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Report 153

Survey of organisation, actual stocks, and procedures of Ex situ conservation of Heritage Sheep Breeds

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ANIMAL SCIENCES GROUP  
WAGENINGEN UR

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### Abstract

This report describes the results of a survey of the organisation and procedures of ex situ conservation of Heritage Sheep breeds in France, Greece, the Netherlands, and the United Kingdom.

**Keywords:** gene bank, ram semen, ex situ conservation, heritage sheep breeds

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# Survey of organisation, actual stocks, and procedures of Ex situ conservation of Heritage Sheep Breeds

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August 2008

## Summary

This report describes the results of a survey of the organisation and procedures of ex situ conservation of Heritage Sheep (HS) breeds in France, Greece, the Netherlands, and the United Kingdom. These four partners presently maintain stocks of cryopreserved semen of a total of 25 different HS breeds (HSB) in their national HSB gene banks.

Most cryopreservation efforts for HSB sheep semen were made quite recently. The main reason for the ex situ conservation were the risks for losing breeds or specific genotypes as a consequence of outbreak of diseases (foot and mouth disease (FMD) and scrapie). Other reasons for cryopreserving specific breeds were the traits of economic importance (in France and Greece), and the degree of endangerment and cultural/historical value (in France and Netherlands).

Costs for the ex situ conservation were/are being paid directly or indirectly by national governments or by industry (France). Semen collection, freezing and storage are undertaken by (or embedded in) commercial organisations (UK, France) or research institutes (Netherlands, Greece). Commercial organisations may operate more cost-efficient than research institutes. Collecting semen on an AI centre is more expensive than on-farm collection. An important cost-reduction is possible by using epididymal semen. The need to adhere strictly to EU regulations (France) incurs higher costs as it is not possible to use on-farm collections or epididymal semen. Housing and training of rams for collecting semen costs time and money. The ease of handling and training of the rams may vary widely among breeds and may be an important factor for cost efficiency. The ban on using laparoscopic insemination (Netherlands) incurs high costs, as cervical inseminations require much more semen per lamb born.

Procedures followed differ between countries. Ejaculated semen is collected from selected rams approximately two times a week. Epididymal semen is obtained from the epididymis of selected rams. The rams are not culled for the semen collection but are culled anyway. Testes are transported from the slaughterhouse and semen is obtained on the day of slaughter with a simple procedure from the caudae of the epididymides. Semen freezing procedures may vary per country, without clear preference for a particular freezing medium or freezing procedure. Freezing media contain egg yolk and glycerol as cryoprotectants. Laparoscopic inseminations generally result in pregnancies over 60%. Cervical inseminations may result in 30% pregnancies. Epididymal semen seems to have at least as good as, or better fertility than ejaculated semen.

Veterinary issues: France needs to follow EU regulations as these were integrated into French national legislation. The Netherlands do not have EU certified sheep AI stations. The Netherlands have predominantly used epididymal semen from testes obtained from slaughterhouses.

Animals were tested serologically for a number of diseases but this varied per country.

In WP4 the partners will make new stocks of frozen semen from two breeds per country. It is recommended that we use this as an opportunity to compare and exchange methods and expertise. If it can be organised, a split sample comparison of freezing media/methods could perhaps be done combined with exchange of researchers/personnel. The comparison of methods can then be done by in vitro assessment of post-thaw semen quality. Partners will not try to do an insemination trial, as this is too costly and logistically very difficult.

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## 1 Introduction

Work package 3 (WP3) of the EU project Heritage Sheep Breeds (HSB) concerns the *ex situ* conservation of HSB and particularly addresses the strategies and practicalities for collection and cryopreservation of HSB ram semen. The objectives of WP3 are:

1. Identification of collection and cryopreservation strategies through a detailed survey.
2. Comparison and evaluation of practicalities in collection and freezing methods, based on questionnaire results and a wider discussion with experts, and including technical, economical and sanitary aspects.
3. Development and implementation of strategies for *ex situ* conservation, tailored for HSBs in Europe.

In 2007, a questionnaire was made and was sent to four partners, i.e., France, Greece, the Netherlands, and the United Kingdom. The information of the returned questionnaires was processed in a draft survey report. The comparison of the information given by the various partners led to a next round of questions to, and discussions with the partners. Finally, the draft report was presented and discussed in a workshop held on April 20, 2008 in Amsterdam. These discussions have then been used as further input for the present final report.

## 2 HSB Gene banks

The following paragraphs of this chapter give an overview of the present stocks of frozen HSB semen, and describe the organisations involved, the rationale for choosing the breeds and the male donors, costs (and ownership), and logistics of semen collection (factors affecting cost-efficiency).

### 2.1 Stocks of cryopreserved HSB semen

The four partners that participated in the survey all had stocks of frozen ram semen from a total of 25 different HS breeds.

**Table 1** Summary of HSB gene bank activities per country

Countries	Breeds	Organisation	Mean males/breed	Mean doses/male
UK	15	Innovis (for NSP semen archive and heritage gene bank)	30	152
NL	6	CGN	26	135
France	3	Nat. Cryobanque + AI org.	30	116
Greece	1	Nagref Research Institute	15	67

**Table 2** Overview of available semen stocks in gene banks per breed

Name of breed	Organisation <sup>1</sup>	When collected	No of rams	No of doses	Sperm/dose <sup>2</sup>	Eja or Epi	Storage sites	Straw or Pellet <sup>3</sup>
Kempen Heath Sheep	CGN	2001-2002	15	2234	200	Eja	2	Str
Veluwe Heath Sheep	CGN	2001-2002	7	715	200	Eja	2	Str
Veluwe Heath Sheep	CGN	2004- - -	27	3036	200	Epi	2	Str
Drenthe Heath Sheep	CGN	2001-2002	2	44	200	Eja	2	Str
Drenthe Heath Sheep	CGN	2004- - -	55	4014	95-200	Epi	2	Str
Mergellander	CGN	2001-2002	21	2538	200	Eja	2	Str
Mergellander	CGN	2004	4	398	200	Epi	2	Str
Schoonebeker	CGN	2001-2002	10	1096	200	Eja	2	Str
Schoonebeker	CGN	2008	7	709	80-200	Epi	2	Str
Zwartbles	CGN	2003	7	168	200	Eja	2	Str
Zwartbles	CGN	2005	1	616 <sup>5</sup>	200	Epi	2	Str
Basco Béarnaise	Ordarp	2005	16	2032	100	Eja	1	Str
Manech tête noire	Ordarp	2005	20	2263	100	Eja	1	Str
Manech tête rousse	Ordarp	2005	53	6017	100	Eja	1	Str
Chios	NAGREF	2006-2007	15	1000	100	Eja	1	Str
Brecknock Hill Cheviot	NSP	2005-2007	18	3499	100	Eja	<sup>4</sup>	Str
Cheviot	NSP	2005-2007	41	7076	100	Eja	<sup>4</sup>	Str
Clun Forest,	NSP	2005-2007	7	1567	100	Eja	<sup>4</sup>	Str
Dalesbred	NSP	2005-2007	6	1154	100	Eja	<sup>4</sup>	Str
Dalesbred	Heritage	April 2001	7	150x2	240	Eja		Pel
Derbyshire Gritstone,	NSP	2005-2007	2	308	100	Eja	<sup>4</sup>	Str
Devon Closewool	NSP	2005-2007	9	1923	100	Eja	<sup>4</sup>	Str
Exmoor Horn	NSP	2005-2007	11	2302	100	Eja	<sup>4</sup>	Str
Herdwick	NSP	2005-2007	37	7262	100	Eja	<sup>4</sup>	Str
Herdwick	Heritage	April 2001	155	2196x2	240	Eja		Pel
Lonk	NSP	2005-2007	1	200	100	Eja	<sup>4</sup>	Str
Lonk	Heritage	April 2001	4	59x2	240	Eja		Pel
Romney	NSP	2005-2007	17	1302	100	Eja	<sup>4</sup>	Str
Rough Fell	NSP	2005-2007	16	2584	100	Eja	<sup>4</sup>	Str
Rough Fell	Heritage	April 2001	20	324x2	240	Eja		Pel
Shetland	NSP	2005-2007	30	13012	100	Eja	<sup>4</sup>	Str
South Welsh Mountain	NSP	2005-2007	20	4173	100	Eja	<sup>4</sup>	Str
Southdown	NSP	2005-2007	3	545	100	Eja	<sup>4</sup>	Str

