retrograde flushing technique. Studies to verify the effects of this extraction technique on goats semen under different experimental condition are reported in Chapter 4. Moreover epididymal semen quality in Camosciata delle Alpi goat breed is influenced by body weight and by secondary sexual trait as, scrotal circumference, horn diameter and length, testicular and epididymal weight.

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Chapter 4

Influence of temperature and post-mortem time on buck epididymal spermatozoa quality

4.1 Abstract

The aim of this work was to evaluate the quality of epididymal buck spermatozoa, extracted from the epididymis with the retrograde flushing technique, in pre-freeze and post-thaw samples, according to storage temperature (environment temperature or 5°C) and time elapsed between animal's death and sperm recovery (0, 24, 48, 72 hours). Testes were collected at the abattoir from 51 Camosciata delle Alpi bucks 1.30 hours on average after slaughter and transported to the laboratory at different temperatures, environmental (E) and refrigeration at 5°C (R). In the two groups (E-R) one testicle from each pair was processed within the first 4 hours after slaughter (T0) forming the control group (T0E-T0R). The contra-lateral testicle, was stored at environmental temperature (21.48 ± 2.22 °C), or 5°C, then processed after 24 (T24E, n=8; T24R, n=8), 48 (T48E, n=8; T48R, n=9) and 72 (T72E, n=9; T72R, n=9) hours.
Immediately after extraction epididymal sperm were cryopreserved in a Tris egg-yolk extender. Sperm concentration, motility, viability and morphology were evaluated on pre-freeze and post-thaw samples. Results show that a good quality of epididymal sperm can be obtained using the retrograde flushing technique in goat species. Moreover, according to our results goat epididymal spermatozoa extracted from testicles stored at 5°C until 48 hours post-mortem are able to maintain good quality in terms of total motility, viability and sperm morphologies, also after cryopreservation.

4.2 Introduction

The interest in developing assisted reproductive technologies (ART) and genetic resource banks for the preservation of biodiversity of endangered species has recently increasead. The recovery and freezing of viable sperm from the epididymes of dead animals (post-mortem recovery) is an interesting option for preserving male gametes (Kaabi et al. 2003). Nowadays concrete examples of application of post-mortem sperm recovery from the epididymis are available for conservation of endangered species (Santiago-Moreno et al., 2006) and in constructing semen banks (e.g. Ehling et al. 2006, Bagirov et. 2009, Leon-Quinto et al., 2009). Preservation of epididymal sperm from dead or slaughtered animals has been reported in several domestic and wild species: boar (Kikuchi et al. 1998), cattle (Martins et al. 2007, Turri et al 2011), goat (Blash et al.2000), ibex (Santiago-Moreno et al. 2006), red deer (Martinez-Pastor et al. 2006; Santiago-Moreno et al. 2009), and sheep (Kaabi et al. 2003; Ehling et al. 2006). Three main methods of post-mortem epididymal sperm recovery have been described

in different domestic and wild species. The retrograde flushing method seems to be one of the most recommendable methods in term of sperm quality recovered (Martinez et al. 2006, Turri et al. 2011). This method has never been reported in goat species.

Several studies have been conducted to determine the entity of decay of epididymal spermatozoa recovered post-mortem depending on some conditions (temperature and post-mortem time) in different species (cattle, Martins et al. 2009; red deer, Martínez-Pastor et al. 2005 and Soler et al. 2003; sheep, Kaabi et al. 2003; wild ruminant, Friedmann et al. 2000, Killian et al. 2000, Bisset and Bernard 2005 and Fernandez-Santos et al. 2011). However no data are available in the literature on the effects of temperature and post-mortem time of testicles conservation on goat epididymal spermatozoa quality.

Given the promising results obtained by the same authors in Chapter 2 (Turri et al. 2011) by using the retrograde flushing technique in cattle, the aim of this work was to evaluate the quality of epididymal buck spermatozoa, extracted from the epididymis with the retrograde flushing technique, in pre-freeze and post-thaw samples, influenced by storage temperature (environmental or 5°C) and by the time elapsed between animal's death and sperm recovery (0, 24, 48, 72 hours). The investigation was carried out within a programme addressed to establish protocols for the recovery and the cryopreservation of viable sperm from the epididymis of slaughtered animals, as a tool in creating semen banks for endangered breeds.

4.3 Material and Methods

4.3.1 Experimental design

Testes were collected at the abattoir from 51 Camosciata delle Alpi bucks (mean age 35.30 weeks, standard error 1.27 weeks), 1.30 h on average after slaughter and transported to the laboratory according to the experimental conditions that underwent: for 25 animals both testicles were transported at environmental temperature (E), instead for the remaining 26 animals, both testicles during transportation were refrigerated at 5°C (R) with a portable fridge. In the two groups (E, R) one testicle from each pair was processed within the first 4 hours after slaughter (T0) forming the control group (T0E, T0R). The contra-lateral testicle was stored at environmental temperature (21.48 ± 2.22 °C) or 5°C, then processed after 24 (T24E, n=8; T24R, n=8), 48 (T48E, n=8; T48R, n=9) and 72 (T72E, n=9; T72R, n=9) hours.

4.3.2 Testicles preparation, sperm recovery and epididymal semen quality evaluation

The procedures of testicles preparation and epididymal sperm recovery were performed as described by Turri et al. (2011) in Chapter 2. Immediately after extraction, sperm concentration, motility, viability, and morphology were evaluated as mentioned in Chapter 2. All assessments were performed also on post-thawed samples.

4.3.3 Freezing and thawing of epididymal sperm

After collection, epididymal semen samples recovered were diluted with a Tris egg yolk extender consisting of 20% v/v egg yolk, 7% wt/vol glycerol, 2.42% wt/vol Tris buffer, 1.0% wt/vol fructose, 1.38% wt/vol citric acid, 5.5 mg tylosin, 27.5 mg gentamycin, 16.5 mg lincospectin, and 33.0 mg spectinomycin per 100 mL (Blash et al., 2000), to obtain a final concentration of $600x10^6$ sperm/ml. Samples were then refrigerated to 5°C and maintained at this temperature for a minimum of 4 hours. After the equilibration time, the diluted sperm suspension was loaded into 0.5 ml CBS™ High Security Straws (IMV Technologies, Piacenza, Italy) by using a semi-automatic system of packaging (Cryo Bio System, IMV Technologies, Piacenza, Italy). For each animal batch cryogenic labels reporting identification code (Animal ID, Date of production) were printed with the labeling printers. Then straws were frozen on nitrogen vapor for 15 min, plunged into liquid nitrogen and stored in liquid nitrogen tanks. After cryostorage for above 4 months, two straws per animal were thawed at 37°C for 2 minutes to determine the post-thaw epididymal semen quality, as above indicated.

4.3.4 Statistical analysis

Statistical analyses were carried out using the SAS™ package v 9.2 (SAS Institute Inc., Cary, NC). The General Linear Model (PROC GLM) procedure was used to analyse the effects of temperature and post-mortem time on buck epididymal spermatozoa quality. In the model fixed effects of farm, experimental group (temperature of conservation and post-mortem time) and sperm status (pre-freeze, post-thaw) were included. Age at slaughter (weeks) and temperature of testicles before epididymal sperm recovery were considered as covariates. Results are expressed as adjusted least squares means ± standard error means (LSM ± SEM).

4.4 Results

Epididymal sperm were successfully collected from all bucks. LSM ± SEM for the sperm parameters assessed in the 51 samples are given in Tables 1, 2, 3 and 4. All the variables with the exception of sperm concentration and cytoplasmatic droplets were affected by farm. Moreover, age at slaughter affected positively volume (covariate 0.08 ml, $p<0.05$) concentration (104.8 $x10^9$, $p<0.01$), total number of sperm (covariate 371.99 $x10^9$, $p<0.001$) and consequently number of doses (covariate 1.23 , $p<0.001$), and affected negatively percentage of proximal droplets (covariate -1.59, $p<0.001$).

4.4.1 Sperm production

Post mortem time had a significant effect on semen volume ($p<0.05$), epididymal yield ($p<0.01$), sperm concentration ($p<0.001$), total number of spermatozoa ($p<0.001$), and number of doses ($p<0.001$) as showed in Table 1. In particular at 48 hours of testicles storage, lower concentration and total number of spermatozoa were observed and consequently a lower number of doses was produced for each epididymides. The same pattern occurred for volume and epididymal yield, but after 48 hours of testicles storage (T48 vs T72, $p < 0.05$).

Table 1. Sperm production (LSM ± SEM) as a function of post-mortem time

Parameters	T0 (n=51)	T24 (n=16)	T48 (n=17)	T72 (n=18)
Volume (ml)	2.39 ± 0.06[a]	2.57 ± 0.12[a]	2.35 ± 0.11[a]	2.00 ± 0.12[b]
Epididymal yield (ml)	0.32 ± 0.03[a]	0.40 ± 0.05[a]	0.26 ± 0.05[a]	0.07 ± 0.05[b]
Concentration (10^9/ml)	1.12 ± 0.05[a]	1.20 ± 0.09[a]	0.85 ± 0.09[b]	0.62 ± 0.09[b]
Number of spermatozoa (10^9)	2.69 ± 0.15[a]	3.22 ± 0.29[a]	1.88 ± 0.27[b]	1.49 ± 0.28[b]
Doses (n.)	8.97 ± 0.51[a]	10.74 ± 0.96[a]	6.28 ± 0.90[b]	4.98 ± 0.96[b]

[1]Values within each row with different letters are significantly different ($p < 0.05$).

4.4.2 Motility

The interaction between the experimental groups and the epididymal semen samples status (pre freeze-post thaw) had a significant effect on total motility ($p<0.001$).

Considering the same temperature of testicles conservation, both for E than R groups, no significant decrease of total motility was observed until 48 h (Table 2). At 72 h a strong decrease of total motility was observed at both temperature (E=$p<0.001$; R=$p<0.01$), most significantly in T72E group.

Comparing the effects of different temperatures at the same time of conservation, a significant decrease ($p<0.001$) occurred at 72 h, considerably in case of R temperature.

Freezing had a significant effect on post-thaw samples in T0E and T24E groups. In case of the R temperature, no differences were observed between pre-freeze and post-thaw samples.

4.4.3 Viability

Also on viability the interaction between the experimental groups and the epididymal semen samples status had a significant effect ($p<0.001$).

Within the E group, no significant decrease of viable epididymal sperm was observed until 48 h of testicles conservation (Table 2); a decrease of 21% in this group occurred at 72 h ($p<0.01$). Also in the R group temperature had a significant effect on epididymal sperm viability, with respect to the control group T0R, at 72 h of testicles conservation ($p<0.05$).

Different temperatures, at the same time elapsed after slaughter, did not affect viability of fresh samples, however in the R groups higher values of live sperm (+8% on average) occurred than in the E groups. A significant reduction of viability from pre-freeze to post-thaw status was evident in all the groups, more evident in case of E temperature: a decreasing on average by 53% against the 32% of the refrigerated samples. Refrigeration temperature, at the same time of conservation on fresh samples, had a positive impact on post-thaw quality of refrigerated samples after 24 h of testicles conservation, increasing viability of R samples on average by 46% with respect to the E samples.

Table 2. Total motility and viability (LSM ± SEM) by experimental group and sperm status

Groups	Testicles (n.)	Total motility (%)		Viability (%)	
		Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
T0E	25	$70.42 \pm 3.14^{a\alpha 1}$	$79.11 \pm 3.14^{a\alpha 2}$	$70.24 \pm 1.92^{a\alpha 1}$	$46.92 \pm 1.92^{a\alpha 2}$
T24E	8	$69.36 \pm 5.56^{a\alpha 1}$	$25.07 \pm 5.56^{b\alpha 2}$	$68.97 \pm 3.40^{a\alpha 1}$	$22.47 \pm 3.40^{bc\alpha 2}$
T48E	8	$60.26 \pm 5.33^{a\alpha 1}$	$47.05 \pm 5.33^{c\alpha 1}$	$64.06 \pm 3.26^{a\alpha 1}$	$30.93 \pm 3.26^{b\alpha 2}$
T72E	9	$19.34 \pm 5.17^{b\alpha 1}$	$20.06 \pm 5.17^{b\alpha 1}$	$49.99 \pm 3.16^{b\alpha 1}$	$19.45 \pm 3.16^{c\alpha 2}$
T0R	26	$78.06 \pm 2.99^{a\alpha 1}$	$84.52 \pm 2.90^{a\alpha 1}$	$73.25 \pm 1.83^{ac\alpha 1}$	$50.35 \pm 1.83^{a\alpha 2}$
T24R	8	$82.33 \pm 5.56^{a\alpha 1}$	$80.33 \pm 5.56^{a\beta 1}$	$78.22 \pm 3.40^{a\alpha 1}$	$49.84 \pm 3.40^{a\beta 2}$
T48R	9	$69.24 \pm 5.87^{a\alpha 1}$	$82.36 \pm 5.87^{a\beta 1}$	$68.31 \pm 3.11^{c\alpha 1}$	$52.78 \pm 3.11^{a\beta 2}$
T72R	9	$50.24 \pm 5.37^{b\beta 1}$	$52.30 \pm 5.37^{b\beta 1}$	$58.62 \pm 3.29^{b\alpha 1}$	$35.00 \pm 3.29^{b\beta 2}$

[1]Different letters (a, b, c) indicate difference ($p<0.05$) within column, at the same temperature of conservation (E-R).

[2]Different letters (α, β, γ) indicate difference ($p<0.05$) within column, at the same time of conservation (0-24-48-72).

[3]Different numbers indicate difference ($p<0.05$) within row, among pre-freeze and post-thaw status.

4.4.4 Sperm abnormalities and cytoplasmatic droplets

Interaction between experimental groups and epididymal semen samples status had a significant effect on sperm abnormalities and distal droplets ($p<0.001$, respectively). Semen samples status had not significant effects on proximal droplets.

Concerning sperm abnormalities within the E group, no significant difference were observed between hours of testicles conservation (Table 3). The same condition occurred in refrigerated samples but in this case after 48 h of testicles conservation a significant increase ($p<0.01$) of sperm abnormalities was observed.

Different temperatures, at the same time of testicles conservation, did not affect sperm abnormalities of fresh/pre-freeze samples until the 48 hours of gonads storage. At 72 hours of gonads storage, a significant increasing ($p<0.001$) of abnormalities occurred again in R samples. At thawing, in general, samples of testicles stored at environment temperature showed an increase of sperm abnormalities, by 83% versus the 30% of samples of testicles stored at refrigerated temperature.

Freezing had a significant effect on post-thaw samples, increasing sperm abnormalities in the testicles stored for 24 hours ($p<0.001$) in the E groups and for 48 hours ($p<0.001$) in the R groups.

Table 3. Sperm abnormalities (LSM ± SEM) by group and sperm status

Groups	Testicles (n.)	Sperm abnormalities (%)	
		Pre-freeze	Post-thaw
T0E	25	$2.17 \pm 1.41^{a\alpha 1}$	$3.10 \pm 1.41^{a\alpha 1}$
T24E	8	$0.43 \pm 2.50^{a\alpha 1}$	$36.74 \pm 2.50^{b\alpha 2}$
T48E	8	$1.51 \pm 2.39^{a\alpha 1}$	$17.90 \pm 2.39^{c\alpha 2}$
T72E	9	$3.96 \pm 2.32^{a\alpha 1}$	$26.97 \pm 2.32^{d\alpha 2}$
T0R	26	$2.52 \pm 1.34^{a\alpha 1}$	$3.04 \pm 1.34^{a\alpha 1}$
T24R	8	$3.22 \pm 2.50^{a\alpha 1}$	$4.51 \pm 2.50^{a\beta 1}$
T48R	9	$7.25 \pm 2.28^{a\alpha 1}$	$17.07 \pm 2.28^{b\alpha 2}$
T72R	9	$18.06 \pm 2.41^{b\beta 1}$	$22.15 \pm 2.41^{b\alpha 1}$

[1]Different letters (a, b, c) indicate difference ($p<0.05$) within column, at the same temperature of conservation (E-R).

[2]Different letters (α, β, γ) indicate difference ($p<0.05$) within column, at the same time of conservation (0-24-48-72).

[3]Different numbers indicate difference ($p<0.05$) within row, among pre-freeze and post-thaw status.

