Annex 8: Work package 3 report – Framework on ex-situ conservation

Heritage Sheep Breeds, WP3

In addition to reporting against WP3, this Annex also delivers against a WP4 requirement to set out the optimal ex-situ conservation methods in each country, deliverable 4.2. It is therefore referred to in Section 2.4 of the main report.

1. 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 then 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 100×10^6 sperm. Collections are generally performed with three days intervals. In the Netherlands, laparoscopic inseminations are forbidden. Therefore NL have generally frozen doses of 200×10^6 sperm/dose. The UK (NSP) have 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 NL it was seen that some Heath sheep breeds simply were to 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 NL 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 200×10^6 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 €200 per ram (100 doses of 200×10^6 sperm per dose) (based on ASG contract research tariffs). 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.

Summarizing remarks for the framework based on section 1 above
A number of factors influence cost efficiency: (1) Collection and cryopreservation of epididymal semen, instead of ejaculate semen, offers huge advantages. (2) The availability of a centre that is doing housing of rams and collection and freezing of semen on a routine and commercial basis may improve cost-efficiency of HSB *ex situ* conservation campaigns. (3) In addition, the number of collections needed to have sufficient number of doses stored is of course of importance. This is dependent on whether or not the possibility to perform laparoscopic inseminations in a country is present.

Apart from mentioning these factors, there were limited possibilities to offer a unified strategy for all HSB partners due to differences in possibilities and veterinary regulations, for instance with regard to collection of semen outside an official AI station, use of epididymal semen, and the use of laparoscopic insemination. With regard to these factors or regulations, the various EU countries widely differed.

2. Procedures used and Semen quality

2.1. 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 4.3.

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 coolbox 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 volumes 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 10^6 sperm/ml.

2.2 Optimal ex-situ conservation strategies for each country: 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 pasteurized 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_2O, 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 pasteurized egg yolk, and 0.766 mol (70.56 g) of glycerol = 5.6 % (v/v).
- First dilution (Single step, so first dilution = final dilution): 400×10^6 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 ±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_2 and transferred to storage tanks.
- As described in paragraph 4.1., epididymal semen is collected from the caudae directly into freezing medium with glycerol at 15 °C, and is then diluted with the medium to 400×10^6 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: 1st extender: lactose + pasteurised egg yolk, 2nd : 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 x 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 400×10^6 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 ±−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 → first dilution = final). For laparoscopic Insemination: $100\text{x}10^6$ spermatozoa. For cervical insemination : $800\text{x}10^6$ spermatozoa/ml (= 200 $\text{x}10^6$ 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_2.
- 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): 400×10^6 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 220×10^6 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 $1000\text{ x }10^6$/ml. Pellets are meant to be 0.22 ml but maybe larger pellets are frozen on dry ice. They like it and think it is better than straws and less variation.

Summarizing remarks for the framework based on section 2 above.

As said, countries differ in the ability to use epididymal semen. Therefore, the methods used for epididymal semen only apply to some countries. As to the methods used for ejaculated semen, the methods for collection and freezing are similar. But in the details of the used semen freezing medium and

the exact steps of the freezing protocol there appear to be some differences. The methods used in UK and NL seem most similar. The methods used in France are somewhat different. This method might be less practical as it involves more then one freezing medium (two-step system). It is therefore recommended to all use one medium and corresponding freezing protocol.

At the same time, however, it was concluded that the locally applied methods all seem to give similar adequate results in terms of post-thaw sperm function. A change of medium or methods could only become acceptable if a split-sample experiment, preferably executed in collaboration with a number of partners, would show that one method would be preferred over the other methods.

As said, we have looked at the possibility to perform such comparisons in the framework of WP4 but this could not be done for practical reasons. Results from tests done in NL showed that the media used in the UK and in NL are virtually identical in practical ease of use and in terms of post-thaw semen quality (reported in the WP4-report).

On the basis of the survey in the four EU partners UK, France, NL and Greece, a number of recommendations were given in chapter 4 of the WP3 report and below.

3. Conclusion and recommendations

Organisation; Financing and Continuation.
Most HSB *ex situ* conservation efforts have been single campaigns, triggered by disease outbreaks (FMD Scrapie). This mean 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*.