Name of breed	Organisation <sup>1</sup>	When collected	No of rams	No of doses	Sperm/dose <sup>2</sup>	Eja or Epi	Storage sites	Straw or Pellet <sup>3</sup>
Southdown	NSP	2005-2007	20	3567	100	Eja	<sup>4</sup>	Str
Welsh Hill Speckled Face	NSP	2005-2007	19	11306	100	Eja	<sup>4</sup>	Str

<sup>1</sup> Organisation that have made the collections and houses and manages this gene bank/semen stock (see also paragraph 2.2.)

<sup>2</sup> Number of sperm per insemination dose, in millions of sperm.

<sup>3</sup> UK Pellets are 0.2 ml pellets each containing 2 doses of 120 million sperm per dose = 1200 million/ml. UK Straws are 0.25-ml straws.

<sup>4</sup> The semen is stored in two locations, the semen is divided into 4 tanks with two tanks stored at each site. Semen in the Northern Ireland Scrapie Plan (NISP) is stored in one location.

<sup>5</sup> Amazing, but true, 616 doses of  $200 \times 10^6$  epididymal sperm/dose from one animal.

## 2.2 Organisations and persons involved

### 2.2.1 Netherlands

#### HSB semen

CGN, Centre for Genetic Resources, The Netherlands

CGN is an independent organisation within Wageningen-UR. It performs statutory tasks on behalf of the Dutch government with regard to genetic diversity in agriculture and forestry. CGN manages a gene bank (ex situ conservation), supports in situ conservation, and gives policy support for the Dutch government and international organisations. The animal genetic resources part of CGN manages a gene bank for livestock species since 2001. CGN-AnGR is physically located at the Animal Sciences Group (ASG) in Lelystad. The gene bank collection is split into two locations (for safety), at Lelystad, and Utrecht, respectively.

#### Stocks of frozen sheep semen of other sheep breeds

Farmers Mr. Wijnker (Swifter), Mr. Bosgoed (Swifter), and Mr. Verduyn (Milk Sheep)

#### Expertise or research with regard to freezing ram semen

Veterinary Faculty Utrecht, Mr Rijnveld

Farmer Mr. Wijnker

Animal Sciences Group of Wageningen UR, Henri Woelders

### 2.2.2 France

#### HSB semen

Ordiarp Cryobanque. CDEO Ordiarp (Centre Départemental de l'Élevage Ovin in Ordiarp) holds stocks of semen from three breeds from the French Basque region.

#### Stocks of frozen sheep semen of other sheep breeds

In France genetic material is stored mostly for meat breeds. The organisations involved are:

Confédération de Roquefort

CIA OVI-TEST

CRIOPYC

INSEM-OVIN

CIA GIE U.S. R.O.M.

CIA de Verdilly

CIAP

(cf. table in Appendix 1)

For some commercial breeds, there is also semen that is cryopreserved in the "Cryobanque Nationale": the doses are kept in 2 sites for patrimonial purpose.

### **Expertise or research with regard of freezing ram semen**

Research is done by INRA of Tours: Pascal Mermillod, Xavier Druart  
Gilles Lagriffoul and Jérôme Raoul are involved in the general management of sheep AI for the "Institut de l'Elevage"

#### *2.2.3 Greece*

### **HSB semen**

NAGREF-Veterinary Research Institute

### **Stocks of frozen sheep semen of other sheep breeds**

None

### **Expertise or research with regard of freezing ram semen**

NAGREF-Veterinary Research Institute. Researchers: Sofia Belibasaki (belibasaki@vri.gr) and Aristotelis Lymberopoulos (lymberopoulos@vri.gr)

#### *2.2.4 UK*

### **HSB semen**

Heritage Gene bank and NSP Semen Archive

In 2001 collections were made by the Heritage Gene bank as an emergency response to the FMD outbreak in April 2001. NSP semen archive collections were made in response to the National Scrapie Plan (NSP) breeding programme. The NSP breeding programme was introduced to eradicate classical scrapie which could have been masking BSE in sheep. The sheep semen archive was introduced to ensure that valuable traits were not lost as a result of the selective breeding programme for scrapie resistance and to enable the reintroduction of genotypes reduced through the NSP if new TSE strains emerged that compromised classically resistant genotypes.

Now that the EU Commission has decided not to proceed with compulsory genotyping and SEAC (Spongiform Encephalopathy Advisory Committee) has concluded that the prevalence of BSE in the UK sheep population is likely to be either zero, or very low, the need for the ram genotyping scheme and the semen archive has diminished. The time has come for them to be drawn to a close, for industry to take over responsibility or for industry to submit a compelling business case as to why the taxpayer should continue to make a contribution, which could be expected to be strictly time-limited and at a lower level.

Studies have now shown that there are no significant production traits associated with the scrapie alleles removed under the NSP programme.

In the UK Innovis holds the NSP semen archive and the Heritage Gene bank collections. Two sites for security in general. NSP semen archives are held at Aberystwith 01970-828236. Britbreed, part of Innovis, performed the collection of semen and embryos for the Heritage Gene bank in 2001. The semen and embryos are still stored on their site in Scotland.

A Semen Archive Management Board oversees the progress of the Archive. Ark Consortium Ltd is the service provider for the Semen Archive under the National Scrapie Plan for Great Britain (NSP) and the Northern Ireland Scrapie Plan (NISPP). ARK consists of two leading UK providers of artificial insemination services (Innovis and AI Services, Northern Ireland).

Currently, the NSP semen archive is paid for by DEFRA (Department for Environmental and Rural Affairs), with anticipated costs of maintaining the archive in the region of £20 – 25,000 per year. This is now under review. The government of the UK has invited a consultation on Responsibility and Cost Sharing for Animal Health and Welfare. Industry and Non Government Organisations have been invited to submit proposals to take over the archive (it seems unlikely that DEFRA will continue to pay the cost of the semen archive).

Innovis™ Ltd is the holding company for three complementary trading companies active in specific sectors of the animal breeding market in the UK. The companies are CBS Technologies, which provides services to the sheep breeding industry in England and Wales, and Genes Direct and ART Porcine, both of which provide services to the pig breeding industry. In 2005 Britbreed in Scotland joined Innovis.

Innovis has three centres in the UK:

- Peithyll Centre, Capel Dewi, Aberystwyth, Ceredigion, SY23 3HU
- Aubreys Farm, Albright Lane, Bromesberrow, Ledbury, Herefordshire, HR8 1RZ
- East Mains, Ormiston, East Lothian, EH35 5HG (Britbreed)

#### Stocks of frozen sheep semen of other sheep breeds

The Rare Breeds Survival Trust (RBST) also has a collection of semen from rare breeds of sheep, Cattle and horses, which is stored with Innovis at its site in Herefordshire.

There are private stores at Dan Fawcett, South West Sheep Breeders. Michelle Hamilton.