Percentage of proximal droplets in pre-freeze samples was not affected by hours of testicles conservation at the same temperature of conservation, with the exception of the T72E group ($p<0.01$), where a significant increasing of proximal droplets occurred (Table 4). Different temperatures, at the same time

of conservation, did not affect the percentage of proximal droplets within groups, but in T72R where a significant decreasing ($p<0.01$) of proximal droplets was observed. In post-thaw samples, freezing had not a significant effect on proximal droplets percentage.
Considering the trend of distal droplets (Table 4) among hours of testicles conservation on fresh samples, no significant variations were observed within the E group, except in the T24E group where higher values were observed ($p<0.001$). Observing the refrigerated group a significant reduction of distal droplets occurred in the control group compared to the T72R group ($p<0.05$). In general in the R group a linear decrease of percentage of distal droplets was observed by increasing post-mortem time.
Different temperatures, at the same time of conservation, affected percentage of distal droplets; in particular the R temperature induced a significant reduction of this kind of sperm morphologies after 24 h of testicles conservation.
Freezing had a significant effect on post-thaw samples, decreasing overall percentage of distal droplets.

Table 4. Proximal and distal droplets percentage (LSM ± SEM) by group and sperm status

Groups	Testicles (n.)	Proximal droplets (%)		Distal droplets (%)	
		Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
T0E	25	$1.76 \pm 1.02^{a\alpha 1}$	$2.07 \pm 1.02^{a\alpha 1}$	$25.70 \pm 2.63^{a\alpha 1}$	$18.85 \pm 2.63^{a\alpha 2}$
T24E	8	$1.95 \pm 1.82^{a\alpha 1}$	$0.32 \pm 1.82^{a\alpha 1}$	$64.46 \pm 4.66^{b\alpha 1}$	$24.18 \pm 4.66^{ac\alpha 2}$
T48E	8	$2.05 \pm 1.74^{a\alpha 1}$	$2.62 \pm 1.74^{a\alpha 1}$	$29.83 \pm 4.46^{a\alpha 1}$	$45.11 \pm 4.46^{b\alpha 2}$
T72E	9	$10.01 \pm 1.69^{b\alpha 1}$	$7.87 \pm 1.69^{b\alpha 1}$	$26.08 \pm 4.32^{a\alpha 1}$	$33.63 \pm 4.32^{bc\alpha 1}$
T0R	26	$0.88 \pm 0.97^{a\alpha 1}$	$1.86 \pm 0.97^{a\alpha 1}$	$22.46 \pm 2.50^{a\alpha 1}$	$19.13 \pm 2.50^{ab\alpha 1}$
T24R	8	$0.24 \pm 1.82^{a\alpha 1}$	$0.19 \pm 1.82^{a\alpha 1}$	$16.87 \pm 4.66^{ab\beta 1}$	$26.87 \pm 4.66^{a\alpha 1}$
T48R	9	$1.32 \pm 1.66^{a\alpha 1}$	$1.85 \pm 1.66^{a\alpha 1}$	$15.17 \pm 4.26^{ab\beta 1}$	$13.93 \pm 4.26^{b\beta 1}$
T72R	9	$1.81 \pm 1.75^{a\beta 1}$	$1.99 \pm 1.75^{a\beta 1}$	$9.87 \pm 4.49^{b\beta 1}$	$16.80 \pm 4.49^{ab\beta 1}$

[1]Different letters (a, b, c) indicate difference ($p<0.05$) within column, at the same temperature of conservation (E-R).
[2]Different letters (α, β, γ) indicate difference ($p<0.05$) within column, at the same time of conservation (0-24-48-72).
[3]Different numbers indicate difference ($p<0.05$) within row, among pre-freeze and post-thaw status.

4.5 Discussion

This study for the first time investigated the decay of quality of epididymal spermatozoa recovered post-mortem, in relation to temperature and post-mortem time in the goat. Moreover, it was the first example of recovery of

epididymal sperm performed by retrograde flushing method in bucks. Then no data are available on literature to compare the effects of decay and extraction technique in goat semen. Blash et al. (2000) reported on epididymal sperm collection in goat but with a different extraction method, the cutting method.
This study revealed that age at slaughter affects positively several parameters of mature sperm production, such as semen volume, concentration, total number of sperm, and consequently number of doses producible per epididymides, and lower incidence of proximal droplets. These results were in agreement with those reported in Chapter 3.

4.5.1 Pre-freeze semen quality

Considering post-mortem time, our data reveal that time elapsed after death, in particular between 24 and 48 hours of testicles conservation, had relevant effects on goat epididymal sperm production, reducing the possibility of extracting epididymal sperm cells by the cauda epididymidis and consequently the number of semen doses (from 9 at T0, to 5 at T72, for each epididimides). This can be explained by the fact that tissues of the vas deferens lumen and of the cauda epididymis, after death progressively undergo to a deterioration process, decomposition and dehydration. This process is more marked in samples stored at environment temperature, that compromises epididymis handling condition during sperm extraction.

Pre-freeze spermatozoa quality parameters as total motility, viability, sperm abnormalities and cytoplasmatic droplets, showed no significant variation until 48 h of testicles conservation, both at environmental and 5°C temperature. However, in this period of time a linear decay of total motility and viability and an increasing of abnormal sperm was observed. At 72 hours of testicles storage a significant decrease of total motility and viability was observed at both temperatures of conservation, with particular incidence in samples stored at environmental temperature (total motility: -67% from T48E to T72E group, respect to -27% from T48R to T72R group; viability: -21% from T48E to T72E group, respect to -14% from T48R to T72R group). Similar trends were observed in refrigerated samples by Martinez-Pastor et al. (2005) in deer. In rams (Kaabi et al., 2003) a general decay of epididymal semen quality was already observed after 24 hours of testicles conservation. Concerning sperm morphologies, an increase of abnormal cells was observed in both experimental group (E, R), more marked under refrigeration conditions ($p<0.01$).

Refrigeration of testicles allowed to obtain a better quality, but only after 48 hours of conservation, especially for total motility, as observed in other rams (Kaabi et al. 2003) and bulls (Martins et al. 2009). This can be explained by the

reduction of the metabolism of sperm cells, in terms of mitochondrial oxidative phosphorylation and glycolytic activities, that occurs at 5°C (Salamon et al., 2000). Viability, although not in a significantly way, was also positively influence by refrigeration temperature. Until 48 hours of testicles conservation, sperm abnormalities did not increase in a significant way under refrigeration. After this period, an increase of abnormal sperm occurred. This behaviour was also observed by Soler et al. (2003) in the red deer and by Bisset et al. (2005) in the eland. The increase of abnormal sperms in testicles kept at 5°C could therefore be due to prolonged exposure to low temperatures. The incidence of cytoplasmatic droplets, both proximal than distal, was significantly lower in refrigerated samples stored for 72 hours. The same condition was observed by Nichi et al. (2007) in bulls, where the loss of sperm droplets was linked to cold shock.

4.5.2 Post-thaw semen quality

Considering the effects of time elapsed between animal death and sperm recovery on post-thaw samples, our data showed that total motility and viability of samples obtained by testicles stored under E temperature have a significant reduction already at 24 hours.

In samples refrigerated at 5°C, a significant decrease of these parameters occurred later, at 72 hours of testicles conservations. Comparing the effects of temperature at the same time of testicles conservation, refrigeration temperature allowed to obtain a significant higher quality, in particular for motility and viability, at 24 hours of testicle conservation.

Reduction in motility after cryopreservation was not observed in this study, as registered by Martins et al. (2007). However freezing induced a significant reduction of viability from pre-freeze to post-thaw status in all groups, more marked in case of the E temperature than refrigerated samples (53% vs 32%). The same conditions were observed in rams (Kaabi et al., 2003) and bulls (Martins et al. 2009). Therefore, the effect of 5°C refrigeration during testicles conservation on fresh samples reflected a positive impact also on post-thaw samples, allowing a lower decline of buck epididymal sperm decay through time. Concerning sperm morphology, freezing had a significant effect also on post-thaw samples, increasing the incidence of sperm abnormalities in testicles stored for 24 hours ($p<0.001$) in the E groups, and for 48 hours ($p<0.001$) in the R groups, and decreasing overall the percentage of distal droplets.

4.6 Conclusions

A good quality of epididymal sperm can be obtained by using the retrograde flushing technique in the goat. Between 24 and 48 hours post-mortem, epididymides can be handled easily, to obtain an acceptable number of semen doses. Goat epididymal spermatozoa extracted by testicles stored at 5°C until 48 hours post-mortem are able to maintain their quality in terms of total motility, viability and sperm morphologies also after cryopreservation. Further studies aiming to the test the fertilizing capacity should be carried out in order to confirm the opportunity of storing epididymal sperm in the creation of semen banks for endangered breeds, that, as shown in Chapter 5, could be less expensive than storing semen collected with the traditional system .

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Chapter 5

Development of the Cryobank for animal genetic resources of the Lombardia region, Italy.

5. Development of the Cryobank for animal genetic resources of the Lombardia region, Italy.

5.1 Abstract

This work reports on several aspects encountered during the creation of the Lombardia Farm Animal Genetic Resources Cryobank (LABank) and during the development of a coordination system between Italian animal genetic resources cryo storages (CRIONET-IT). Among others, aims, choice of breeds and donors, health management, costs, access and ownership of the stored material are presented and discussed. At present in the LABank genetic material of Varzese cattle, Brianzola sheep and Frisa, Orobica and Verzaschese goats is stored. In order to reduce costs and overcome organisation difficulties, in the case of Brianzola sheep, epididymal spermatozoa were collected instead of conventional semen collection. Information on donor animals and the material stored are managed through the specific software CryoWEB. CRIONET-IT is made of two major components. The first is a website, open to the public, where the aims of the Network are illustrated, Partners and Collaborators are given, and a summary of the material stored by all partners is presented. The second is the CryoWEB reserved area, in which Partners can manage their own data by using a CryoWEB software somehow modified. Data and information collected in the development process of the two systems is intended as a contribution to the future creation of an Italian national gene-bank, and more generally to the *ex situ* management of farm animal genetic resources as a complementary tool to *in situ* conservation strategy.

5.2. Introduction

Within the framework of conservation and sustainable use of livestock genetic resources (AnGR), conservation strategies are categorized as *in situ*, that is the keeping of animals within the production systems in which they were developed, and *ex situ*. *Ex situ* conservation mainly refer to cryoconservation of semen, ova, embryos or tissues. The Convention on Biological Diversity (CBD, 1992) emphasizes the importance of *in situ* conservation and considers *ex situ* conservation as an essential complementary strategy to *in situ*. The choice of the technique, or of a combination of techniques, depends upon the conservation aims, considering that *in situ* and *ex situ* techniques differ in their capacity to achieve different objectives (Gandini and Oldenbroek, 2007).

In the European Union, in order to rationalise cryo-storage of AnGR, some countries have started to create national gene banks or analogous centralised operations. These include: Austria (Austrian Gene Bank for Farm Animals, 1997), France (Cryobanque Nationale, 1999), Netherland (Center for Genetic Resources - Gene Bank, 2001) and Nordic Countries (Denmark, Finland, Iceland, Norway and Sweden: Viking Genetics, the Danish-Swedish-Finnish AI-centre).

In Italy, in the last two decades, semen of local livestock breeds has been collected in the framework of different conservation programs, by the Associazione Italiana Allevatori (AIA), Ministero delle Politiche Agricole, Alimentari e Forestali (MiPAAF), ConSDABI, some regional administrations, Universities and research institutes. Because lack of coordination at the national level, the acquisition process has been most of the time opportunistic, that is without searching specific animals within specific gene-banking aims.

In 2008 the Directorate General (DG) for Agriculture of the Lombardy Region and the National research Council (CNR) started the creation of a cryobank for the animal genetic resources of the Lombardia Region, the Lombardia Farm Animal Genetic Resources Cryobank (LABank). Being aware that conservation of animal genetic resources can not only be planned at the regional level, the work was also addressed to create an information system to share the information on the genetic material stored in the different Italian collections, the "Network of the Italian cryobanks of farm animal genetic resources" (CRIONET-IT), as a first step toward the development of a national gene-banking system.
This chapter presents and discusses the scientific aspects of the process of development of both LABank and CRIONET-IT.

5.3 Development of the Lombardia Farm Animal Genetic Resources Cryobank (LABank) - Methodology

5.3.1 Cryo banking objective

Gandini and Oldenbroek (2007) review the different aims of gene-banking of AnGR. These includes: to reconstruct the breed in case of extinction or loss of a major portion of its diversity; to create new lines/breeds; as a back up to quickly modify or reorient the breed evolution process; to support cryo aided management schemes of small populations; as a resource for research. Considering the list of possible objectives and the high cost associated to cryo-conservation, gene-banks should have a multifunctional character.

Among the other aims, breed reconstruction requires the highest quantity of material, thus covering somehow other aims (Boettcher et al, 2005).
Breed reconstruction was chosen as objective of the collections stored in LABank. Because the limited budget available, the work developed has to be considered an initial phase.

5.3.2 Access and ownership of the genetic material

All cryo-storage objectives are related to the future use of genetic material stored. Then, rules of access to the stored material and property rights of the material should be clearly defined (Hiemstra, 2003).
In the case of LABank, material to be stored included both semen collected in the past under different conditions, and semen collected within the project in various farming contexts. To accomplish the different requirements raising from this multifaceted framework, various access and ownership rules were adopted for different breeds and different genetic stocks within the same breed.

The following overall principles were adopted:
1) the genetic material is stored exclusively for the conservation and sustainable use of breed genetic diversity, that is material for routine or commercial use is not considered;
2) rules and decisions on material acquisition and use are taken by the LABank management committee, but all major stakeholders should participate to define both processes of acquisition and use of the genetic material;
3) access and ownership rules should be as much as possible simple to facilitate their implementation and the sustainable and equitable use of the genetic material stored.

The Varzese cattle and Verzaschese goat cases provide an example of the complexity of the process undertaken.
Written agreements were stipulated between the owner of each Varzese bull donor and LABank. The semen collection was paid by the project. LABank is the owner and manager of the genetic material. In particular in this case the material will be managed in agreement with the Associazione Provinciale Allevatori di Pavia, peripheral office of Varzese cattle breed Herbook according to the indications provided from Ministry of Agricultural, Food and Forestry Policies or Regions thought the DM n° 403/2000, Art.20. On average ten percent of doses produced by each bull will be donated to the farmers owner of the donors for routine use as defined above. In addition, Varzese semen from 3 bulls was bought from a research Institution that collected large amounts of material some year ago. Also in this case LABank owns the material.

In Verzaschese goat breed a written agreement between LABank and the Comunità Montana Valli del Verbano was stipulated in order to acquire the genetic material collected and stored within the project Interreg IIIA Italia - Svizzera - 2000-2006. The Comunità Montana was interested to find a low cost solution for the long maintenance of the material. The agreement implies that the Comunità Montana Valli del Verbano remains the owner of the genetic material transferred to LABank. LABank will manage the material, with the Comunità Montana having a veto option if breed conservation aims are not met. A limited amount of the material, if required, can be allocated for the routine management of the breed.

Thus, ownership and access rules of the material stored in LABank can vary. We deliberately avoided to have the donors owner keeping ownership on the semen, in order to avoid complex management issues in the future.

5.3.3 Storage sites

According to the Guidelines for the Constitution of the National Cryopreservation Programmes for Farm Animals (ERFP, 2003) and Guidelines for the Cryoconservation of Animal Genetic Resources (FAO, 2011) a primary storage site has been created, and with the aim to duplicate the material collected for insurance against catastrophic events a "secondary storage" site of cryoconserved germplasm is under construction, The primary storage site is located at the Parco Tecnologico Padano (PTP), in Lodi. The second storage site is planned to be built at an AI center.

Particular attention was given to the safety both of personnel and genetic material stored. The gene bank rooms are equipped with a oxygen saturation alarm system (Select S.r.l, Milano, Italy) for the detection environmental oxygen saturation levels. Liquid nitrogen tanks (IC-38RX-10, International Cryogenics Inc., IMV Technologies, Piacenza, Italy) are monitored by probes that, in case of a significant temperature variation, sends through an alarm system (Chessell 6100A- Eurotherm, IMV Technologies, Piacenza, Italy), signals via web and telephone to the gene bank manager (Fig 1-2). The LABank personnel was trained within a liquid nitrogen safety course held by UOFAA s.c.r.l, Pavia.

5.3.4 Choice of species, breeds, animal donors and genetic material

The choice of species and breeds to be stored was carried out through four steps, with the collaboration of the breeders' associations and breed experts.

Step 1: Analysis of the list of the "local animal breeds threatened with extinction" in the Lombardia region Rural Development Programme (PSR)

2007-2013. In the Lombardia region a total of nine local breeds are still farmed, mostly in marginal areas, as hills and mountain, in small herds. In Table 1 the nine breeds are given by species: cattle, sheep and goat. The status of risk of extinction was taken by DAD-IS (www.fao.org/dad-is) and verified according to the most recent census. Six of the nine local breeds (66,6%) are classified as "Critical" and "Endangered". Some priority was given to those breeds classified as critical or endangered.

Table 1. Lombardia livestock species and breed according to PSR 2007-2013 in year 2008.

Species	Breed	Farm areas	Population size	Risk Status FAO
Cattle	Bianca Val Padana	BS	670	Endangered
Cattle	Varzese-Ottonese	AL, MI, PV	95	Critical
Sheep	Brianzola	CO, LC, MI	912	Endangered
Sheep	Corteno	BS	322	Endangered
Goat	Bionda dell'Adamello	BG, BS, CO, LC	3426	Not at risk
Goat	Frisa Valtellinese	SO	1043	Endangered
Goat	Livo	CO	2517	Not at risk
Goat	Orobica	BG, CO, LC, SO	1071	Endangered
Goat	Verzaschese	CO, VA	1931	Not at risk

[1]Population size in year 2008 as reported by DAD-IS FAO database (cattle) and ASSO.Na.Pa (sheep and goat).

[2]Risk status FAO, 1) Critical: the total number of breeding females is less than or equal to 100 or the total number of breeding males is less than or equal to five; or The overall population size is less than or equal to 120 and decreasing and the percentage of females being bred to males of the same breed is below 80 percent. 2) Endangered: the total number of breeding females is greater than 100 and less than or equal to 1000 or the total number of breeding males is less than or equal to 20 and greater than five; or The overall population size is greater than 80 and less than 1000 and increasing and the percentage of females being bred to males of the same breed is above 80 percent; or The overall population size is greater than 1000 and less than or equal to 1200 decreasing and the percentage of females being bred to males of the same breed is below 80 percent. 3) Not at risk: a breed is categorized as Not at Risk if none of the above definitions apply and: The total number of breeding females and males are greater than 1000 and 20, respectively; or If the population size is greater than 1200 and the overall population size is increasing.

Step 2: A census of the existing collections established in previous project was carried out in order to avoid duplication and optimize the conservation value of the material to be collected.

Step 3: No data to estimate genetic variation within and between breeds and to perform a proper breed selection based on genetic variation were available. All information available on breeds' past and recent history, including some genetic distances, were collected. It was decided to give priority to breed uniqueness and to high diversity among breeds.

Step 4: Information on past and current conservation programmes were collected. The opportunity of contributing, with the cryo-storage activities, to enhance an existing conservation programme was given some priority.

Considering the aspects above described, the following breeds were chosen to be included in the cryo-bank: Varzese-Ottonese cattle, Frisa Valtellinese goat, Orobica goat, Verzaschese goat, and Brianzola sheep.
When we consider each of the five breeds selected, sampling of donors should aim to maximise genetic variation in the storage (e.g. Caballero and Toro, 2002). Pedigree information were available only for the Varzese-Ottonese cattle, and the group of donors was selected by minimising average relationship using the Minbreed software (Gandini and De Filippi, 1998). Minimisation was carried out considering also the material previously stored. When no pedigree information were available, information on exchange of animal among herds were collected and donors were sampled as much as possible from different unrelated herds. For each donor, morphological traits, photographs and farm location were recorded.

In this first step of creation of the LABank, it was decide to store semen because the lower costs and the lower operation difficulties. In the next steps oocytes and embryos collection will be considered.
Concerning semen, the collection from the cauda epididymides of slaughtered animals was also used in order to reduce costs (Gandini et al., 2007) and in those cases where it was particularly difficult to have animals trained to semen collection. Blood and hair samples were also collected for future DNA screening.

5.3.5 Health management

Sanitary criteria has to be followed to allow in the future the safe use of the genetic material stored. Law n. 30, 15 january 1991, on "Disciplina della riproduzione animale", and subsequent amendments, establish criteria for the management of animal reproduction in the major livestock species. Semen has to be collected in authorised centres from donors farmed in controlled herds. However D.M. 403/2000, art. 20 allows to collect genetic material from local breeds directly on farm. All semen samples were collected under this regulation and cattle donors were tested for Bovine brucellosis (B. melitensis, B. abortus), Bovine tuberculosis, Bovine paratubercolosis, Bovine viral diarrhoea-mucosal disease (BVD-MD), Bovine Leptospirosis, Infectious bovine rhinotracheitis (IBR), Enzootic bovine leukosis (EBL), Campylobacter foetus, Trichomonas foetus, Blue Tongue. For local sheep and goat breeds semen collection does not

exist a national health protocol, then a protocol was developed with the Official Veterinary Services.
For local sheep and goat breeds does not yet exist a protocol regarding donor status health investigation in case of semen collection directly on farm. To overcome this gap, for these species farm and flock disease status was checked and certified by Veterinary Services.

Semen was packaged with 0,5 ml CBS™ High Security Straws (IMV Technologies, Piacenza, Italy) in order to exclude contamination from straw to straw due to different donors' health status and to provide a dual sanitary warranty: the straw content cannot be contaminated by the outside environment, and the environment cannot be contaminated by the straw content (Guérin, 1998).

5.3.6 Semen collection, evaluation and cryopreservation

Bulls semen collection and cryoconservation were performed by a commercial firm.

Bucks semen was collected with repeated semen collections, by our team using an AV and estrous females as teasers. After collection, semen samples obtained were diluted with Ovixcell extender (IMV Technologies, Piacenza, Italy) at a ratio of 1:1 (semen to Ovixcell extender, v:v) at environment temperature. The diluted sperm suspensions were cool at 5 °C within 30 minutes after collection with a portable refrigerator and transported to our laboratories in Lodi. After collection sperm concentration, motility, viability and morphology were evaluated following the methodology given by Turri et al. (2011). After semen evaluation the diluted semen at a final concentration of $300x10^6$ sperm/ml was loaded into 0.5 ml CBS™ High Security Straws (IMV Technologies, Piacenza, Italy) through a semi-automatic system of packaging (Cryo Bio System, IMV Technologies, Piacenza, Italy).

Artificial Insemination is not routinely used in sheep breeding in Italy so no trained rams were available to collect semen directly on farm. Epididymal sperm was recovered post mortem from slaughtered rams, following the protocol developed in our laboratories (see Chapter 2). Cauda epididymis and ductus deferens were then isolated and spermatozoa were collected using the float-up methods as described by Turri et al. (2011). Epididymal sperm suspension obtained from the two washing steps were filtrated through 200 μm stainless sieve and collected in a glass tube. Immediately after extraction also in this case sperm concentration, motility, viability and morphology were evaluated following the methodology given by Turri et al. (2011). After collection, sperm

suspension were diluted with the cooling diluent (Fiser et al., 1987) to obtain 1,5x10^9 sperm/ml and cooled progressively to +5°C over 2 hours (0.2°C/min). After the period of equilibration the freezing diluents was added (Fiser et al., 1987) to obtain final concentration of 500x10^6 sperm/ml.

For each donor of both species cryogenic labels reporting an identification code (Breed code, Animal ID, Date of production) were printed with the labeling printers (LABXPERT™ Brady, Saronno, Italy). They were then frozen in nitrogen vapour for 15 min and transferred to a liquid nitrogen tanks (International Cryogenics Inc. - IMV Technologies, Piacenza, Italy).

5.3.7 Costs

Very little information is available on expected costs in AnGR cryo banking (e.g. Gandini et al. 2007, McClintock et al. 2007). For this reason we recorded costs associated to the different phases of building the bank and the collections.

5.3.8. LABank Information system

CryoWEB, an Open Source software, created by 'Institute of Farm Animal Genetics – Mariensee (http://cryoweb.tzv.fal.de/), was installed at the LABank, within the EFABIS-Net project "An integrated network of decentralized country biodiversity and genebank databases", co-financed by European Commission (Duchev et al., 2010). CryoWEB allows to store information and documentation of national genebanks of cryopreserved domestic animals. This software is currently installed in 14 European Countries, including Italy, and in two non European countries, Vietnam and Bhutan.

5.4 Development of the Lombardia Farm Animal Genetic Resources Cryobank (LABank) - Results

5.4.1 Samples collected

A total of 2295 semen doses, 286 ml of blood and 69 g of hair were collected from a total of 35 donors of cattle, goat and sheep local breeds (Table 2).

Table 2. Number of donors, type of material an number of doses by breed, stored in LABank.

Breed	Donors	Type of material	N° of doses
Varzese	Bull 1	Semen	406
Varzese	Bull 2	Semen	293
Varzese	Bull 3	Semen	104
Varzese	Bull 4	Semen	40
Varzese	Bull 5	Semen	379
Bulls Total	*5*		*1222*
Brianzola	Ram 1	Epididymal sperm	38
Brianzola	Ram 2	Epididymal sperm	42
Brianzola	Ram 3	Epididymal sperm	25
Brianzola	Ram 4	Epididymal sperm	2
Brianzola	Ram 5	Epididymal sperm	13
Brianzola	Ram 6	Epididymal sperm	28
Brianzola	Ram 7	Epididymal sperm	35
Brianzola	Ram 8	Epididymal sperm	12
Brianzola	Ram 9	Epididymal sperm	12
Rams Total	*9*		*207*
Frisa	Buck 1	Semen	7
Frisa	Buck 2	Semen	8
Frisa	Buck 3	Semen	22
Frisa	Buck 4	Semen	7
Frisa	Buck 5	Semen	7
Subtotal	*5*		*51*
Orobica	Buck 1	Semen	28
Orobica	Buck 2	Semen	15
Orobica	Buck 3	Semen	19
Orobica	Buck 4	Semen	11
Orobica	Buck 7	Semen	9
Orobica	Buck 8	Semen	11
Orobica	Buck 9	Semen	5
Subtotal	*9*		*120*
Verzaschese	Buck 1	Semen	103
Verzaschese	Buck 2	Semen	222
Verzaschese	Buck 3	Semen	34
Verzaschese	Buck 4	Semen	317
Verzaschese	Buck 5	Semen	7
Verzaschese	Buck 6	Semen	8
Verzaschese	Buck 7	Semen	4
Subtotal	*7*		*695*
Bucks Total	*21*		*866*
TOTAL	***35***		***2295***

Cattle: Varzese-Ottonese breed

Sperm was obtained from three sexual mature bulls (mean age 21 months). A total of 1078 number of doses and 12 ml/bull of blood were collected. One hundred forty-four doses from two bulls, recovered in previous projects, were acquired. A total of 1222 doses of Varzese breed, summing the semen doses acquired from ConSDABI, were recovered and stored within LABank project at IBBA-PTP storage site.

Goat: Frisa Valtellinese, Orobica and Verzaschese breeds

Sperm was obtained from 17 sexual mature bucks (mean age 33 months). Six hundred and seventy-six doses from four Verzaschese breed bucks, recovered in previous projects, were acquired. A total of 866 doses from Frisa Valtellinese, Orobica and Verzaschese breeds bucks were recovered and stored within LABank project (Table 2). Overall the quality of the buck sperm recovered was acceptable (Table 3), considering viability (51.96% ± 14.11), total motility (69.83% ± 19.93) and the almost absence of morphological abnormalities (2.54% ± 2.86).

Sheep: Brianzola breed

Testicles from 9 sexual mature Brianzola rams (mean age 21 months) and 12 ml/ram of blood were collected at the slaughterhouse. A total of 207 doses were obtained and stored within LABank project (Table 2). Overall the quality of the sperm recovered was acceptable (Table 3), considering viability (60.92% ± 12.50) and the almost absence of morphological abnormalities (2.09% ± 4.69).

Table 3. Quality of rams and bucks fresh sperm and epididymal semen collected.

Breed	Donors (n.)	Volume (ml)	Total n. of sperm ($x10^9$)	Total Motility (%)	Viability (%)	Morphological abnormalities (%)
Brianzola	9	1.5 ± 1.0	4.7 ± 4.0	32.8 ± 28.4	60.9 ± 12.5	2.1 ± 4.7
Frisa	5	2.7 ± 1.1	2.4 ± 2.2	58.0 ± 20.3	49.2 ± 11.7	3.6 ± 3.4
Orobica	2	1.0 ± 0.7	2.6 ± 1.8	51.8 ± 25.8	47.9 ± 14.7	0.1 ± 0.2
Verzaschese	3	0.9 ± 0.4	1.2 ± 0.2	84.5 ± 5.7	57.1 ± 9.2	3.9 ± 4.9

[1]Values are expresses as means ± S.D.

Table 4. Number of units of blood (ml) and hair (g) collected.

Breed	Donors	Type of material	Units
Varzese	Bull 1	Blood	12
Varzese	Bull 1	Hair	3
Varzese	Bull 2	Blood	8
Varzese	Bull 2	Hair	3
Varzese	Bull 5	Blood	12
Varzese	Bull 5	Hair	3
Blood Subtotal			*32*
Hair Subtotal			*9*
Brianzola	Ram 1	Blood	8
Brianzola	Ram 1	Hair	3
Brianzola	Ram 2	Hair	3
Brianzola	Ram 7	Blood	30
Brianzola	Ram 7	Hair	3
Brianzola	Ram 8	Blood	12
Brianzola	Ram 8	Hair	3
Brianzola	Ram 9	Blood	12
Brianzola	Ram 9	Hair	3
Blood Subtotal			*62*
Hair Subtotal			*15*
Frisa	Buck 1	Blood	12
Frisa	Buck 1	Hair	3
Frisa	Buck 2	Blood	12
Frisa	Buck 2	Hair	3
Frisa	Buck 3	Blood	12
Frisa	Buck 3	Hair	3
Frisa	Buck 4	Blood	12
Frisa	Buck 4	Hair	3
Frisa	Buck 5	Blood	12
Frisa	Buck 5	Hair	3
Orobica	Buck 1	Blood	12
Orobica	Buck 1	Hair	3
Orobica	Buck 2	Blood	9
Orobica	Buck 2	Hair	3
Orobica	Buck 3	Blood	15
Orobica	Buck 3	Hair	3
Orobica	Buck 4	Blood	15
Orobica	Buck 4	Hair	3
Orobica	Buck 5	Blood	15
Orobica	Buck 5	Hair	3
Orobica	Buck 6	Blood	15
Orobica	Buck 6	Hair	3
Orobica	Buck 9	Blood	15
Orobica	Buck 9	Hair	3
Verzaschese	Buck 5	Blood	12
Verzaschese	Buck 5	Hair	3
Verzaschese	Buck 6	Blood	12
Verzaschese	Buck 6	Hair	3
Verzaschese	Buck 7	Blood	12
Verzaschese	Buck 7	Hair	3
Blood Subtotal			*192*
Hair Subtotal			*45*
TOTAL Blood			**286**
TOTAL Hair			**69**

5.4.2 Costs

Effective costs related to setting-up of the cryobank have been reported considering building and maintaining activities (Tab. 7), and germplasm collection activities (Tab. 8).

Table 7. Effective costs (€) for LABank building and maintaining activities.

Item	Euro
System for detecting oxygen saturation	4,650.00
System for detecting liquid nitrogen levels	7,800.00
Cryogenic tanks	3,600.00
First Refill Cryogenic tanks	163.00
Liquid nitrogen costs per 3 years	2,428.18
TOTAL building and maintaing cost	**18,641.18**

Table 7 reports costs for building the primary storage site and for maintaining the stored semen in the first three year.

In Table 8 costs for semen collection and freezing are shown. These include: travel costs, labour costs, service for semen collection on farm, health test, laboratory material supplying and other materials cost. As travel costs, we considered all expenses incurred by IBBA staff (two operators on average) to travel from Milano to the collection site. Travel costs for goat breeds were higher due to the longer distance than in the other species from Milano to the farming area. Labor costs have been evaluated considering the number of working days required for collection, evaluation and freezing procedures for each operator. Also in this case expenses for goat were higher than other species, due to the high number of donors managed and consequently for the high number of working-days dedicated to this species. Expenses for service of semen collection on farm and for health tests have been incurred only in cattle species; for other species expenses related to services for third parties were avoided because semen collection and processing were performed by IBBA staff. Laboratory costs include all the expenses related to the material supplying for the laboratories activities as chemicals and disposable products necessary only for sheep and goat breed semen freezing procedure, because, as mention before, cattle semen processing has been done in outsourcing. For cattle species other material costs include the acquisition of doses of two bulls previously collected by ConSDABI. In sheep species other material costs are related to the material necessary for the epididymal extraction as forceps, scalpel, Petri dish, needle and CBS™ straws whereas for goat species expenses concerns the AV with graduate tubes and CBS™ straws acquisition.

From this list of expenses, costs necessary for store genetic material for each species were calculated.