#### Expertise or research with regard of freezing ram semen

Dr. Bill Holt. (WV Holt). ZSL Institute of Zoology, Regent's Park, London NW14RY, UK. [Bill.holt@ioz.ac.uk](mailto:Bill.holt@ioz.ac.uk)

### 2.3 Why these breeds were chosen?

**Table 3** Criteria that were used for prioritizing the breeds in different countries

	Degree of endangerment	Disease outbreak	Adaptation to a specific environment	Traits of economic importance	Unique traits	Cultural and historical value
Netherlands	+++	++++	+++	+	+	++
France	++	++++	+	++++	not	++
Greece	not	+++	not	++++	not	not
UK	not	++++ <sup>1</sup>	not	not	not	not

Importance of criteria (++++, +++, ++, +, not)

<sup>1</sup> The collections made by the Heritage Gene bank in 2001 were made as an emergency response to the FMD outbreak in April 2001. For the other UK breeds (NSP semen archive) the collections were made in response to the National Scrapie Plan (NSP) breeding programme (see also paragraph 2.2.).

### 2.4 Selection of donor animals

Genetic/phenotypic/morphology considerations, reproductive/health considerations. Practical and costs considerations.

#### 2.4.1 Netherlands

##### I. Ejaculated semen

Rams were selected by a breeding inspector of the Rare Breeds Foundation (SZH) together with CGN. The rams were chosen from redundant rams offered after completion of the breeding season. The genetic diversity and requirements of the herd book were the primary selection criteria used.

##### II. Epididymal semen

Rams were accepted when they were culled for other reasons than fertility problems or genetic problems. Only rams that had been approved by the herd book (morphology, compliance with standard breed characteristics) were offered after having served in the herd. In the first years we also got young rams that had not been tested or had served. No selection was made on the Scrapie genotype.

#### 2.4.2 France

In France for HSB breeds, the "elites" rams are selected on the basis of genetic considerations, essentially milk production (EBV values). It is just checked that they do not have any morphologic defects and that their sanitary status is compatible with the EU regulations of ram collection in an AI centre.

### 2.4.3 Greece

In Greece the breed was selected for genetic, phenotypic and morphology considerations. Further for reproductive and health considerations.

### 2.4.4 UK

Innovis: Selection criteria for the rams: 1) Financial, 2) Performance and 3) Rarity  
Initially, the NSP semen archive proposed to collect 20 rams for each scrapie allele ARQ/AHQ/ARH/VRQ. ARR alleles were not collected. However, this was reviewed to collect the alleles according to the frequency they occurred naturally in the breed. For example Suffolks do not typically have the AHQ allele.

## 2.5 Costs and ownership

### 2.5.1 Costs for collecting and freezing semen

#### Netherlands

In the Netherlands CGN (subsidised by Dutch Ministry of Agriculture, Nature and Food Quality) pays for collecting and freezing semen of heritage sheep breeds.

#### Ejaculated semen (2001-2002)

According to the budget of the project, the costs of housing, training, collection and freezing of semen were €260,000 for five breeds, or €52,000 per breed. However, this was only partly successful because the rams were very wild and it took a lot of effort to train them, or training failed altogether: Semen from only 55 out of the planned 129 rams was collected and cryopreserved (7,000 straws). Per ram this is almost €5,000, and per straw this is €37.

#### Epididymal semen

Much depends on logistical possibilities and number of animals that can be done on the same day. This may vary between years. Currently, costs of collecting and freezing epididymal semen may be approximately €300 per ram (100 doses of 200×10<sup>6</sup> sperm per dose). However, costs for additional rams collected at the same day are much lower. For 25 males per breed, this would amount to €5,000 per breed.

#### France

In France, ONILAIT (Interprofessional national organisation for milk and milk products) and CDEO Ordiarp (the AI centre) pay for collecting and freezing semen of heritage sheep breeds.  
The cost for this campaign for the 3 breeds in 2005 was €25,000 (89 rams collected).

#### Greece

NAGREF or through any other projects that are running.

#### UK

Personal funds or NSP for selective breeds and genotypes.  
The average costs in the past 5 years were: Approximately £300/ram.

In summary, France and UK can operate much more cost-efficient (€300/ram) than the Netherlands (€5,000/ram), but the high price in the Netherlands was also due to training difficulty for collecting semen. In addition, it may be very difficult to compare countries. Costs may be defined differently. CGN only collects (and freezes) semen for the purpose of gene banking. So all the costs are for CGN. In other countries routine operations are run in commercial or co-operative facilities.

### 2.5.2 Costs for storing semen

#### Netherlands

In the Netherlands CGN (subsidised by Dutch Ministry of Agriculture, Nature and Food Quality) pays for storing the semen of heritage sheep breeds. The average costs in the past 5 years (per breed) were: Assuming 25 rams with 100 doses per ram = 2,500 straws = 8.3 goblets = € 100 per breed per year.

#### France

In France The National Cryobanque pays for storing the semen of heritage sheep breeds. The average costs in the past 5 years (per breed) were: Liquid nitrogen tank (single expense: 1,000€) and liquid nitrogen (1,200€/year)

#### Greece

NAGREF covers the expenses for storing the semen. The tank cost is € 500,- and an additional cost has to be paid for the liquid nitrogen which is 300/year.

#### UK

Personal funds for personal clients + DEFRA for NSP

The average costs in the past 5 years were: Approximately 25€/dose/year.

Currently, the NSP semen archive is paid for by DEFRA (Department for Environmental and Rural Affairs), with anticipated costs of maintaining the archive in the region of £ 20 – 25,000 per year.

### 2.5.3 Ownership

Who is the owner of the stored semen of heritage sheep breeds?

**Netherlands:** CGN

**France:** The organisation that puts semen in the gene bank remains the owner of the stock. The AI centre are owners of the stored semen.

**Greece:** The owner is the NAGREF-Veterinary Research Institute.

**UK:** Heritage Gene Bank semen owned by Sheep Trust. NSP semen owned by DEFRA (but the future ownership is undecided).

Is the semen of heritage sheep breeds made available to be used or is it just preserved for possible long term future use? If so, what are the conditions. (For example, it can be that the semen may be used to support breeding schemes for small populations in order to minimise inbreeding).

**Netherlands:** Just preserved for long term future use.

**France:** Both:

- An active stock which can be used in any case (sanitary problem...), in order to have semen of rams still used by the farmers.
- A stock in the Cryobanque: it can be used for example to add genetic variability in a breed, or for research purpose.

**Greece:** Preserved for long term future use (sanitary problem, etc) and also for research purpose

**UK:** 10% of NSP and HGB semen officially is owned by the owner of ram. However, it is never used.

## 2.6 Logistics of semen collection (factors affecting cost-efficiency)

In all countries, gene banking of ram semen seems to have been done predominantly in specific campaigns, triggered by FMD and the scrapie sensitivity selection programmes. The UK NSP semen archive activities have stretched over several years (2005-2007), but continuation is expected to be time-limited and at a lower level. In the Netherlands gene banking activities have also been continued for several years in order to reach the originally set goals of the first campaign.