As show in Table 8 they constitute the 37% of the total cost of LABAnk. Costs by species are 4863.00 €, 2222.88 € and 4018.00 € respectively for cattle, sheep and goat species. Related to the total cost by species, 11104.66 €, they constitute respectively the 44%, 20% and 36% of the expenses. From each costs by species cost per straw and per donor were evaluated. Cattle species shows the lowest cost per straw (3.39 €), due to the higher number of doses collected and for the absence of laboratory material supplying expenses, but at the same time the highest cost per donor (1219.31 €) considering costs necessary for the service for semen collection on farm and the health tests required by sanitary regulation legislation. In this case acquisition of doses costs doesn't concur in the calculation of the cost per straw and per donor. Sheep case-study evidence that cost per straws is less than goat one, and this depends on a lower labor, travel and other material costs, obtainable thanks to the epididymal extraction technique that allows a quicker samples collection and lower travel costs. Goat species instead shows the lowest cost per donor, because travel and labor costs were shared among many animals collected in the same working day. However due to small number of doses produced by each buck donor the cost per straw was higher than in other species, 84% more than in cattle and 50% more than in ram.
Considering costs for the settlement of the genebank site and collection activities at present a total amount of 29745.84 € were necessary for LABanks.

Table 8. Effective costs (€) for LABank collection activities.

Item	Cattle	Sheep	Goat	Total cost
Biological materials collected	S[1]	ES[1]	S[1]	
Working Days	10	7	13	
N. animals collected	3	9	21	
N. straws collected	1078	207	190	
Travel costs	337.93	229.36	1006.85	
Labor costs	1400.00	980.00	1820.00	
Service for semen collection on farm	1320.00	0.00	0.00	
Health tests	600.00	0.00	0.00	
Laboratory material supplying	0.00	900.00	900.00	
Other materials cost	1205.60	113.52	291.40	
Costs by species	*4863.53*	*2222.88*	*4018.25*	
TOTAL cost by species				**11104.66**
Cost per straw	3.39	10.74	21.15	
Cost per donor	1219.31	246.99	191.35	
TOTAL LABank cost[2]				**29745.84**

[1]S: semen; ES: epididymal semen.
[2]TOT. LABank cost: Tot. building and maintaing cost + Tot. cost by species

5.4.3 LABank information system

Detailed and accessible documentation related to donors and materials stored is essential for the future use of any stored gene bank material. LABank uses CryoWEB software as information system, on a dedicated server.
The following data blocks are available: Contacts, Animals, Samples, Protocols, Storage facilities, and Samples distribution. The Contacts block includes information on people and organizations involved with the gene bank. In the Animals block, data about the donors, animal ID, birth date, species and breed, sex, pedigree information and pictures are recorded. In the Storage Facilities block the user specifies the structure of the localization of doses in tanks and freezers. In the Samples and Protocols blocks data about production and freezing of the material are entered. In Samples distribution, distribution within the storage, moving and usage of material are recorded. Several printable outputs are available.

5.5 Creation of CRYONET-IT

In Italy, the implementation of AnGR conservation policies belongs to the regional administrations, and it is financed by the National Strategic Plan (PSN). As above mentioned, Italy does not have a national gene bank or other forms of national coordination in cryo preservation of AnGR.

In 2008 the DG for Agriculture of the Lombardy Region, in collaboration with the Institute of Agricultural Biology and Biotechnology of National Research Council (IBBA-CNR), within the framework of the project "Risorse biologiche e tecnologie innovative per lo sviluppo sostenibile del sistema agro-alimentare", started the development of the Lombardia Farm Animal Genetic Resources Cryobank (LABank), to preserve the AnGR biodiversity still present in this region. During this project it was decided to develop a coordination system of Italian collections, to share the information on cryo-preserved genetic material through a virtual bank, to create a national network of institutions involved in cryo-preservation of AnGR.
To accomplish these goals, it was created the "Network of the Italian cryobanks of farm animal genetic resources" (CRIONET-IT) available online at http://www.genrescryonet.unimi.it/. Aims of CRIONET-IT include the promotion of collaboration among collections, sharing information among collections and Institutions working in farm animal genetic resources conservation, development of cryoconservation programmes, as indicated by the Strategic Priority 8 of the Global Plan of Action for Animal Genetic Resources,

adopted by the International Technical Conference on Animal genetic resources, Interlaken, September 2007. CRIONET-IT is made of two major components. The first is a website, open to the public, where the aims of the Network are illustrated, Partners and Collaborators are given, and a summary of the material stored by all partners is presented. The second is the CryoWEB reserved area, in which Partners can manage their own data by using a CryoWEB software somehow modified to allow its multiple use by all partners (http://cryoweb.vete_vsa.unimi.it/).

CRIONET-IT has the following six Partners (at 31-12-2011):

1. Associazione Nazionale Allevatori Bovini di Razza Reggiana (AnaBoRaRe), Banca Genetica;
2. Banca delle Risorse Genetiche Animali Lombarde, LABank;
3. Riserva genetica della razza Verzaschese, Comunità Montana Valli del Verbano;
4. Criobanca del Germoplasma Animale "Giuseppe Rognoni", IBBA-CNR;
5. Riserva genetica della razza Burlina, Associazione Provinciale Allevatori di Treviso;
6. Riserva genetica della razza Cabannina, Associazione Provinciale Allevatori di Genova.

Information about the 286 donors and the 38227 semen doses of four species, currently registered in CRIONET-IT, are shown in Table 5.

Table 5. Partners of the "Network of the Italian cryobanks of farm animal genetic resources" and material stored (at 31-12-2011).

Partners	Storage site	Species	Breed	Donors (n°)	Material stored		
					Semen (n. doses)	Blood (ml)	Hair (g)
1	AnaBoRaRe[1]	Cattle	Reggiana	167	7269		
2	IBBA-PTP[2]	Cattle	Varzese	5	1222	32	9
		Goat	Frisa	5	51	60	15
			Orobica	9	120	96	21
			Verzaschese	3	19	36	9
		Ovine	Brianzola	7	207	62	15
3	IBBA-PTP[2]	Goat	Verzaschese	4	676		
4	IBBA-PTP[2]	Pig	Casertana	11	3804		
			Cinta Senese	14	5188		
			Mora Romagnola	1	478		
			Nero Siciliano	15	5785		
	INSEME[3]	Pig	Casertana	*10	2981		
			Cinta Senese	*14	5325		
			Mora Romagnola	*1	464		
			Nero Siciliano	*7	2292		
5	Co.Mi.Zo[4]	Cattle	Burlina	29	1866		
6	CIZ[5]	Cattle	Cabannina	16	480		
TOTAL				**286**	**38227**	**286**	**69**

[1] Associazione Allevatori Bovini di Razza Reggiana, Reggio Emilia; [2] Parco Tecnologico Padano, Lodi; [3] INSEME, Zorlesco

[4] Consorzio Miglioramento Zootecnico, Treviso; [5] Consorzio Incremento Zootecnico, Pisa.

* Duplication of donors number and respectively doses number from IBBA-PTP to INSEME storage site for insurance against catastrophic events. For this reason number of donors of INSEME storage site were not consider for the calculation of the total number of Network donors.

Donors that mostly contribute to the Network are cattle (75%), followed by pig (14,5%), goat (7,27%) and sheep (3,11%) donors, as shown in Table 6. If we consider the number of doses, at first place there is the pig (68,8%), followed by cattle (28,3%), goat (2,2%) and sheep (0,5%) .

Table 6. Donors and doses contribution by species and breeds in the "Network of the Italian cryobanks of farm animal genetic resources" (at 31-12-2011)

Species and breed	Donors (n.)	Doses (n.)	Donors contribution within Network (%)	Doses contribution within Network (%)
Cattle: Varzese	5	1222	1.73	3.20
Cattle: Burlina	29	1866	10.03	4.88
Cattle: Cabannina	16	480	5.54	1.26
Cattle: Reggiana	167	7269	57.79	19.02
Cattle	*217*	*10837*	*75.09*	*28.35*
Goat: Frisa	5	51	1.73	0.13
Goat: Orobica	9	120	3.11	0.31
Goat: Verzaschese	7	695	2.42	1.82
Goat	*21*	*866*	*7.27*	*2.27*
Pig: Casertana	11	6785	3.81	17.75
Pig: Cinta Senese	14	10513	4.84	27.50
Pig:Mora Romagnola	1	942	0.69	2.46
Pig: Nero Siciliano	15	8077	5.19	21.13
Pig	*41*	*26317*	*14.53*	*68.84*
Sheep: Brianzola	9	207	3.11	0.54
Sheep	*9*	*207*	*3.11*	*0.54*
TOTAL	**289**	**38227**	**100.00**	**100.00**

5.6 Discussion and conclusion

We reported here the creation of the Lombardia Farm Animal Genetic Resources Cryobank (LABank) and of the "Network of the Italian cryobanks of farm animal genetic resources" (CRIONET-IT).
During the development of LABank some aspects as cryobanking objective, access and ownership of the genetic material, storage sites, choice of species, breeds, animal donors and genetic material, health management, costs, access and ownership of the stored material were considered.

To create a genetic reserve for the local breeds still present in Lombardia region, male gametes were collected and stored from donors of Varzese cattle, Brianzola sheep and Frisa, Orobica and Verzaschese goat breeds. In order to reduce costs and overcome organisation difficulties, in the case of Brianzola sheep, epididymal spermatozoa were collected instead of conventional semen

collection. For each donor samples of blood and hair were also collected, as source of genetic material for future DNA research.
All the information related to donors and genetic material collected and stored, were recorded and managed by CryoWEB Software.
Protocols for access and ownership of the genetic material stored were developed.
From the analysis of the cost associated to the different phases of LABank creation we concluded that the principal investment in a genebank creation is constitute by the establishment facilities. Other costs varies according to species, kind of the biological material collected, number of donors and number of semen doses managed.

Parallel at the creation of the LABank a coordination system of Italian collection was developed, to share the information on cryo-preserved genetic material through a virtual bank, to create a national network of institutions involved in cryo-preservation of AnGR, through the creation of the "Network of the Italian cryobanks of farm animal genetic resources" (CRIONET-IT). Six partners, including research institutes, breeders associations and local authorities, joined at CRIONET-IT.

At present a total of 2295 semen and epididymal sperm doses, 286 ml of blood and 69 g of hair were collected from 35 donors of Varzese cattle, Brianzola sheep and Frisa, Orobica and Verzaschese goat breeds and stored in the LABank.
The Lombardia Farm Animal Genetic Resources Cryobank presented here is an important tool to avoid irreversible loss of biodiversity that is occurring at regional level ant it would be an example of Regional model for the *ex situ* management of regional farm animal genetic resources.
Moreover the creation of the CRIONET-IT is a contribution to the future creation of an Italian national gene-bank, and more generally to the *ex situ* management of farm animal genetic resources as a complementary tool to *in situ* conservation strategy.

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Chapter 6

Motives and values in farming local cattle breeds in Europe: a survey on fifteen breeds

G. Gandini[1], L. Avon[2], D. Bohte-Wilhelmus[3], E. Bay[4], F.G. Colinet[4], Z. Choroszy[5], C. Díaz[6], D. Duclos[2], J. Fernández[6], N. Gengler[4], R. Hoving-Bolink[3], F. Kearney[7], T. Lilja[8], A. Mäki-Tanila[8], D. Martín-Collado[6], M. Maurice-vanEijndhoven[3], M. Musella[1], F. Pizzi[9], K. Soini[8], M. Toro[10], F. Turri[1], H. Viinalas[11], the EURECA Consortium[12] and S. J. Hiemstra[3]

[1]Department VSA, University of Milan, Via Celoria, 10, 20133 Milan, Italy;
[2]Institut de l'Elevage, Paris, France;
[3]Centre for Genetic Resources, the Netherlands (CGN), Wageningen University and Research Centre, Lelystad, the Netherlands;
[4]Animal Science Unit, Gembloux Agro-Bio Tech, University of Liege, Gembloux, Belgium;
[5]National Research Institute of Animal Production, Poland;
[6]Departamento de Mejora Genética Animal, INIA, Madrid, Spain
[7]Irish Cattle Breeding Federation, Bandon, Ireland;
[8]MTT Agrifood Research Finland, Jokioinen, Finland;
[9]IBBA-CNR, Lodi, Italy;
[10]Departamento de Producción Animal, ETS Ingenieros Agrónomos ,Madrid, Spain;
[11]Estonian Agricultural University, Estonia;
[12]http://www.regionalcattlebreeds.eu/

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6.1 Abstract

Within the EURECA project (*Towards self-sustainable EUropean REgional CAttle breeds)*, we interviewed a total of 371 farmers of 15 local cattle breeds in eight European countries. Besides collecting data on farmers, land use, herd composition, and economic role of cattle, we aimed to understand farmers' motives and values in keeping local cattle. The most frequent first reason to keep the local breed was productivity, followed by tradition. When comparing the local breed to a mainstream breed, only in four breeds was productivity considered the same, while in three breeds more than 50% of farmers valued the local breed as more profitable. The local breed was valued as always superior or the same on functional traits. Farmers were asked which type of appreciation they thought representatives of various stakeholders had on their local breed: a positive appreciation was observed in 33% of farmers. On average across breeds, 39 % of farmers expect to increase the size of their herd in the next years, 5% to give up farming. The degree of dependence of farmers on economic incentives was estimated by asking farmers their expected behavior under three scenarios of change of subsidies. Most farmers demanded activities for promoting local breed farming. Results are discussed in terms of breed sustainability and conservation.

6.2 Introduction

The worldwide process of erosion of animal genetic resources for food and agriculture (AnGR) has been recently analyzed by FAO (FAO, 2007); in Western Europe it started with the industrialization of agriculture after the second world war, more recently in Eastern and Central Europe countries, it followed the political change and economic restructuring they undergone after the eighties. The European Union recognizes the importance of conserving AnGR, and since 1992 started a policy of economic incentives for farmers keeping endangered breeds under EC Regulation 2078/92, followed by Regulation EC 1257/99. Despite the erosion during the last decades, Europe still hosts a large variety of local cattle breeds, though many are endangered (e.g. EFABIS, 2009).

In Europe local cattle breeds are distributed across a wide variety of political, social, economical, cultural and environmental contexts. It is reasonable to think that this variety corresponds to a consistent diversity of farming structures, methods and motivations. In addition, both the erosion processes of the last decades and the more recent recovery processes observed in some breeds, driven by a variety of actions, possibly affected farming structure by creating additional variation within and between breeds. Thus several questions can be posed: what

kind of variation is present today among local cattle farming in Europe? What are the conditions affecting sustainability of local breed farming? Is it advisable to have common EC rules for conservation of local breeds? Can the current EC policy, based on incentives payments - to compensate farmers for the lower profitability of the local breeds compared to substituting these breeds with more profitable mainstream breeds - and on some additional funds for applied research (GENRES, 2008), effectively contribute to AnGR conservation? The EURECA project - Toward self-sustainable EUropean REgional CAttle breeds - supported by the European Council (EURECA, 2009) was developed to contribute answers to these questions, and more generally to contribute methods and data that will be of value when new policies on farm animal genetic resources and rural development, as well as conservation programmes, are designed.

Within EURECA, this investigation aimed to understand: who is today the farmer of the endangered local cattle breeds in Europe, what are the reasons for keeping local breeds instead of/besides mainstream ones, does the farmer feel understood or neglected by society, which kind of help would the farmer like to have, what is the programme for the size of the herd in the next years? Farmers currently keeping local breeds are in a key position to guarantee sustainability of breeding, and for that reason it is necessary to understand their values and motives. This paper reports data collected by interviewing farmers of fifteen European cattle breeds and proposes a first analysis of differences and similarities among breeds and countries. Other papers will investigate breed farming sustainability and will provide an analysis of strengths, weaknesses, opportunities and strengths (SWOT) to reach and/or maintain sustainability.

6.3 Material and methods

Farmers of fifteen local cattle breeds, in the eight European countries partner of the EURECA project, were interviewed. Interviews were mostly conducted face-to-face during a farm visit, or by telephone or email. The questionnaire included: i.) questions related to background information on the interviewed farmer, his/her family, land use, production system and economic role of the farm, ii.) questions addressed to investigate farmers' perceptions on roles and values of the breed now and in the future, to understand farmers' perceptions on how the society values the breed, iii.) questions aimed to analyse actions taken by the farmer in the past and expected in the future. A semi-structured questionnaire was used, including both structured and open-ended questions, for a total of 44

questions. This paper reports results on the 25 structured questions of the questionnaire.

Table 1 reports, by country, names of the fifteen breeds analysed, breed codes used in the presentation of results, number of herds surveyed (i.e. farmers interviewed) per breed and degree of completeness of the questionnaires returned. One breed was analysed in Estonia (Estonian Native, code EEEN) and Ireland (Kerry, code IEKE), two breeds were analysed in Belgium (Dual Purpose Belgian Blue, code BEBM; Dual Purpose Red and White, code BEPR), Finland (Eastern Finn Cattle, code FNES; Western Finn Cattle, code FNWS), France (Ferrandaise, code FRFE; Villard de Lans, code FRVI), Italy (Modenese, code ITMO; Reggiana, code ITRE), Spain (Avileña-Negra Ibérica, code EASN; Alistana-Sanabresa, code ESAS), and three breeds in the Netherlands (Deep Red, code NLDR; Groningen White Headed, code NLGW; Meuse- Rhine- and Yssel, code NLMR).

Table 1. Breeds surveyed by country, n. of herds analysed and completeness of returned questionnaires.

Country	Breed	Breed code	N. herds analyzed	Completeness %	N. of cows	Trend
Belgium	Dual Purpose Belgian Blue	BEBM	23	92.9	4,400	s
	Dual Purpose Red and White	BEPR	18	84.2	3,000	d
Estonia	Estonian Native	EEEN	30	94.1	1,500	d
Finland	Eastern Finn Cattle	FNES	30	77.2	790	i
	Western Finn Cattle	FNWS	31	78.3	2,950	d
France	Ferrandaise	FRFE	19	94.7	730	i
	Villard de Lans	FRVI	15	88.9	340	s
Ireland	Kerry	IEKE	20	85.6	1,200	i
Italy	Modenese	ITMO	26	80.9	650	s
	Reggiana	ITRE	30	89.9	1,500	i
The Netherlands	Deep Red	NLDR	21	92.8	454	i
	Groningen White Headed	NLGW	22	92.0	1,500	s
	Meuse-Rhine-Yssel *	NLMR	24	83.5	14,400	d
Spain	Avileña-Negra Ibérica *	ESAN	31	83.7	100,000	s
	Alistana-Sanabresa	ESAS	31	84.2	2,000	i

Trend: i =increasing; s = stable; d = decreasing. * = Breeds that, although are not endangered following EU criteria, after the fifties experienced severe declines.

The set of the fifteen breeds surveyed across the eight countries was selected among those classified as endangered following EU criteria (5,000 or 7,500 cows, for breeds respectively numerically stable or declining; EC Regulations 1257/99 and 445/02) with the additional criteria of including breeds numerically declining, stable or increasing, but two breeds above 7,500 cows, Avileña-Negra Ibérica and Meuse-Rhine-Yssel, that after the fifties experienced severe declines. Breed sizes, as number of cows, and demographic trends are given in table 1. All

breeds are classified as dual purpose, but two are primarily dairy breeds (EEEN, ITRE) and two are beef breeds (EASN, ESAS).

We aimed to interview an equal number (30) of farmers per breed, representing from 5% to 75% of the herds of the breed. An average of 24.7 farmers per breed was interviewed, with a minimum of 15 to a maximum of 31, for a total of 371 farmers across the fifteen breeds. Farmers were chosen at random. If the farmer community presented some specific structure with different typologies, then a stratified random sampling was used.

Across the twenty-five questions and the fifteen breeds, average level of responses was satisfactory (86.9% completeness), with some variation among breeds (range 84-95%) and questions. Analysis of variance and Pearson chi-square tests were used to compare results across breeds (SAS, 2004).

6.4 Results and discussion

Tables 2 to 5 provide information on the famers interviewed and their farms. Table 2 reports on farmers and their family. Average farmer age across breeds is 48.7 years (SD 11.4), with some variation among and within breeds from a minimum of 43.3 years (SD 9.8) in FRFE to a maximum of 53.5 years (SD 14.6) in EEEN. Considering all breeds, most farmers (53.8%) have a middle education level, 29.2% have a basic education and 17.0% a university education. Education level differs somehow among breeds. In four breeds, ESAS in Spain, FRFE in France and ITMO and ITRE in Italy, the majority of farmers (from 43 to 70%) have a basic education. In the other ten breeds the middle level is the most common, from 35% in IEKE to 83% in NLMR. In five breeds the percentage of farmers with a university level is above 27%, up to a maximum of 36.7% (EEEN, ESAN). Information at the national levels is scarce and comparisons between farmers of mainstream breeds and our findings on local breeds are not possible. Age of the famer provides indications on the process of transferring farming activities to the next generation, on opportunities for breed survival in the next years. However, we did not ask farmers how they foresee the transfer of their farming activities. A recent survey in Belgium indicates that only 15,8 % of farmer older than 50 claim they have a presumed successor; 57,8% of them claim they have no successor and 26.4 % of them do not know yet (DGARNE, 2009).

Average family size across breeds is of 3.6 (SD 1.9) ranging from 2,4 (SD 0.9) in EEEN to 4.6 (SD 1.7) in NLGW. On average 64.9% (SD 29.6) of family

members contributes to farming activities, with some variation from 42.9% (SD 23.4) in ESAN to 89.7% (SD 19.4) in EEEN.

Table 3 reports data on land use. Average farm size across breeds is 151.3 ha (SD 15.8), 49.6 % (SD 2.1) of property. Farm size ranges from 30.1 ha. (SD 28.1) in NLDR to 760.7 (SD 633.9) in ESAN, percentage of property from 1.6 (SD 1.4) in IEKE to 80.9 (SD 17.9) in FNWS. The percentage of the land used for grazing (Spanish data missing) across breeds is 48.0 (SD 2.3), ranging from zero in ITRE to 92.1 (SD 15.0) in FRFE. Table 3 also provides the type of land on which farms are located, in terms of both soil productivity compared to the country average, and orographic structure. Across breeds the soil occupied by farming activities is approximately equally distributed across the three categories of low (32.8%), medium (44.2%) and high productivity (23.0%). Low or medium soil productivity is prevalent in all but two breeds, ITRE and NLGW with respectively 66.7 and 50% of high productivity soil. Only in four breeds mountain terrains are used by at least 25% of the herds, from 26.7% in FRVI to 46.2% in ITMO. Self-sufficiency in cattle feedstuff and organic production were also analysed (data not reported in Table 3); percentage of self-sufficiency in production of feedstuff for the local cattle herd (Spanish data missing) on average was 91.8% (SD 19.7) for roughage, with little variation across breeds (82%-100%), and 22.44% (SD 36.5) for concentrate, with higher variation ranging from 2% in ITRE to 62% in EEEN. The percentage of farms producing organic was on average 13.2%, with a consistent variation, from zero in ESAS and ITRE to 25% or higher in EEEN, FRVI, IEKE and NLDR. It is worth noting that at least a quarter of farmers of four breeds from four different countries in Eastern, Southern, Central and Northern Europe add value to the local cattle by producing organic milk or meat.

Among the 371 farmers interviewed, 145 (39.2%) keep on their farm only the local breed that is the object of this investigation, the remaining 226 (60.8%) keep also cows of one or more additional breeds. Considering all 371 herds, the average size of the local cattle herd across all breeds is of 37.4 (SD 55.1) cows with some differences among breeds, ranging from 7.2 (SD 5.5) in FNES to 141.3 (SD 101.2) in EASN (Table 4). Considering both the local breed under investigation and the other cattle kept on farm, the average cattle herd size is of 61.2 (SD 82.9) cows, ranging from 10.6 (SD 5.9) in FNES to 170.3 (SD 118.9) in ESAN. Considering the 226 farms with two or more breeds, the average farmer keeps on his farm, in addition to the analysed local breed, 1.5 (SD 0.8) breeds, ranging from 1 to a maximum of 2.6 in FNES, for a total, within each local breed, of 1 (NLGW and NLMR), 4 (ITRE and NLDR), 5 (FNWS, FRVI and

Table 2. General data on the farmer and his family, by breed. Lower area, breed comparisons.

Country	Breed	N	Age of farmer			Farmer's education level (%)			Family size			Workers of family (%)		
			Mean	SD	Range	Basic	Middle	University	Mean	SD	Range	Mean	SD	Range
Belgium	BEBM	23	47.2	11.29	29-63	4.4	82.7	13.0	4.1	1.56	2-7	75.4	27.48	20-100
	BEPR	18	51.9	10.64	38-75	5.6	88.8	5.6	3.2	1.40	1-6	61.6	30.93	20-100
Estonia	EEEN	30	53.5	14.60	17-72	10.0	53.3	36.7	2.4	0.86	1-4	89.7	19.44	50-100
Spain	ESAN	31	49.2	10.34	34-72	43.3	20.0	36.7	3.5	2.27	1-13	42.9	23.40	0-100
	ESAS	31	48.0	12.12	26-67	70.0	20.0	10.0	2.7	1.08	1-4	70.8	33.34	0-100
Finland	FNES	30	48.2	9.71	30-64	23.3	73.3	3.4	3.4	1.68	1-7	68.2	28.48	20-100
	FNWS	31	46.1	10.49	26-65	25.8	61.3	12.9	3.8	1.83	1-9	66.7	28.86	20-100
France	FRFE	19	43.3	9.75	25-61	45.4	27.3	27.3	2.9	1.37	1-5	65.9	32.66	20-100
	FRVI	15	45.5	10.18	29-61	14.3	71.4	14.3	3.9	1.58	1-7	43.9	25.32	14-100
Ireland	IEKE	20	50.9	11.51	27-78	35.0	35.0	30.0	3.6	1.93	1-9	75.1	30.48	20-100
Italy	ITMO	26	51.0	13.09	28-76	61.5	26.9	11.5	3.6	1.86	1-9	71.2	27.89	11-100
	ITRE	30	47.2	13.14	27-83	66.0	33.3	6.7	4.4	2.61	1-15	60.2	22.91	25-100
Nederland	NLDR	21	48.4	9.26	30-66	0.0	71.4	28.6	3.8	2.09	1-7	54.7	32.94	0-100
	NLGW	22	48.6	10.06	30-67	4.5	72.7	22.7	4.6	1.71	1-8	54.3	27.15	20-100
	NLMR	24	48.9	10.34	28-68	4.2	83.3	12.5	3.8	1.95	1-9	64.2	23.71	33-100
Total		371	48.7	11.43	17-83	29.2	53.8	17.0	3.6	1.86	1-15	64.9	29.60	0-100

	BEBM	BEPR	EEEN	ESAN	ESAS	FNES	FNWS	FRFE	FRVI	IEKE	ITMO	ITRE	NLDR	NLGW	NLMR
BEBM															
BEPR															
EEEN	ab	cd													
ESAN	cd	cd	bcd												
ESAS	bd	d	cd	cd											
FNES			bcd	cd	d										
FNWS			abc	cd	bd										
FRFE	bd	ad	acd	cd											
FRVI	c		abc		bcd	c	c	c							
IEKE	d	d	b	c	d	d		a	c						
ITMO	d	d	bcd	c	b	d	d	a	c						
ITRE	cd	bd	abcd	bcd	b	bd	d	b							
NLDR	c		bc	d	bcd	d	d	d		cd	cd	d			
NLGW	c	b	bc	bd	bcd	bd		bd		cd	cd	d			
NLMR			bc	cd	bd			d	c	d	d	d			

Presence of letters corresponds to significant differences between breeds (< 0.05). ANOVA significance (age of farmer: a; family size: b; workers of family: c), Chi-square significance (farmer's education level: d).

Table 3. Land use, by breed. Lower area, breed comparisons.

Country	Breed	N	Total Ha			% property Ha			% grazing			Soil productivity (%)			Terrain type (%)		
			Mean	SD	range	Mean	SD	range	Mean	SD	range	Low	Medium	High	Plain	Hill	Mountain
Belgium	BEBM	23	82.4	33.71	28-140	40.77	19.72	10-91	62.4	19.54	15-90	52.2	8.7	39.1	39.1	60.9	0.0
	BEPR	18	43.5	26.81	8-110	12.2	14.14	0-46	94.4	18.30	25-100	72.2	27.8	0.0	11.1	88.9	0.0
Estonia	EEEN	30	219.6	368.18	7-1800	61.3	27.32	0-100	55.7	27.68	0-100	50.0	50.0	0.0	100.0	0.0	0.0
Spain	ESAN	31	760.7	633.93	84-2740	49.1	43.10	0-100	/	/	/	70.0	20.0	10.0	13.3	70.0	16.7
	ESAS	31	188.9	277.33	8-1300	36.8	27.88	0-93	/	/	/	56.7	36.7	6.6	19.4	41.9	38.7
Finland	FNES	30	130.7	158.71	25-882	70.9	30.40	0-100	12.9	18.12	0-84	3.4	73.3	23.3	100.0	0.0	0.0
	FNWS	31	114.8	67.02	11-283	80.9	17.91	47-100	5.0	6.72	0-21	9.7	77.4	12.9	100.0	0.0	0.0
France	FRFE	19	60.5	37.14	13-133	25.4	35.23	0-80	92.1	15.01	50-100	42.1	57.9	0.0	11.1	50.0	38.9
	FRVI	15	51.9	41.74	8-170	62.9	42.96	0-100	86.4	16.75	50-100	46.7	53.3	0.0	40.0	33.3	26.7
Ireleand	IEKE	20	39.0	27.16	6-120	1.6	1.44	0.1-5	90.6	13.57	50-100	20.0	50.0	30.0	65.0	30.0	5.0
Italy	ITMO	26	67.9	78.95	11-300	30.4	45.51	0-100	3.6	10.05	0-45	46.2	19.2	34.6	34.6	19.2	46.2
	ITRE	30	64.5	74.88	7-330	28.8	54.01	0-100	0.0	0.0	0-0	10.0	23.3	66.7	66.7	23.3	10.0
Nederlands	NLDR	21	30.1	28.09	1-100	57.7	38.28	0-100	71.7	23.64	20-100	19.0	52.4	28.6	100.0	0.0	0.0
	NLGW	22	63.9	64.49	15-330	74.5	29.41	0-100	78.7	17.65	40-100	4.5	45.5	50.0	100.0	0.0	0.0
	NLMR	24	40.5	11.09	20-66	69.7	21.96	27-100	66.8	17.66	20-89	0.0	66.7	33.3	100.0	0.0	0.0
Total		371	151.3	15.82	1-2740	49.6	2.12	0-100	48.0	2.29	0-100	32.8	44.2	23.0	54.5	31.2	14.3

	BEBM	BEPR	EEEN	ESAN	ESAS	FNES	FNWS	FRFE	FRVI	IEKE	ITMO	ITRE	NLDR	NLGW	NLMR
BEBM															
BEPR	bcde														
EEEN	abde	abce													
ESAN	e	ab	ade												
ESAS	de	abe	be	a											
FNES	bcde	bcd	cd	abd	bd										
FNWS	bcde	bcd	bcd	abd	bd	d									
FRFE	cde	e	ace	ad		bcde	bcde								
FRVI	cde	be	ace	ad	b	cde	cde	b							
IEKE	bcd	de	abcde	abde	abde	bce	bce	de	bcd						
ITMO	bce	cde	abcde	abe	d	bcde	bde	cd	bcd	bce					
ITRE	bcde	cde	abcde	abde	abde	bcde	bde	cde	bcd	bcd	de				
NLDR	bde	bcde	acd	ade	abde	c	bc	bcde	cde	bce	bcde	bcde			
NLGW	bcde	bcde	acd	abde	bde	c	cd	bcde	de	bce	bcde	bce	b		
NLMR	bde	bcde	acd	abde	abde	c	c	bcde	cde	bce	bcde	bcde		cd	

Presence of letters corresponds to significant differences between breeds (< 0.05). ANOVA significance (total Ha: a;% property Ha: b; % grazing: c; soil productivity: d; terrain type: e).

IEKE), 6 (BEBM, ESAN and FNES), 7 (ESAS), 8 (BEPR, FRFE and ITMO) and 10 (EEEN) additional breeds. These additional breeds include mainstream breeds such as Holstein, Brown Suisse, Limousine, Simmental, Belgian Blue Beef, Charolaise, regional and local breeds, and crosses. In the average farm keeping more than one breed, the percentage of local cows of total cows is 46.4% (SD 29.2), ranging from 28.3 in ITMO to 82.9 in FNWS. The presence on the farm of breeds additional to the local one can be linked to a precise strategy to increase profitability (e.g Belgian breeds), to the country tradition of having more breeds on farm (e.g. Finland breeds), to the cultural affection of farmers of mainstream breeds to the local breeds of their parents (e.g. Italian breeds, where some successful Holstein farmers keep a few Reggiana or Modenese cows, and French breeds), to the willingness of contributing to the conservation of the endangered breed (e.g. French breeds). In some cases local cows are preferred for their better fertility, rusticity and maternal ability but they are mated to mainstream breed cows to produce F1 veals (e.g. Spain breeds).