The efficiency of collecting semen and maintaining the collection depends on various factors. If, for instance, the rams are housed at an AI station and semen is collected anyway for other than gene banking purposes, this could make the gene banking activities more cost-effective. A commercial or cooperative organisation that has a daily

routine in collecting and freezing ram semen is likely to have the expertise, facilities, equipment and personnel (Scale, Automation, Good Technique) to operate highly cost-effective. Factors that lead to higher sperm yield per ram or to better post-thaw sperm quality, including ram management, procedures for handling and freezing of the semen, and post-thaw sperm evaluation (chromosome integrity, Greece) improve cost-efficiency. In Greece and the UK, semen from the HSB may also be frozen for regular (commercial) use. In the other countries the rams were housed in a central facility for practical reasons (facilities, expertise) and veterinary requirements. In the UK and France, semen collection and freezing is done by sheep AI organisations, whereas in Greece and the Netherlands, operations are performed by research institutes (NAGREF, and ASG, Lelystad + Veterinary Faculty, Utrecht for CGN, respectively). Collecting semen on an AI centre (which is necessary to comply with EU regulations) is more expensive than in farm collection.

Another factor is the number of collections needed. Preservation of 100 insemination doses of 25 males per breed may be sufficient for recovering a lost breed by backcrossing. Overall it seems that 2 to 5 collections per ram were needed for obtaining 100 doses of  $100 \times 10^6$  sperm. Collections are generally performed with three days intervals. In the Netherlands, laparoscopic inseminations are forbidden. Therefore, the Netherlands have generally frozen doses of  $200 \times 10^6$  sperm/dose. The UK (NSP) has frozen much more than 100 doses per ram. So in both cases more collections were needed. Generally, collections can be finished within one to 4 weeks. However, in the Netherlands it was seen that some Heath sheep breeds simply were too wild to be trained for efficient semen collections and the number of collections needed was high and the number of doses obtained were not sufficient for all breeds. Generally, there may be breed differences in handling ease and semen yield.

Semen collections can be done all year round (Greece) but are done predominantly in/around the breeding season, i.e. July through to March. It is felt that the semen quantity and quality is poorer out of the breeding season. Epididymal semen was also collected in March-July but again it was believed to have a lower quantity and quality.

Epididymal semen can be a cost-efficient alternative. In the Netherlands only epididymal semen was used since 2004. Epididymal ram semen seems to have a freezability and post-thaw fertility equal to or surpassing that of ejaculated semen. Enough semen can be obtained from one male (two epididymides) to prepare 100 doses of  $200 \times 10^6$  sperm. No housing and training of the rams is required. The rams used have to be culled after having served anyway. The costs involved are €300 for one ram (100 doses of  $200 \times 10^6$  sperm per dose) (based on ASG contract research tariffs), but costs for material collected from other rams at the same time are lower. The other countries generally do not use epididymal semen because of EU regulations and/or because they can arrange a cost-efficient collection of ejaculated semen.

### 3 Procedures used and Semen quality

#### 3.1 Procedures of semen collection

##### I. Ejaculated semen

Animals are housed in pens bedded with straw with natural lighting and ambient temperature. The animals get the veterinary treatment that they may need or is prescribed by (EU) regulations. Blood samples are taken to run serological tests for a number of diseases as described in paragraph 3.3.

There is a training period before the real collection.

The ram is taken to the teaser ewe that can be in natural heat or brought into oestrus by hormone treatment. When the ram is willing to mount the worker may gently rub his underbelly. Once the ram mounts, this procedure is repeated until the ram permit semen to be collected into an artificial vagina filled with warm water (40-42°C) with lubricant in the end where intromission of the penis occurs, and a graduated collecting glass tube at the opposite end.

In France a dummy is effectively used instead of a teaser ewe. Procedure: cleaning of the abdomen of the ram, letting the ram 5-6 minutes with the dummy, doing 1 or 2 false mounts (which improves the quality and quantity of the semen) and then collection of 2 ejaculates (with a few minutes between the ejaculates).

Collections can be done twice weekly. Electro-ejaculation may be done (UK) in the few (4%) cases that the rams are too wild and resist training.

##### II. Epididymal semen

Directly after slaughter, the testes are removed in the presence of CGN personnel in order to verify the animal ID of the testes. The testes are packed together with the label with the animal ID code in a polythene bag and placed in a cool box on top of ice or freezing elements, but isolated from that by a towel. Thus, the temperature of the testes will decrease during transport (1½ h) to approximately 15 °C.

The further processing is performed the same day. All further processing is done at 15 °C. The cauda of the epididymis can be easily dissected from the testis and the rest of the epididymis with one single cut by cutting with a slaughter knife through the epididymis at the transition between corpus and cauda and proceed cutting, while sliding the knife downward (distally) along the side of the testis, thus freeing the cauda from the testis, separated proximally from the corpus and distally from the ductus deferens. In this way, the cauda contains very little blood. This procedure is done with both caudae. The caudae are rinsed with Tris-egg yolk freezing medium, blotted dry with tissue paper, and placed in a 15cm Petri dish. Approximately 13 ml of freezing medium is poured over the cauda. Then the cauda is cut many times (more or less 'minced') with a scalpel. One can see the thick yellowish very concentrated semen oozing out of the incisions. The cauda is 'washed' in the medium by moving it about using a pair of tweezers.

Washing while massaging the cauda is repeated twice in a new volume of freezing medium. The semen/medium from the first volume is sieved through a 212 µm screen, which is then washed with the second and the third volume. The second cauda is treated in the same way. In total we now have approximately 80 ml of semen in freezing medium. Sperm concentration is estimated turbidimetrically with an adapted calibration curve and sperm motility is assessed microscopically. The semen is then diluted with freezing medium to 400 x 10<sup>6</sup> sperm/ml.

#### 3.2 Procedures of semen handling and freezing

##### The Netherlands

- Single step procedure.
- Medium: Tris-egg yolk freezing medium. The medium is prepared from medium concentrate, from Gibco BRL Life technologies, Breda, The Netherlands, and pasteurised egg yolk from Eiproma, Wormerveer, The Netherlands. One litre of medium contains 0.200 mol (24.22 g) Tris(hydroxymethyl)aminomethane, 0.0644 mol (13,44 g) of citric acid.1H<sub>2</sub>O, 0.0555 mol (10.0 g) of D- fructose, 0.05 g of Tylan, 0.25 g of Gentamycin sulphate, 0.676 g of 'Lincospectin 100' (lincomycin/spectinomycin), 200 ml of pasteurised egg yolk, and 0.766 mol (70.56 g) of glycerol = 5.6 % (v/v).
- First dilution (Single step, so first dilution = final dilution): 400×10<sup>6</sup> sperm/ml. The semen collection tube with semen is placed in a 30 °C water bath. Subsequent ejaculates of the same ram are pooled. Semen

concentration is measured turbidimetrically and the semen is then extended. Then, the percentage motile sperm is estimated microscopically.

- Cooling: The tubes are placed in an open rack in a 5 °C thermostat cool box, which has low-intensity forced ventilation. The effective cooling rate of the semen was  $\pm 0.2$  °C/min. When all the rams are done, the tubes are transported from the barn to the laboratory. Total holding (including the time during cooling and after reaching 5 °C) is approximately 1-2h.
- Package: 0.5-ml straws (IMV)
- Identification on the straws: Ink-jet straw printing: Breed; Country code (528); Farm number (UBN); Animal ID code; Date; CGN.
- Freezing: Straws are filled, sealed, and placed on racks (30-40 straws/rack) and placed in a nitrogen vapour freezer with forced ventilated nitrogen vapour at -80 °C during 10 minutes (average effective cooling rate inside the straws over the range +5 °C to -60 °C is approximately 30 °C/min. Maximum cooling rate after dissipation of heat of fusion = approximately 60 °C/min), then plunged in LN<sub>2</sub> and transferred to storage tanks.
- As described in paragraph 3.1., epididymal semen is collected from the caudae directly into freezing medium with glycerol at 15 °C, and is then diluted with the medium to  $400 \times 10^6$  sperm/ml. The semen is then placed at 5 °C. Other procedures are identical to those for ejaculated semen.
- Post-thaw quality control: From every ejaculate, or epididymal semen sample, one straw is thawed and % motile sperm is assessed microscopically. In case of apparent poor quality, a second straw is done. We do not discard the semen but will note the quality in our Cryo Information System (Cryo IS) database

### France

- Multi step freezing procedure
- Medium: 1<sup>st</sup> extender: lactose + pasteurised egg yolk, 2<sup>nd</sup> : skim milk + 4 % (v/v) glycerol.
- First dilution: 1:1. So sperm concentration is half the initial sperm concentration. The semen is then diluted further with that same medium to  $667 \times 10^6$  sperm/ml.
- Cooling: 10 minutes after collection the tube is placed in a glass full of water and put in a 4 °C refrigerator during 2h20min.
- Addition of glycerol medium: 0.6 volume of diluted semen receives 2 x 0.2 volume of skim milk freezing medium with 10% glycerol with 10 minute interval to reach  $400 \times 10^6$  sperm/ml and 4% glycerol.
- Continued holding for 90 minutes
- Package: Semen is packed in 0.25-ml straws (IMV).
- Identification on the straws: Collection centre, Ram ID and Breed.
- Freezing: 0.25-ml straws in static vapour of  $\pm 75$  °C. The cooling rate may be relatively slow.
- Post-thaw quality control: The semen is put at 38°C and after 2 hours the number of live spermatozoa and their motility are estimated. The semen is rejected if less than 10% of the spermatozoa are alive.

### Greece

- Single step and multi-step procedures according to the extender.
- Medium: Egg-yolk based extender (home-made), milk based (home-made), soybean lecithin-based extender (commercial medium).
- First dilution: (single step  $\rightarrow$  first dilution = final). For laparoscopic Insemination:  $100 \times 10^6$  spermatozoa. For cervical insemination :  $800 \times 10^6$  spermatozoa/ml (=  $200 \times 10^6$  per insemination).
- Cooling: The semen after dilution is stored at a cold cabinet for a 2 hrs cooling period at a rate of 0.5°C/min.
- Addition of glycerol medium: When a multi step procedure is used, glycerol is added at the temperature of 4°C, and the semen is subsequently held for 1 hr at 4°C.
- Package: Straws of 0.25 and 0.5 ml.
- Identification on the straws: Name of the ram, Date of collection.
- Straws are cooled slowly (5 °C/min) to -25 °C. Below -25 °C the cooling rate is higher (-50°C/min) until -130 °C. The straws are then plunged into LN<sub>2</sub>.
- Post-thaw quality control: Sperm motility (CASA), evaluation of membrane integrity, mitochondrial membrane potential, capacitation status, genomic integrity, sperm-oocyte interaction and ultra-structure with Electron Microscopy.

## UK

- Single step procedure.
- Medium: Commercial Sheep medium from IMV+ 20% egg yolk, or Triladyl.
- Sperm concentration after extending (= final sperm concentration):  $400 \times 10^6$  sperm/ml).
- Cooling: The extended semen at 30 °C is cooled to 4°C during 2 hours and subsequently held at 4 °C for another hour.
- Package: Semen is packed in 0.25-ml straws (IMV) (in 2001, for the Heritage gene bank, 0.22-ml pellets with  $220 \times 10^6$  sperm had been used).
- Identification on the straws: Ram no., Ram name, Breed, Date, Collection centre.
- Freezing: Digitcool programme by IMV: first ramp 10 °C/min to -10 °C, followed by a very fast ramp of -80 °C/min till -55 °C. Further cooling till -140 is a bit slower, then plunging into LN2. Probably ice formation starts somewhere between -11 and -16 °C, then it takes 20 seconds for the ice to grow along the length of the straw. This means that the cooling rate inside the straw after dissipation of heat of fusion will probably be even higher than 80 °C/min and part of the straw may become severely supercooled.
- Post-thaw quality control: Progressive motility assessed visually by experienced technician using contrast TV monitor and heated stage.

For pellets, the semen is diluted to  $1,000 \times 10^6$ /ml. Pellets are meant to be 0.22 ml but may be larger pellets are frozen on dry ice. They like it and think it is better than straws and less variation.

### 3.3 Veterinary/Sanitary issues

1) Do you follow EU semen regulations?

#### The Netherlands

No. We do not have EU certified sheep AI stations. Furthermore, we have predominantly used epididymal semen from testes obtained from slaughterhouses.

#### France

Yes, the EU semen regulations were transcribed in the French Legislation.

#### Greece

Yes

## UK

There is no legislation apart from that covering intra-community trade.

Under the Balai Directive. See:

[http://www.defra.gov.uk/animalh/int-trde/imports/iins/livebalai/bal\\_live\\_1.htm](http://www.defra.gov.uk/animalh/int-trde/imports/iins/livebalai/bal_live_1.htm)

[http://www.defra.gov.uk/animalh/int-trde/imports/iins/livebalai/bal\\_live\\_7.htm](http://www.defra.gov.uk/animalh/int-trde/imports/iins/livebalai/bal_live_7.htm)

Statutory Instrument 1993 No. 3248 The Artificial Breeding of Sheep and Goats Regulations 1993

2) Are there additional national regulations that you (need to) follow?

- **NL:** No
- **France:** No
- **Greece:** Yes

3) What diseases do you routinely check for before collection?

#### The Netherlands

- Maedi Visna
- Brucella infections (abortus, melitensis, ovis)
- Scrapie
- BTV (Blue Tongue) since 2007

**France**

- Brucellosis
- Contagious agalaxia (*Mycoplasma agalactiae*)
- Paratuberculosis
- Caseous lymphadenitis
- Epididymitis (*Brucella ovis*)
- Scrapie
- Pulmonary adenomatosis
- Maedi-visna

**Greece**

In Greece there are diseases that are checked for, no description.

**UK**

NSP semen archive: Routine blood test for Border Disease after the first collect has passed our post thaw test. This is the only test we do, if we have a ram in for private collection then we take an A. seminus test on the semen sample.

Brucella and Contagious Agalactiae do not occur in the UK.

For the bluetongue animals we can only take rams from the surveillance zone (rams from protection zone are not allowed to move for 2 yrs). With these animals we are taking a blood sample for bluetongue testing on the day of the last semen collection. There is a repeat test 21-60 days after this test.

**3.4 Documentation****Table 4** Data is recorded of the donor animal

	NL	France	Greece	UK
Breed	Yes	Yes	Yes	Yes
Date of Birth	No	Yes	Yes	No
Collection date	Yes	Yes	Yes	Yes
Identification number of donor animal	Yes	Yes	Yes	Yes
Breeder	Yes	Yes	Yes	Yes
Owner	Yes	Yes	Yes	Yes
Pedigree	No	Yes	Yes	No
Veterinary status	Yes	Yes	Yes	Yes
Location of the sample (storage)	Yes	Yes	Yes	Yes
Year of birth	Yes	?	?	Yes
Quality of semen (post thaw)	Yes	?	?	Yes
Number of cells/dosis	Yes	?	?	Yes
Colour of sheep	Yes	?	?	No
Other <sup>1</sup>		Yes		

<sup>1</sup> Status of breed or animal (rare breed, genetically original, 'representative animal')

**3.5 Insemination results**

Insemination results with the frozen-thawed HSB semen are obtained in the Netherlands, France, Greece and UK.

**The Netherlands**

The frozen-thawed sheep semen is normally not used in the Netherlands. Laparoscopic insemination is not allowed. The HSB semen in the CGN gene bank was frozen exclusively for the long term securing of the germ plasm of these breeds. One small-scale trial has been done with Veluwe Heath Sheep breed semen, used in cervical and laparoscopic insemination on synchronised Swifter ewes, comparing epididymal semen from 4 males and ejaculated semen from 4 (different) males.