Multi-functionality was investigated by asking roles and functions of the local cattle on the farm. Besides the obvious roles of milk to be sold or processed as cheese on farm, meat, and dual purpose, the grazing role (identified by farmers as a specific role, not as simply a cattle activity) was recognised, across all breeds, by 11% of farmers, in particular 30% in IEKE, 33% in FNES and 71% in NLDR. Only 4% of farmers, across all breeds, mentioned a tourism role, 60% of those in the NLDR. Other roles included, e.g. in the Netherlands, nature management and energy production. Some local breed farmers are moving from traditional products to new opportunities for increasing profitability, but this approach seems still limited, for example in tourism, as we will see also from data reported in Table 5.

Table 5 reports data on the economic role of the local cattle. Farmers were asked to identify the percentage of the total family income covered by the farming activities, using the following classes: from 76 to 100% (high), from 51 to 75% (medium), from 26 to 50% (low), less than 26% (minimal). As average across breeds, the percentage of income from the farm is high in 66.6% of cases, with consistent variation among breeds ranging from 20.0% in IEKE to 94.4% in BEPR. In two breeds the income from the farm covers on average less that 25% of the total family income in a consistent percentage of the interviewed farmers, in IEKE (35% of farmers) and in NLDR (48% of farmers, most of them using cows just for nature management). The local cattle breed share of the total farm income is across breed 57.4% (SD 38.3), with a minimum of 3.3% in NLDR to a maximum of 87.2% in BEPR.

The average number of external workers, measured as the sum of full time person and part time/seasonal multiplied by .25, is 0.8 (SD 4.2), ranging from 0.0 in BEBM to 1.0 in both ESAN and ITRE. Farmers were asked to partition the farm income into income from animal food products, from non-feed crop production, from forestry, from work services for other farms, from grazing as landscape management, from tourism services, and from welfare and educational services. Considering all breeds, as above mentioned, multi-functionality seems limited, with an high percentage (87.6) of the income deriving from animal food products, followed by 3.4% from non-feed crop production and a total of 9.0% from the other types of income. Percentage of income from animal food products is above 83% in all breeds, but FNWS, where 12.4% of the income derives from non-feed crops, and NLDR, where 3.5%, 12.8%, 10.5% and 5.0% of the income derives respectively from work services for other farms, from grazing as landscape management, from tourism services and from welfare and education services. It might be worthwhile to create opportunities to exchange ideas, to promote institutional support in order to foster multi-functionality in other breeds as a tool to increase their productivity and sustainability.

Farmers were also asked to identify the percentages of the production from either the local breed and the total herd (in case of presence on farm of two or more breeds) sold as raw material on farm, as processed material on farm, to the local market, or to the industry. For the local breed, on average most (39.9%) of the production is sold to industry, followed by local markets (25.4%), by on farm as raw material (9.6 %) and by on farm as processed material (5.9%). Additional investigations are needed to understand the role of both industry and local market on farmer profitability, that seems to vary from case to case. In some cases the industry guarantees a good promotion of the breed product (e.g. Spain breeds), in other cases the local market adds value to the product (e.g. Italian breeds). When we consider the total herd production, the percentage sold to industry increases to 44% and quotas sold on farm decreased by half.

Table 4. Herd size and composition, by breed. Lower area, breed comparisons.

Country	Breed	N	All herds						Mixed herds					
			Local breed			All cattle			% Local cattle			N.breeds excluded the local		
			n. cows	SD	Range	n. cows	SD	Range	Mean	SD	Range	Mean	SD	Range
Belgium	BEBM	23	54.4	23.41	23-100	93.8	51.51	25-255	47.1	23.59	20-100	1.6	0.65	1-3
	BEPR	18	41.0	18.18	12-81	53.7	31.47	20-150	55.2	18.63	27-100	1.4	0.54	1-2
Estonia	EEEN	30	11.9	16.62	1-85	77.9	160.15	1-727	37.1	31.23	2-100	1.5	0.51	1-2
Spain	ESAN	31	141.3	101.16	31-470	170.3	118.88	31-500	68.7	26.12	29-100	1.3	0.49	1-2
	ESAS	31	52.7	82.84	2-450	76.5	90.20	7-450	41.4	24.42	10-100	1.2	0.43	1-2
Finland	FNES	30	7.2	5.49	2-25	10.6	5.93	3-25	48.5	22.58	17-100	2.6	1.25	1-5
	FNWS	31	14.6	11.52	2-50	15.4	11.34	2-50	82.9	11.41	57-100	2.5	0.99	1-4
France	FRFE	19	13.4	11.56	2-41	30.6	22.89	4-75	35.2	19.55	6-100	1.0	0.00	1-1
	FRVI	15	9.0	7.02	1-22	18.7	14.27	4-60	17.9	15.74	5-100	1.0	0.00	1-1
Ireland	IEKE	20	14.7	12.05	2-45	21.9	23.81	0-100	38.6	22.26	6-100	1.0	0.00	1-1
Italy	ITMO	26	22.0	22.16	1-80	94.0	93.79	4-452	28.3	26.81	0-100	1.2	0.41	1-2
	ITRE	30	32.7	33.40	2-170	57.1	47.52	12-170	41.3	30.86	3-100	1.1	0.32	1-2
Nederland	NLDR	21	15.6	18.65	2-60	20.0	19.96	2-60	58.5	25.49	23-100	1.0	0.00	1-1
	NLGW	22	39.5	28.53	2-101	53.0	28.08	8-107	47.4	26.42	6-100	1.0	0.00	1-1
	NLMR	24	58.7	37.64	2-130	73.8	28.96	14-130	59.3	35.87	2-100	1.0	0.00	1-1
Total		371	37.4	55.12	1-470	61.2	82.86	0-727	46.4	29.18	0-100	1.5	0.83	1-5

	BEBM	BEPR	EEEN	ESAN	ESAS	FNES	FNWS	FRFE	FRVI	IEKE	ITMO	ITRE	NLDR	NLGW	NLMR
BEBM															
BEPR															
EEEN	a	ac													
ESAN	abc	a	abc												
ESAS			ab	abc											
FNES	abd	abd	bd	abcd	abd										
FNWS	abcd	acd	bcd	abd	abcd	ac									
FRFE	abd		bd	abc	ab	d	cd								
FRVI	abcd	ac	bd	abc	ab	cd	cd								
IEKE	abd		b	abc	ab	d	cd								
ITMO	ac	c		abc	a	bcd	bcd	b	b	b					
ITRE	d		d	abc		bd	bcd		c						
NLDR	abd		abcd	ab	ab	ad	acd	ac	c		bc				
NLGW	d		ad	abc		abd	acd	a	ac		bc				
NLMR	d		acd	ab		abd	abcd	ac	abc	ab	ac	a	ab		

Presence of letters corresponds to significant differences between breeds (< 0.05). ANOVA significance (local breed: a; n. total cows: b; % local cattle/total cattle: c; breeds farmed in addition to the local breed: d).

Table 5. Economic role and activities of the farm, by breed. Lower area, breed comparisons.

Country	Breed	N	Economic role of the farm						Income shares from farm activities (%)				Income share from local cattle (%)	
			Farm income / Family income (%)				N. external workers		Animals	Non-feed crop	Forestry	Other		
			High	Medium	Low	Minimal	Mean	SD	Mean	Mean	Mean	Mean	Mean	SD
Belgium	BEBM	23	87.0	8.7	4.3	0.0	0.0	0.0	89.4	0.0	0.0	10.6	68.2	30.56
	BEPR	18	94.4	0.0	5.6	0.0	0.1	0.0	97.8	0.0	0.0	2.2	87.2	26.30
Estonia	EEEN	30	60.0	10.0	20.0	10.0	5.3	13.56	85.8	2.0	0.7	11.5	38.6	37.07
Spain	ESAN	31	58.6	13.8	17.2	10.3	1.0	2.07	95.0	0.6	0.2	4.2	79.3	28.97
	ESAS	31	37.0	18.5	33.3	11.1	0.3	1.14	91.7	7.6	0.0	0.7	70.3	35.78
Finland	FNES	30	86.3	0.0	3.3	10.0	0.2	0.40	75.8	12.4	2.4	9.4	29.1	29.44
	FNWS	31	83.9	12.9	0.0	3.2	0.1	0.18	89.5	1.0	5.7	3.8	59.6	38.66
France	FRFE	19	57.9	26.3	15.8	0.0	0.1	0.32	100.0	0.0	0.0	0.0	50.8	36.33
	FRVI	15	40.0	26.7	26.7	6.6	0.3	0.56	82.7	0.0	0.0	17.3	44.3	41.40
Ireland	IEKE	20	20.0	10.0	35.0	35.0	0.1	0.00	89.0	4.2	4.2	2.6	46.1	35.06
Italy	ITMO	26	80.8	7.7	3.8	7.7	1.0	2.96	92.	4.7	0.0	3.1	44.3	39.59
	ITRE	30	73.3	23.3	3.4	0.0	0.3	0.83	95.5	4.5	0.0	0.0	65.6	35.34
Nederland	NLDR	21	23.8	14.3	14.3	47.6	0.5	1.63	44.7	0.7	0.2	54.3	32.3	37.71
	NLGW	22	81.8	18.2	0.0	0.0	0.8	2.57	83.7	7.8	0.0	8.6	61.0	34.44
	NLMR	24	91.6	4.2	0.0	4.2	0.1	0.00	96.4	0.3	0.0	3.3	81.5	31.58
Total		371	66.6	12.6	11.5	9.3	0.8	4.24	87.6	3.4	1.0	8.1	57.4	38.27

	BEBM	BEPR	EEEN	ESAN	ESAS	FNES	FNWS	FRFE	FRVI	IEKE	ITMO	ITRE	NLDR	NLGW	NLMR
BEBM															
BEPR															
EEEN	af	abf													
ESAN					f										
ESAS	aceg	cg	acef	cd											
FNES	acf	bcdf	acg	bcdfg	defg										
FNWS	d	dfg	adf	fg	cdg	bcdf									
FRFE	deg	fg	abe	f	c	bcdfg	d								
FRVI	fg	befg	a	be	cef	cdg	deg	e							
IEKE	df	dfg	adg	dg	cdf	bcg	g	dg	de						
ITMO	f	f	a		cfg	bcd	d		eg	dg					
ITRE	e	f	aefg		cg	bcdeg	d		beg	dg	f				
NLDR	efg	befg	abeg	beg	bcefg	bcdeg	bdefg	beg	be	bde	beg	befg			
NLGW	ac	bcf	acfg	bcf	bg	dfg	cd	bcg	ceg	dg			bcefg		
NLMR			abfg	fg	cg	bcdf	df	f	efg	dfg	f	f	fg	bcef	

Presence of letters corresponds to significant differences between breeds (< 0.05). ANOVA significance (total external workers: a; % animals farm activity: b; % non-feed crop farm activity: c; % forestry farm activity: d; % other farm activity: e; local cattle breed's share of the total farm income %: f). and Chi-square significance (% income: g).

Beside information on farmers and their farms, our survey aimed to understand values and motives of farmers for keeping their local breed, their perception of the attitude of the society toward them continuing to farm local breeds instead of turning to mainstream ones, and farmers' plans on the size of their herds. Figures 1 to 7 reports on these aspects.

Farmers (Figure 1) were asked to identify and rank the three main reasons to keep their local breed among the following: tradition (of the farm and/or of the farming area), multi-functionality (i.e. opportunities for multi-functional farming including tourism, production of niche products, vegetation management), external support (presence of economic incentives and/or conservation programs), functional traits, social value (including: image value for the farm, link to other people who have such breeds or values, bringing pleasure to the family), other reasons. Across all breeds, as first reason the most frequent answer was functional traits (36%) strictly followed by tradition (35.4%), that was the most frequent answer in eight breeds (BEPR, EEEN, FNWS, FRFE, FRVI, ITMO, IEKE, NLDR). In ESAS the most frequent first reason was external support (30%), in IEKE farmers indicated with equal frequency (35%) tradition and presence of external support. When tradition was given as first reason, the second reason was functional traits in five breeds (BEPR, EEEN, FNWS, IEKE, ITMO), tradition again in two breeds (FRFE, FRVI) and multi-functionality or social, with equal frequency, in one breed (NLDR). In addition, the social reason was given as most frequent third reason in three breeds (IEKE, NLGW, NLMR). The importance of tradition, beside productivity, suggests the importance of conservation programmes keeping in consideration the cultural aspects of local cattle farming (Gandini and Villa, 2003). The relative low importance of support from conservation programmes might reveal the average inadequacy of these to contribute to maintaining the farming of the local breed.

Farmers were asked to compare their local breed (as poor, same, good) to a mainstream breed they knew, for productivity, economic profitability and functional traits (Figure 2). Farmers were asked to analyze the following functional traits, fertility, longevity, management requirement, robustness, and docility, and Figure 2 reports rounded averages across the five traits. Productivity was considered poor by the majority of farmers in all breeds, as the same by about 30% of farmers in FNES, FNWS, NLMR. In IEKE 20% of farmers valued productivity as good with respect to the mainstream breed. Comparison in terms of economic profitability increased the value of the local breed. In fact in only six breeds the majority of farmers considered their breed less profitable than the mainstream breed. In four breeds (BEBM, BEPR, FRFE, FRVI) productivity was considered the same by a vast majority of farmers.