## Synchronisation protocol:

Day 0. Progesterone sponge for 12 days

Day 10. Prostaglandins

Day 12. Sponges removed + eCG injection

Day 14. HCG + antibiotics (52 ± 1 hours after removal)

Day 14. Cervical AI after 57 ± 1 hours

Day 14. Laparoscopic AI after 59 ± 1 hours

**Table 5** Post-thaw semen quality and fertility results of frozen-thawed semen of the Veluwe Heath Sheep breed

	Ejaculated semen		Epididymal semen	
	% motile	% live	% motile	% live
	42.0 ± 4.5	48.5 ± 2.1	60 ± 0	62.3 ± 5.6
	pregnant	lambs/ewe	pregnant	lambs/ewe
Cervical	0/11	---	4/10	2.0
Laparoscopic	6/10	2.3	7/10	3.1

While the laparoscopic results were fine, and results of cervical inseminations with epididymal semen also seemed adequate, the results of cervical inseminations with ejaculated semen were of course disappointing (0/11 pregnant). In later insemination trials with frozen thawed ram semen from commercial breeds we had better results with ejaculated semen. Initially the results had been poor again (12% pregnancy) but in later years we obtained better results, up to approximately 30% pregnancies. Because of the initial poor results with ejaculated semen from commercial breeds we do not think that the poor results with ejaculated semen in Table 5 were due to the Veluwe breed. We think that it may reflect at least in part that we had little experience in semen handling, freezing, and insemination protocol, while we improved on these point in later years (2004-2006). Anyway, the above results show that pregnancies and lambing is possible with the Veluwe sheep semen in the CGN gene bank and that epididymal semen performs at least as good as (or better than) ejaculated semen.

**France**

The heat synchronisation is done with sponge. The method of insemination is always laparoscopic for the frozen semen (cervical for fresh semen).

In 2004, the fertility measured on all the breeds using frozen semen (almost 3,700 AI) was 63.3%.

**Greece**

The synchronisation is done with Progestagen pessaries 12 days and PMSG at sponge withdrawal. Laparoscopic inseminations at 52 – 60 hours gave pregnancy rate of 66%.

**UK**

Various Innovis

Cervical AI is not used with frozen semen unless natural (unsynchronised) oestrus, and then only with care.

For laparoscopic AI, ewes are synchronised with a Progestagen pessary 12 days and PMSG at sponge withdrawal.

Laparoscopic AI at 52 – 60 hours. Standard UK dose size is 100 million with min post thaw 30%.

Pregnancy/conception at 65% (approximately 10,000 inseminations/year).

## 4 Conclusions

### Organisation financing and continuation

Most HSB ex situ conservation efforts have been single campaigns, triggered by disease outbreaks (FMD Scrapie). This means that financial support may not be guaranteed for the future. In France until now, only three Basque breeds have been preserved. In Greece only one breed. The other breeds in other regions may still be at risk, as the breeds as a whole or the within-breed genetic diversity have no ex-situ back-up yet. Another issue is the updating of the gene bank collections in the future, and continuation of the storage of the present collections. In the UK it is undecided who will remain owner and will maintain the stocks in the future. Internationally, efforts to organise ex situ conservation under the responsibility of industry and other stake-holders proved to be only partially successful, and does not provide a secure situation especially for the smaller breeds. The loss of alleles is a virtually irreversible process. The alleles that may be lost from the live population due to selective breeding and the scrapie programmes will still be available in the ex situ gene banks. Maintaining these stocks may therefore be very valuable, and suits the commitments agreed in the convention on biodiversity (CBD) of Rio de Janeiro 1992. The costs of maintaining the present collections are not very high, for instance for the UK, DEFRA estimates that the costs for securing the heritage gene Bank and NSP semen archive for the next hundred years would be less than 2 million pounds.

### Restrictions following from EU- or national regulations

On farm collection of ejaculated semen and collection of epididymal semen is banned in some countries (e.g. France), as it does not comply with EU regulations. However, in a strict sense, these regulations pertain to semen that is to be exported to other (EU) countries. In the Netherlands, the use of laparoscopic inseminations is forbidden for animal welfare reasons. The question raised during the May 2008 HSB workshop is whether the HSB consortium should advocate that for the specific use of safeguarding and possible future retrieval of genetic diversity, there should be derogations to these restrictions, as these measures have a large impact on the efficacy and cost-efficiency of ex situ conservation.

### Sperm dosage

The issue of needed sperm dose per insemination has been addressed in the discussions with experts and the May 2008 Workshop. There seems to be little scientific evidence to support the presently used sperm dosages for laparoscopic and cervical inseminations, respectively. If we could use less sperm per dose this would enhance the cost-efficiency of ex situ conservation. However, we will not have the capacity within this project to do the necessary research to support a change from presently accepted procedures.

### Freezing procedure

Semen freezing procedures may vary per country, without clear preference for a particular freezing medium or freezing procedure. Freezing media contain egg yolk and glycerol as cryoprotectants. The scientific literature is very unclear on which medium or procedure is best, largely because of confounding factors that hamper comparisons of media and procedures between or within studies.

### Recommendations for WP4

In WP4 the partners will make new stocks of frozen semen from two breeds per country. It makes sense to use this as an opportunity to compare and exchange methods and expertise. If it can be organised a split sample comparison of freezing media/methods could perhaps be done combined with exchange of researchers/personnel. The comparison of methods can then be done by in vitro assessment of post-thaw semen quality. Partners will not try to do an insemination trial, as this is too costly and logistically very difficult. While the details of any experiment still have to be considered, emphasis could be on differences between methods employed in the various countries, and more generally on factors that are expected to affect either the post-thaw sperm quality, or the ease of the procedure. Important factors may be the initial handling, dilution and cooling of the semen, the semen holding time, and the actual freezing rate, or freezing programme. As to the medium, a comparison could be done of *Milk-lactose-egg yolk* versus *IMV-egg yolk* or *Tris-egg yolk*.

## Appendices

### Appendix 1 Other organisations in France that keep semen

Organisation	Address	Telephone	Breeds of the rams (number of doses in stock)
CIA Verdilly stverdilly@wanadoo.fr (AI centre)	Verdilly 02400 CHATEAU THIERRY  M. LEMAIRE (Chief Centre)	03.23.69.15.94 Fax : 03.23.83.33.62	Ile de France (11 266) Berrichon du Cher (10332) Hamsphire (535) Est à Laine Mérinos 6 027) Boulonnaise
GEN'OSE (organisation for genetic selection) /ADEO(AI centre)  genose@wanadoo.fr	17 allée des Genêts 04200 SISTERON Siège social : 8ter, rue du Capitaine Bresson 05000 GAP M. PINEAUT (Director) M. TRINQUIER	04.92.61 55 63 Fax : 04 92 61 19 37  04.92.52.53.00 Fax : 04.92.52.53.09	Préalpes du Sud Ile de France, Mérinos d'Arles
Confédération Roquefort (organisation for genetic selection)  confederation- roquefort.cia@ wanadoo.fr confederation-roquefort. elevage@roquefort.fr	Le Bourguet Vabre l'Abbaye 12400 SAINT-AFFRIQUE Siège social : Confédération de Roquefort - BP 348 36, avenue de la République  12103 MILLAU CEDEX M. BRIOIS (Dir.)	05.65.98.10.80  05.65.59.22.00 Fax : 05.65.60.28.58	Lacaune lait Lacaune viande Charollais Rouge de l'ouest Suffolk
CIA OVI-TEST (AI centre)  unotec@unotec.net	La Glène 12780 SAINT LEONS Siège social : OVITEST Les Balquières Route d'Espalion 12850 ONET LE CHATEAU M. BELLOC (Director) M. ALBARET M. GIROU (Chief Centre)	05.65.61.86.22  05.65.67.89.40 Fax : 05.65.67.89.48	Lacaune lait (14 180) Lacaune viande Berrichon du Cher (6 393) Charollais Rouge de l'Ouest (6 298) Suffolk
CORSIA uprabrebiscorse@ worldonline.fr (AI centre)	Domaine de Casabianda 20270 ALERIA Ph. TEINTURIER (administration) S. HARLAUX (Preparation)	04.95.57.10.91 Fax : 04.95.57.14.48	Corse
CRIOPYC criopyc@caramail.com (AI centre)	Route de Langlade 31450 POMPERTUZAT M. BELLIURE Director CIA M. RICHARD (Preparation)	05.61.81.75.88 Fax :	Berrichon du Cher INRA 401 Tarasconnaise