Figure 1. The three main motivations of farmers to keep the local breed

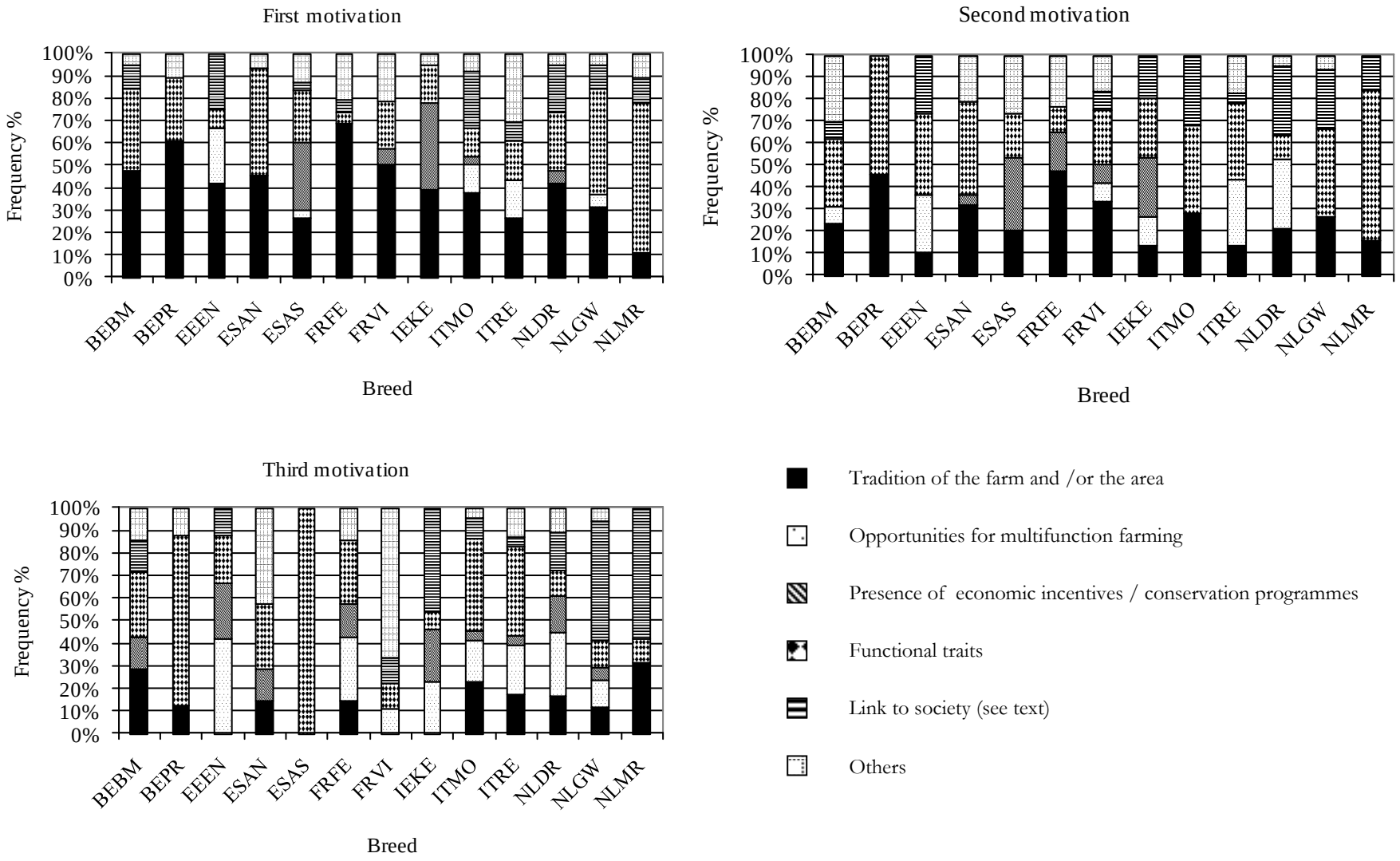
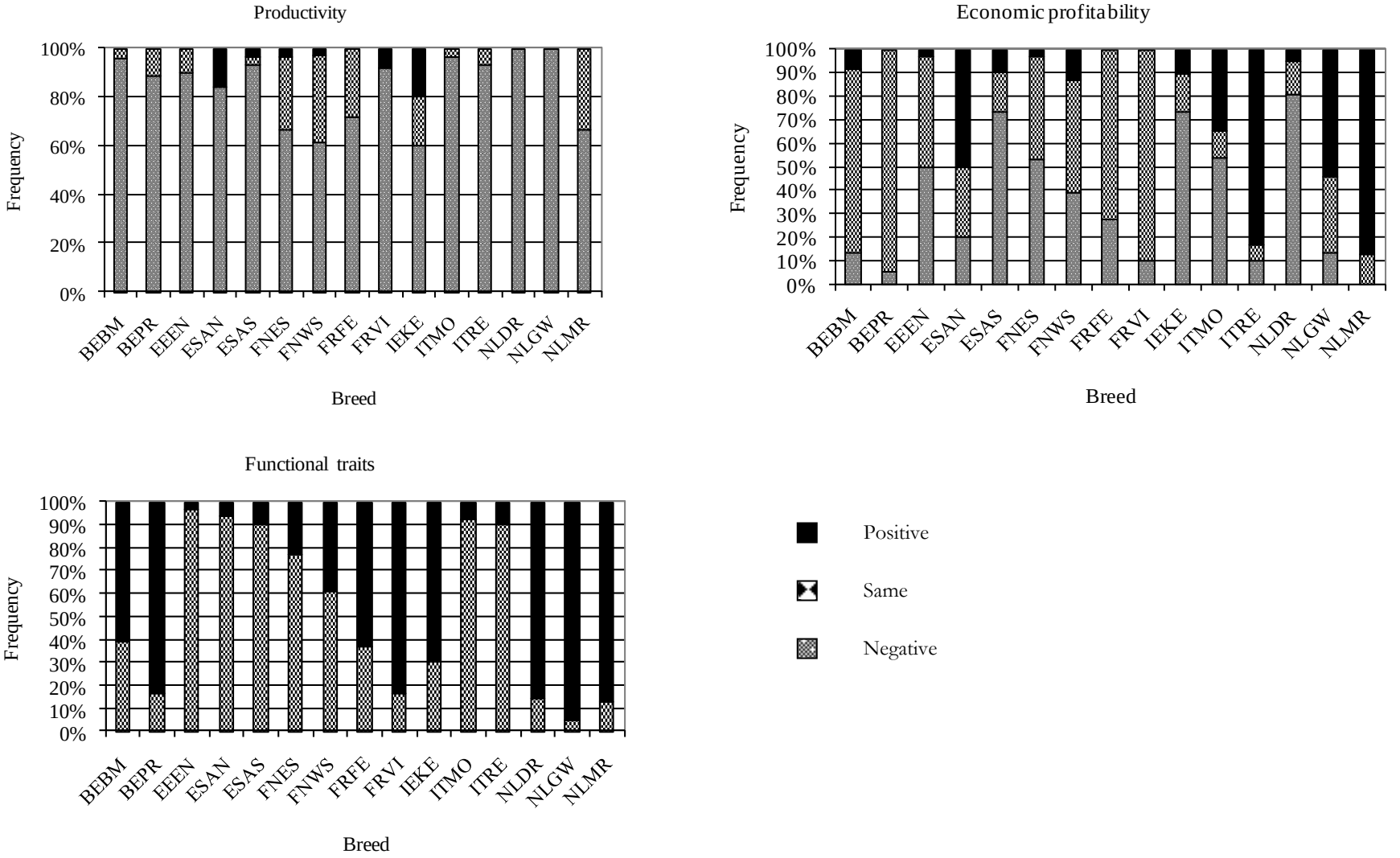


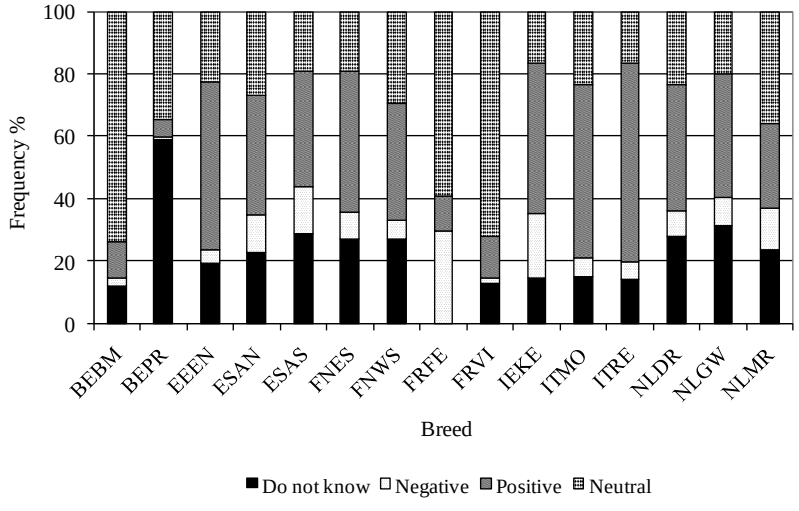
Figure 2. How farmers compare their local breed to the mainstream breed on productivity, economic profitability and functional traits



In three breeds (ITRE, NLGW, NLMR) more than 50% of farmers valued their breed as more profitable than the mainstream breed. For the Reggiana breed (ITRE) the high profitability is linked to the success of a branded Parmigiano Reggiano cheese that is sold at a high price (Gandini et al, 2005). The local breed was valued always superior or the same when comparison was on functional traits. In particular, five breeds (BEPR, FRVI, NLDR, NLGW, NLMR) were considered by 80% or more of the farmers positive with respect to the mainstream. Profitability comparisons based on farmers estimates can be misleading if production costs are not correctly kept in consideration. However, they provide some indications on the interest of farmers for their breeds and consequently on opportunities for breed survival.

The two following questions were based on the assumption that acknowledgement by society of a positive image of the farmer of local breeds can contribute to maintaining these breeds. Farmers were first asked which type of appreciation (positive, neutral, negative, do not know) they thought the following eighteen categories of persons and entities have on their local breed and their products: extension persons, inseminators, veterinarians, breeding organization, farmers' associations, agricultural authorities, environmental authorities, regional authorities, food industry, research institutes, farmers of main stream breeds, farmers without animals, non-farmer neighbors, tourists, tourism agencies, cultural societies, consumers, media. As average over the eighteen categories and the fifteen breeds (Figure 3) a positive appreciation was observed in 35.2% of farmers, but with rather low values in the Belgian and French breeds, BEPR (5.4%), FRFE (11.3%), BEBM (11.6%), FRVI (13.3%), and a maximum in ITRE (63.9%).

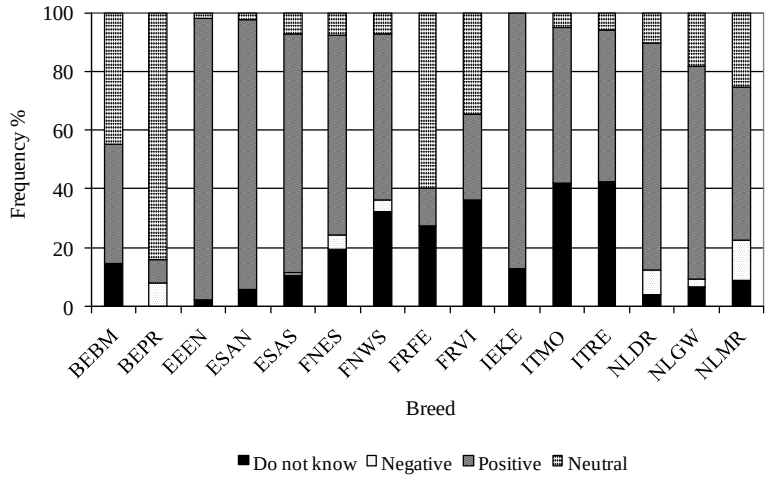
Figure 3. Farmers' view on the appreciation of their local breed and its products by eighteen stakeholders categories (see text): average across the eighteen stakeholder categories.



A neutral appreciation is expected on average from 32.9% of farmers, with a minimum in IEKE (16.5%) and a maximum in BEBM (73.7%). A negative appreciation is on average expected by 9.6% of farmers, with two situations above 20%, in IEKE (20.9%) and FRFE (29.5%). A negative appreciation is expected from most farmers (37.2%) in the case of the category "farmers of main stream breeds", and with a rather high percentage (19.3%) in the category "food industry". The percentage of farmers that did not have a precise idea (do not know) was on average 36.8%, ranged from zero in FRFE to 58.7% in BEPR. A negative appreciation was seen with <7% occurrence in 9 categories.

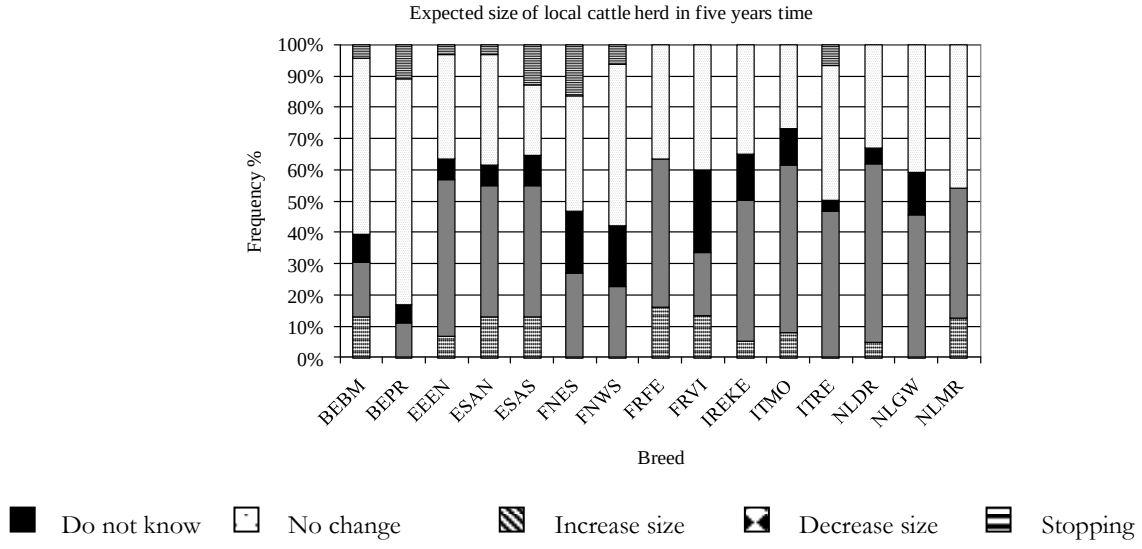
In order to further understand the farmer perception of the attitude of the society toward the local breed, farmers were then asked to give their opinion on how (positive, neutral, negative, do not know) they think the society values the following five breed attributes: quality of products, specific traits, cultural heritage, landscape conservation, source of genetic variation. As average over all farmers, the highest positive opinion was expected for the cultural value (68.1%), followed by the genetic value (65.56%), the quality of products (63.7%), landscape conservation (60.5%), and specific traits (48.3%). Considering an average above the five breed attributes (Figure 4), some differences were observed among breeds, with 6 breeds where 75% or more of the farmers that think the society has an overall positive attitude toward the local breed and 9 breeds were no farmers think that the society has an overall negative attitude. Some variation is observed also on the percentage of the farmers that did not have a precise idea (do not know), ranging from 0% in BEPR to 41.7% in ITMO and in ITRE. If we assume that a positive recognition of the society of the work of the farmer can enhance the interest in maintaining local breed farming, it could be advisable to promote through the media the importance of local breed conservation and the communication among farmers and the society as a whole.
Farmers were asked on the level of cooperation among them, in terms of participation in the activities of the breeding association, and of marketing of products and services. On average, across breeds, collaboration with the breeding association is rather high with an average across breed of 66.9%, and a percentage below 50% only in five breeds (BEBM, BEPR, FNES, FNWS, FRVI). Cooperation in marketing of products or services, on the contrary, is rather low with an average across breeds of 23.5% and with only three breeds (ESAN, IEKE, ITRE) above 40%. The farmers of only four breeds, EEEN, FRFE, ITMO, ITRE, said they participated to inbreeding control centralized programs and to cooperative programs for development of niche products. For inbreeding control the level of appreciation was above 85% in three cases but the ITMO (47%). Programs on niche products were judged as failure or low appreciated, but in ITRE with 100% of good level of appreciation.

Figure 4. Farmers' view on the value attributed by society to their local breed: average over five breed attributes (see text).



Farmers were asked the size of their local cattle herd expected in five years time, with respect to the current size. On average (Figure 5) 38.5 % of farmers expect to increase the size (from 11.1% in BEPR to 57.1% in NLDR), 6.7 % to decrease (from 0% in BEPR, FNES, FNWS, ITRE, NLGW to 15.8% in FRFE), 39.9 % to keep the same size (from 22.6% in ESAS to 72.2% in BEPR) and 4.9 % to give up farming (from 0% in seven breeds to 16.7 in FNES). Ten percent of farmers said they could not predict herd size in the next five years. The high proportion of NLDR farmers expecting to increase herd size is also due to the fact that many farmers started to keep this breed in the last five years and they still need to reach an appropriate herd size.

Figure 5. Changes of herd size planned by the farmer in the next five years, with respect to current size.



Eleven of the fifteen breeds analyzed benefit from economic incentives. The degree of dependence of farmers on economic incentives was estimated by asking farmers their expected behavior (to give up farming, to decrease herd size, to keep the same herd size, to increase herd size, do not know) under three scenarios of change of subsidies: 50% increase, 50% decrease, removal. In Figure 6, for each breed, proportions of farmers' expected behaviors are illustrated for the three scenarios. Almost all farmers seemed to know how they would react to subsidy changes, but a high proportion of French farmers. In case of 50% increase of subsidies, in four breeds (BEBM, EEEN, FNES, ITRE) most farmers will not change herd size, in ESAS and IEKE farmers will increase herd size and in ESAN, FNWS and ITMO farmers are equally distributed among no change and increase. Under the hypothesis of subsidies removal, in BEBM, EEEN, ITMO, ITRE most farmers will not modify herd size and in good proportion will even increase it (FNES, FNWS). Farmers of the Spanish breeds (ESAN, ESAS) and IEKE are in good proportion ready to give up farming of the local breed or to decrease herd size. The answer in case of 50% decrease of subsidies is close to the case of having the subsidies removed, although slightly negative. Then, seven breeds (BEBM, EEEN, FNES, FNWS, ITMO, ITRE) seem to be fairly independent of subsidies.

Signorello and Pappalardo (2003) observed that, in spite of EU support to farmers, it still remains unprofitable to rear local breeds. In seven breeds we asked farmers how much subsidies per cow per year they would think to be reasonable to cover the lower income profitability compared to the mainstream breeds. Responses were rather different both within and between breeds. In the Netherlands the requested incentives were on average 100 Euro (SD 164.3; range 0-500) in NLMR, 172.7 Euro (SD 254.3; range 0-800) in NLGW and 303.9 (SD 256.1; range 0–1000) in NLDR. In Spain 187 (SD 50.6; range 120-300) in ESAN and 396.1 (SD 116.5; range 200-600) in ESAS. Farmers of EEEN requested on average 370.1 Euro (SD 128.8; range 256-770) and farmers of IEKE 381.3 Euro (SD 183.4; range 150-1000). Our survey detects poor homogeneity among requests, possibly different ideas among farmers how local breed farming should be supported, and the necessity of better investigating amount and roles of economic incentives.