Organisation	Address	Telephone	Breeds of the rams (number of doses in stock)
CIA (AI centre)- GIE U.S. R.O.M. (organisation for genetic selection) giebmc@wanadoo.fr	Paysat Bas  Mazeyrat d'Allier 43300 LANGEAC Siège social : IMACO Route de Thiers - BP 13 63370 LEMPDES M. PERRIN (Director) M. BOYER (Chief Centre)	04.71.77.14.14  Fax : 04.71.77.08.02  04.73.92.74.07 Fax : 04.73.92.76.87	Blanc Massif Central  Rava Limousine
UPRA C.D.L. (organisation for genetic selection)	46240 Fontanes du Causse Siège social : Chambre d'Agriculture BP 199 - 46004 CAHORS CEDEX M. ISSALY H. M. CARRON (Preparation)	05.65.31.12.05 05.65.23.22.00 Fax : 05.65.23.22.19	Causses du Lot
GENESIA genesia@gr-agena.com (AI centre)	BP 47 - Le Suquet 63370 LEMPDES M. LACROIX (Dir. CIA) M. AUBERT (Preparation) ; M. BESSEAS	04.73.42.17.16 Fax : 04.73.91.35.60	Charollais, Ile de France
CIOP cdeo.ordiarp@ wanadoo.fr (AI centre)	CDEO – Quartier Ahetzia 64130 ORDIARP  M. SOULAS (Dir. ) M. CACHENAUT (Chief Centre) M. FIDELE (Chief Centre)	05.59.28.05.87 Fax :  05.59.28.19.90	Basco-Béarnaise Manech Tête Noire  Manech Tête Rousse Berrichon du Cher Charollais Suffolk
INSEM-OVIN insemovin@ wanadoo.fr  (AI centre and organisation for genetic selection)	Maison Neuve ; 11 allée du Breuil 87430 VERNEUIL/VIENNE  Siège social : idem Siège adm. : Toutedjoie 86500 MONTMORILLON  M. KUPPEL (Dir.) M. FERNANDEZ (Chief Centre)	05.55.00.14.62 Fax :  05.55.00.12.04  05.49.83.30.46	Berrichon du Cher Charollais (11 031) Charmoise (1 850) Ile de France Rouge de l'Ouest (5 029) Suffolk (6 007) Texel (8 873) Vendéen (8 702)
CECNA cecna@ucacig.fr (AI centre)	3 rue Jules Rimet 89400 MIGENNES  M. MONTIGNY (Chief Centre) Mlle JARLOT ; M. BOURGEOIS	03.86.73.22.51 Fax : 03.86.73.22.07	Charollais Ile de France

Gilles Lagriffoul and Jérôme Raoul are involved in the general management of sheep AI for the “Institut de l'Élevage”.

## Appendix 2 Approval of centres and teams

1. The appropriate Minister, upon being satisfied that a semen collection centre complies with the provisions of Council Directive 92/65/EEC<sup>21</sup> laying down animal health requirements governing trade in and imports into the Community of animals, semen, ova and embryos not subject to animal health requirements laid down in specific Community rules referred to in Annex A(1) to Directive 90/425/EEC (in particular Chapters I, IIB and C and III of Annex D) (so far as it relates to ovine and caprine semen), shall, for the purposes of export of ovine and caprine semen to another member State, approve the semen collection centre.
2. The appropriate Minister, upon being satisfied that a collection team is capable of complying with the provisions of Directive 92/65/EEC in relation to the collection of ova and embryos of sheep and goats may approve that team for the purposes of these Regulations.

And

### Intra Community trade

- 1 No person shall collect, process, store or transport for the purpose of export to another member State any ovine or caprine semen, ovum or embryo unless it meets the conditions laid down in Article 11 of Directive 92/65/EEC.
- 2 No person shall use semen for the insemination of any sheep or goat for the purpose of production of ova or embryos for export to another member State unless
  - (a) the sheep or goat meets the conditions laid down in Chapter IV of Annex D to Council Directive 92/65/EEC, and
  - (b) the semen meets the conditions laid down in Article 11(2) of that directive.
- 3 No person shall collect any ovum or embryo from any sheep or goat for the purpose of export to another member State unless he is a member of a team approved under Regulation 2(2) above.

For approved training centres see:

<http://circa.europa.eu/irc/sanco/vets/info/data/semen/ms-sc-ov-cp.html>

## Appendix 3 More details of the freezing procedures

### A3.1 UK

#### The UK procedure

Innovis use medium from IMV, and the ovine freezing curve supplied by IMV for the IMV digit cool. They have no rationale other than that they are also distributor for IMV products, and have good/adequate results with it.

Innovis use single step for practical reasons. The semen is diluted 5-10 times in total. This means that the glycerol (and other components) concentration may vary between 80-90% of that in the medium, but that may be quite acceptable.

Innovis use Red Ovine Freezing Buffer (IMV, France). This is a clear synthetic diluent, to which they add 20% egg yolk (10 ml of egg yolk to a 40-ml bottle). On the basis of the final osmolality it is assumed that the final medium contains approximately 5% glycerol. The NSP semen was done with a 'concentrate' (add 3 volumes of water + one volume of egg yolk to one volume of concentrate).

They place the raw semen in a 30 °C bath, then do sperm concentration measurement with IMV's Accucell. This apparatus tells them how much medium they have to add to the semen to dilute to  $400 \times 10^6$  sperm/ml (this means total dilution 5-10 times). The semen is then placed in a large wide beaker with water and this is then placed in a refrigerator at  $\pm 4$  °C.

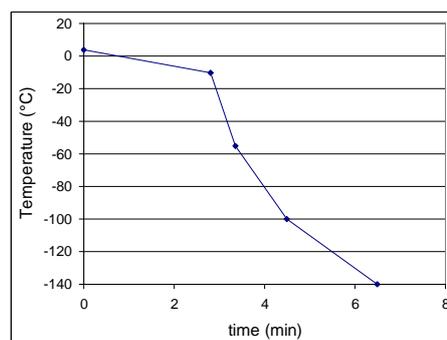
Cooling is said to take 90 minutes, but after that the semen is held another 90 minutes. This may be even longer, maybe also shorter, depending on how busy they are. As many other things, it seems that also the total holding time is not a real 'law'.

#### The freezing curve

The cooling rate between -10 and -55 is quite high!. Probably ice formation starts somewhere between -11 and -16 °C, then it takes 20 seconds for the ice to grow along the length of the straw. This means that the cooling rate inside the straw after dissipation of heat of fusion will probably be even higher than 80 °C/min and part of the straw may become severely supercooled.