Beside subsidies, we investigated which elements would support keeping the local breed on the farm. Farmers were asked to value (as positive, neutral, negative, do not know) the following six activities: increasing breed productivity, developing/promoting food products associated to the breed, promoting other - less traditional - breed roles such as vegetation management, support to social or therapy activities and cultural, increasing technical assistance,

Figure 6. How farmers will react to changes in subsidies amount. Responses in those breeds that currently benefit of subsidies

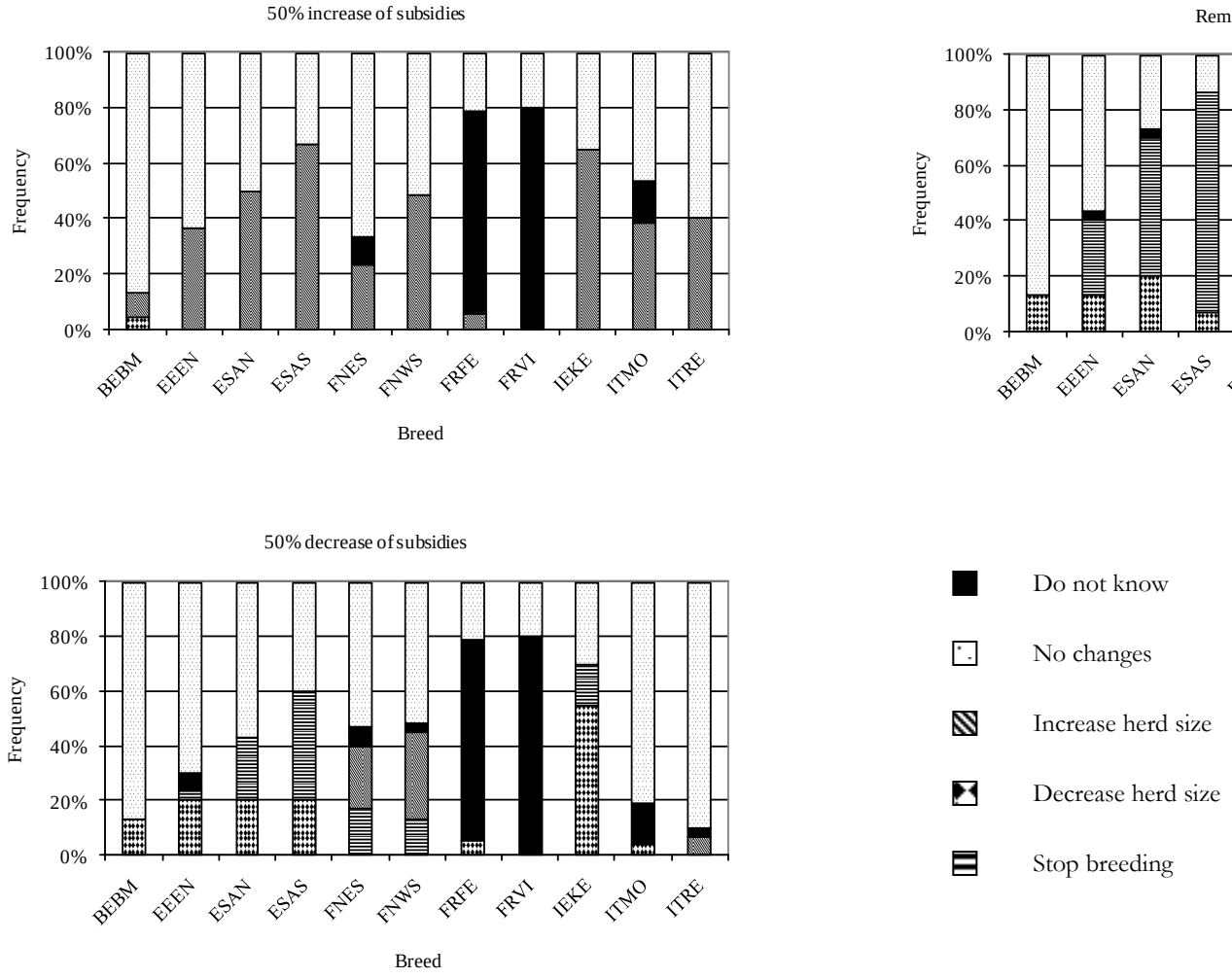
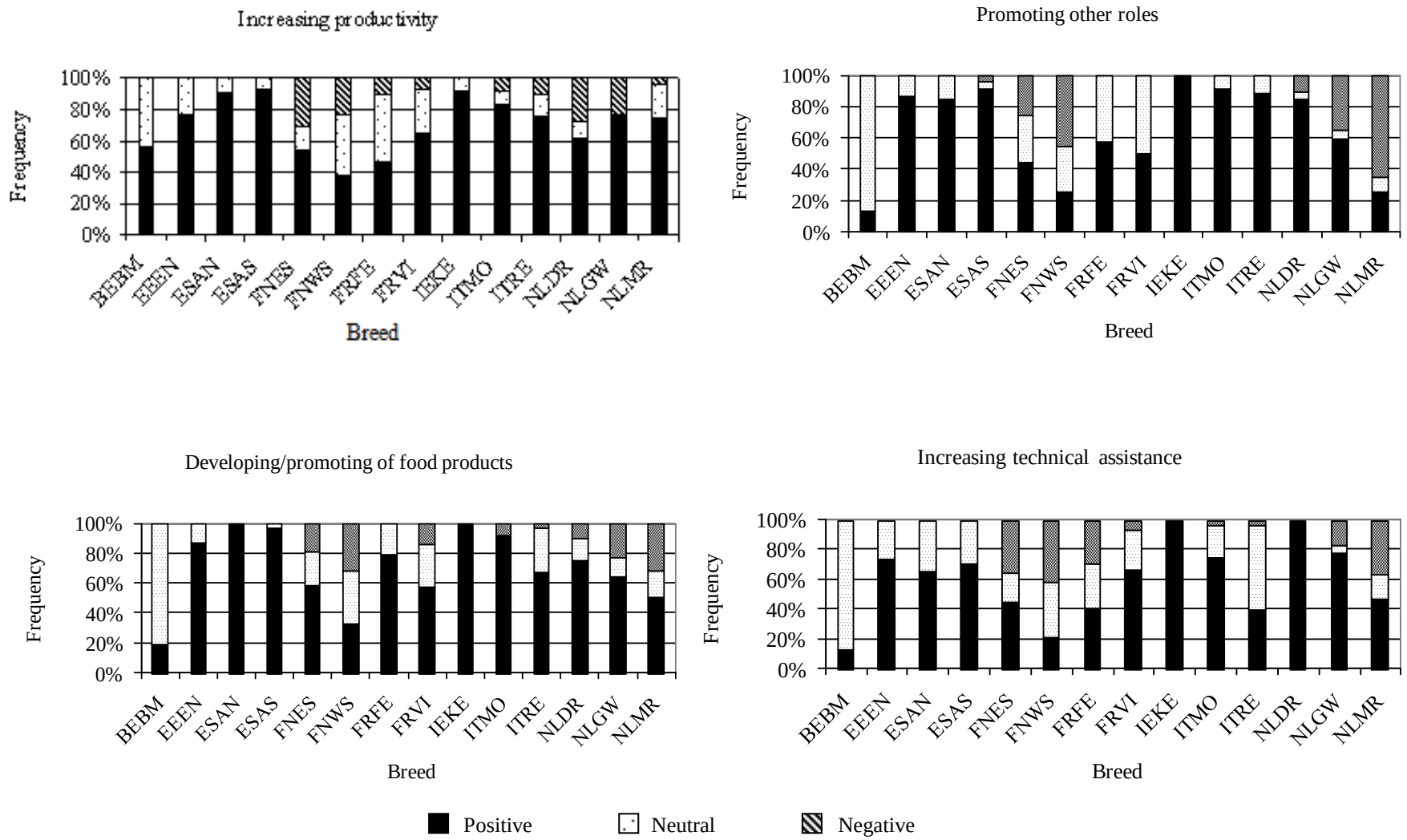


Figure 7. Opinion of the farmer on four possible activities as support to continue to keep the local breed on farm.



developing non-food products associated to the breed, and increasing consumers' awareness. Results are given in Figure 7 for the first four activities above listed. Results concerning the activities improving technical assistance and increasing consumers' awareness were very similar, respectively, to promoting less traditional roles and to developing/promoting food products, and are not reported in Figure 7. All activities were most often valued positively. Considering all breeds, increasing consumer awareness (not reported in Figure 7) was valued positive with the highest average percentage (67%) and with seven breeds above 85%. High positive responses were also given on average to increasing productivity (63.5%) and to developing food products associated to the breed (63%). The highest frequency of negative responses were from Dutch and Finnish farmers. A large majority of BEBM's farmers valued all activities neutral.

6.5 Conclusions

This survey revealed a large variation between and within breeds for most of the analysed aspects. In particular it is worth noting that almost all local breeds are kept, by a certain percentage of farmers, together with other breeds. On average the income from the local cattle covers 57% of the farm income. In some cases the local breed represents a small percentage of the total cattle farm herd and it can be questioned if this type of farming risks having the local breeds at the edges of the production system, kept as hobby activity.

Many farmers indicated the family tradition or the area tradition as an important motivation to continue keeping the local breed, and it is reasonable to wonder if this motivation will be transferred to the next generation and if other motivations will be capable to replace tradition.

Considering that on average local breeds are producing less milk and/or meat than mainstream breeds, beside the optimisation of the low input-output production system, multifunction farming systems capable of adding value to local breeds has been often advocated. Apart from a few cases, the survey revealed that multi-functionality is still poorly adopted.

Our survey strategy was aimed to detect the average situation of the fifteen breeds, but also to achieve the greatest possible amount of information from each breed, and to be considered a case study (e.g. Flyvbjerg, 2006). Here we can conclude that in many breeds, e.g. ESAS, ITRE, NLDR, the traditional farmer coexists with more recent production systems, characterised by more extensive systems, greater attention to quality products or to farming for specific functions such as nature management.

Some aspects investigated provide indications on sustainability of local cattle farming. The degree of sustainability in the short term can be directly derived by the changes in herd size expected by the farmer in the next five years. Most farmers provided this information and answers are optimistic for the survival of the fifteen breeds surveyed, considering that only 13% of the farmers declared planning to reduce herd size or to discontinue local cattle farming. In a larger context, other parameters such as age of the farmer, farmers' view on the appreciation from the society for the local breed, and comparison of the local cattle with the mainstream breed provided positive elements for the survival of the breeds. Today most farmers receive some EU subsidies, but the survey on the fifteen breeds revealed some degree of independency of farmers from public economic support.

Most farmers of the local cattle breeds demand the development of activities promoting and helping local breed farming. In particular they would favor opportunities to increase productivity and profitability through promoting non conventional roles, and developing food products associated with the breed. However, the large variation observed among breeds suggests the need to develop conservation actions capable of being flexible and adaptable to the local situations, among and within breeds. The presence of successful experiences in different countries and breeds suggests also the necessity of exchanging information about successes and failures of conservation and promotion initiatives. Finally, we suggest that the information on local cattle farming should not be restricted to the farming society, but it should extended to the whole society in order to increase general knowledge, awareness and appreciation of the work done by the farmers of local cattle breeds.

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Summary

Summary

The general aim of this thesis is to contribute to increase our knowledge in the area of conservation and sustainable use of farm animal genetic resources (AnGR). The thesis also provides elements for the development of the Global Plan of Action for AnGR (GPA-AnGR), led by FAO and signed by 109 country in 2007, including Italy.
At this respect the work carried out can be divided into three parts. First, Chapters 2, 3 and 4 that, in line with Strategic Priority 11 of GPA-AnGR, relate to the development of approaches and standards for AnGR conservation. Second, Chapter 5, in line with Strategic Priorities 9 and 10 of GPA-AnGR, focuses on *ex situ* conservation programmes and national and regional long-term conservation strategies. Third, Chapter 6, in line with Strategic Priorities 2 and 6 of GPA-AnGR, relates to the development of methods for local breeds evaluation and comparison.

Chapters 2, 3, 4

These chapters report about three research investigations aimed to establish a protocol for recovery and cryopreservation of viable sperm from the epididymis of slaughtered animals, as opportunity in constructing semen banks.

Chapter 2 reports on bull epididymal sperm extraction performed with two different techniques, the float-up and the retrograde flushing, in combination with two different extenders, with or without egg yolk. The different techniques were compared in term of sperm quality. Results suggest that in cattle retrograde flushing technique is the recommendable method to obtain viable sperm from the epididymis. Flushed samples had higher quality probably due to less contamination by blood. Moreover, the addition of egg yolk was found to have a significant beneficial effect on protecting epididymal sperm collected in float-up method, whereas in flushing its action was not substantial. Results also suggest that the use of a medium with egg yolk addition can have a positive effect in the early stages of semen cryo-preservation, during extraction and the recovery phase, before cooling and freezing process.

Given the promising results obtained in the cattle species with the use of retrograde flushing technique, epididymal buck semen recovery was performed by using the same epididymal sperm extraction method. In Chapter 3, the

relationship between body weight, secondary sexual traits and epididymal semen quality in Camosciata della Alpi young bucks is evaluated. This study reveals that good quality epididymal sperm can be obtained also in goat by using the retrograde flushing technique. Moreover, scrotal circumference, horn length and testicular development occur in parallel to the increase of live body weight. Epididymal semen quality in the Camosciata delle Alpi goat is influenced by body weight, scrotal circumference, horn diameter and length, testicular and epididymal weight.

In Chapter 4, the quality of epididymal buck spermatozoa, extracted from the epididymis with the retrograde flushing technique, in pre-freeze and post-thaw samples, is investigated. The effects of testicles storage temperature (environmental temperature or 5°C), and time elapsed between animal's death and sperm recovery (0, 24, 48, 72 hours) were studied. The study showed that between 24 and 48 hours post-mortem epididymides can still give an acceptable number of epididymal semen doses. Moreover, goat epididymal spermatozoa extracted by testicles stored at 5°C until 48 hours post-mortem are able to maintain their quality in terms of total motility, viability and sperm morphologies, also after cryopreservation.

These three experiments demonstrate that both bull and buck epididymal spermatozoa can be successfully collected by using the retrograde flushing technique. Additional research is needed to the test the fertilizing capacity of goat epididymal sperm recovered by retrograde flushing technique, in order to confirm the opportunity to store epididymal sperm in semen banks.

Chapter 5

Chapter 5 illustrates the work carried out to create the Lombardia Farm Animal Genetic Resources Cryobank (LABank), and to develop the "Network of the Italian cryobanks of farm animal genetic resources".

To create a genetic reserve for the local breeds of the Lombardia region, male gametes were collected and stored from donors of Varzese cattle, Brianzola sheep and Frisa, Orobica and Verzaschese goats. In order to reduce costs and overcome organisation difficulties, in the case of Brianzola sheep, epididymal spermatozoa were collected instead of conventional semen collection. For each donor, samples of blood and hair were also collected, as source of genetic material to perform future DNA research. At present a total of 2,295 semen and epididymal sperm doses, 286 ml of blood and 69 g of hair are stored in the

LABank. Information related to donors and genetic material stored were recorded by using the CryoWEB Software. Protocols for access and ownership of the genetic material were developed.

In addition, a coordination system of Italian collections, the "Network of the Italian cryobanks of farm animal genetic resources" (CRIONET-IT), was developed, to share the information on cryo-preserved genetic material through a virtual bank, and to promote collaboration among institutions involved in cryo-preservation of AnGR. Six partners, including research institutes, breeders associations and local authorities, have joined CRIONET-IT. CRIONET-IT aims to be a contribution to the future creation of an Italian national gene-bank, and more generally to the development of the ex-situ management of farm animal genetic resources in Italy.

Chapter 6

In Chapter 6 farmers' motives and values in keeping local cattle breed are investigated trough 371 interviews of farmers of 15 local cattle breeds, in eight European countries. The work was part of a larger investigation on local cattle breed sustainability, co-financed by EU.

Surprisingly, the most frequent first reason to keep the local breed was productivity, followed by tradition. When comparing the local breed to a mainstream breed, in four cases (breed) productivity was considered to be the same, in three cases more than 50% of farmers valued the local breed more profitable; moreover, the local breed was always valued as superior, or the same, on functional traits. Farmers were asked which type of appreciation they thought various stakeholders had on their local breed: a positive appreciation was observed in 33% of farmers. On average across breeds, 39 % of farmers expect to increase the size of their herd in the next years, 5% think to give up farming. The degree of dependence of farmers from economic incentives was analysed by asking farmers their expected behavior under three scenarios of change of subsidies. Overall, the analysis was addressed to identify elements for the development of national and regional cattle conservation programmes.

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