For pellets, the semen is diluted to  $800-1,000 \times 10^6$ /ml. Pellets are meant to be 0.22 ml but I saw one that seemed to be larger, perhaps 0.5 ml. Pellets are frozen on dry ice. They like it and think it is better than straws and less variation.

	Time (min)	T start (°C)	T end (°C)	Rate (°C/min)
Ramp 1	0	4	-10	-5
Ramp 2	2.80	-10	-55	-80
Ramp 3	3.36	-55	-100	-40
Ramp 4	4.49	-100	-140	-20
End	6.49	-140	plunge	



### A3.2 France

Below is according to a manual written for the FAO

I think that according to Delphine's info the only thing different is that semen is frozen at  $400 \times 10^6$  sperm/ml.

#### ÉTUDE FAO PRODUCTION ET SANTÉ ANIMALES 83

Manuel de formation pour l'insémination artificielle chez les ovins et les caprins

par

G. Baril, P. Chemineau, Y. Cognie, Y. Guérin, B. Leboeuf, P. Orgeur et J.-C. Vallet

Station de la physiologie de la reproduction

Institut national de la recherche agronomique (INRA)

Nouzilly, 37380 Monnaie, France

FAO Organisation des Nations Unies pour l'alimentation et l'agriculture  
Rome, 1993

Chapitre 4. Collecte et conservation de la semence  
<http://www.fao.org/docrep/009/t0121f/T0121F06.htm#tab.14>

While fresh semen is prediluted, diluted, and stored chilled in an isotonic skim milk solution, for freezing, predilution and first dilution are done with a lactose egg yolk medium. This is followed by a glycerolated milk medium. The milk medium is made like the extender for fresh semen but with more milk powder (hypertonic).

### Freezing media

Medium 1: Lactose egg yolk

10.3 gram of lactose (monohydrate?) plus 100 ml of water ( $\rightarrow \pm 282 \text{ mM} = 311 \text{ mOsm/kg}$ )  
80% of this solution plus 20 ml of egg yolk

Medium 2: Hypertonic milk plus glycerol. It is described to make first the recipe according to the fresh semen milk extender, then add 4.0 gram of extra milk powder. This isn't entirely clear. The 'extra' milk powder should probably also have to be heated 'au bain Marie' as is described for the recipe for the 'fresh extender' So it is probably best that the 'extra' 4 gram of milk powder is added together with the 11.1 gram of the fresh extender recipe. So the recipe then will be:

Medium 2: Hypertonic milk plus glycerol

100 ml of water + 0.33 gram of sulfamides (see below) plus 14.1 gram of skim milk powder  
15 minutes in bain-Marie  
Cool down, Add Pen-strep  
Adjust pH with  $\text{Na}_3\text{citrate}$  to 6.6 - 6.7  
Add glycerol to 10% (v/v)

It says that the osmolality of the medium before adding glycerol will be 450 mOsm/kg water.

Sulfamides. It is not clear what that means but elsewhere I found the term Exoseptoplax, and again elsewhere this was described as **4-aminobenzenesulfonamide**, or  $\text{C}_6\text{H}_8\text{N}_2\text{O}_2\text{S}$ . Seems to be a preservative/disinfectant. 3 gram per litre would mean  $\pm 17 \text{ mM}$ . Perhaps strange that disinfectant and antibiotic are present in the second medium and not in the first. It may be that these agents are supposed to have a function after insemination, i.e. in the ewe?

### Procedure

- Within 30 sec after collection, the semen is prediluted with an equal volume of medium 1 (32°C)
- The sperm concentration and motility are determined.
- After 10 min the semen is diluted with medium 1 (28-30°C) to  $1500 \times 10^6/\text{ml}$
- Cool in a beaker of water placed at 6 °C
- At 2h10 0.6 volume of diluted semen receives 0.2 volume of Medium 2
- At 2h30 again 0.2 volume of Medium 2 is added
- At 4h Fill 0.5-ml straws
- Freeze over static LN2 vapour: Cooling rate inside the straw after dissipation of heat of fusion: approximately 7 °C/min. It says that at 8 min -50 is reached. It also says at -50 it is plunged in LN2.

Sperm concentration would thus be  $900 \times 10^6$  sperm/ml (according to the FAO manual)

But in the present work  **$400 \times 10^6$  sperm/ml** was used, so dilution with medium 1 should be to  $667 \times 10^6$  sperm/ml. Also in the present work 0.25-ml straws are used.

Final concentrations (assuming raw semen had  $4600 \times 10^6$  sperm/ml):

- $400 \times 10^6$  sperm/ml
- 4% (v/v) glycerol
- $\pm 10\%$  egg yolk
- 144 mM lactose, or 51% of isotonic lactose solution
- 36% hypertonic milk solution

The reason why the medium 2 (before adding glycerol) has a hypertonic osmolality (450 mOsm) is not specified. But after adding glycerol the osmols non permeable solutes per litre of medium (osmolarity) is lowered by 10%. The rationale of having a high osmolality may be to limit the reswelling of the sperm after initial shrinking and perhaps to prevent them from swelling to a volume higher than  $V_0$ . To 0.6 ml isotonic diluted semen one adds 0.36 ml of hypertonic medium + 0.04 ml of glycerol. The 0.6 ml of diluted semen + 0.36 ml of hypertonic medium would result to only slight hypertonicity ( $\pm 350$  mOsm/kg), which then would be just enough to make the cells reswell to exactly  $V_0$  after addition of 4% glycerol.

### **A3.3 The Netherlands**

In the research done in 2006, we used two step dilution. Actually one medium with and without glycerol. We added media without and with glycerol shortly after each other at 30 °C! So the only reason to do a two step is to make sure that the end concentration of glycerol is always 5%. The medium with glycerol is made slightly hypertonic (like that of) France to prevent swelling of the sperm above  $V_0$ . We could probably just as well have diluted with three volumes of 7.5% glycerol with even less elevated osmotic pressure. Our semen concentrations were relatively low ( $\pm 3,000 \times 10^6$  sperm/ml; 2,300 (Wijnker) – 3,670 (Utrecht)) and we used a final concentration of 400, so in total  $\pm 7.5$  times diluted

- Semen collected at 8:30 tot 10:30
- One volume of semen receives one volume medium without glycerol and then two volumes of medium with 10% glycerol.
- Cooled to 4 °C
- $\pm 3.30$  h after collections (13:00h), all semen pooled, diluted with medium with 5% glycerol to  $400 \times 10^6$ /ml.
- $\pm 5.30$ h after collection Semen frozen in 0.5-ml straws
- 0.5-ml straws are frozen in a strongly ventilated LN2 vapour freezer at a constant temperature of -95 °C. Alternatively we used a programmable freezer with a cooling rate after dissipation of heat of fusion of 50 °C/min

The long holding was because of all the work, the transport, semen evaluation (flow-cytometry). Not because we think long holding is necessary.

We compared three different recipes:

1. Tris-egg yolk according to Salomon and Maxwell (modified), 14% egg yolk
2. Milk medium according to Paulenz (modified), 5% egg yolk
3. Our own recipe, 14% egg yolk

All with 5% glycerol final concentration

The Post thaw % live and % motile was

- 1>3>2 (Wijnker)
- 1>3>2 (Bosgoed)
- 3>1>2 (Utrecht)

Post-thaw motile/live at Wijnker and Bosgoed were quite low, with less then 20% in medium 2.

Pregnancy rates in 37 ewes per group were similar (34, 30, 29% ( $\pm 15\%$ )). Numbers too low to see any difference.