Animal Genetic Resources Conservation in The Netherlands and Europe: Poultry Perspective[1]

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ABSTRACT Increased global use of highly productive breeds of farm animals has been coupled to loss of genetic diversity in most species. In European countries, various governmental, non-governmental, and private organizations try to preserve genetic diversity of livestock in situ (e.g., by stimulating the use of indigenous, rare breeds by farmers; in nature reserves; or in noncommercial farms). In the case of poultry, maintaining in situ populations of the noncommercial (fancy) breeds largely relies on hobby farmers. In addition to in situ conservation, gene banks are being established for ex situ conservation. In at least 2 countries, France and The Netherlands, there are limited collections of frozen semen of rare poultry breeds. Since 2003, the CGN has started with a more systematic effort to collect, freeze, and store semen of indigenous Dutch poultry breeds. At present, the CGN gene bank contains semen of 11 Dutch rare poultry breeds. Also, CGN has performed research on the methodology for cryopreservation of fowl semen. This recent work was focused on finding a suitable replacement for glycerol, which is contraceptive in the hen, as a cryoprotectant. For reasons of hygiene and sample identification, we favored straw freezing, as opposed to the highly effective pellet freezing method. A significant interaction was found between cooling rate and cryoprotectant concentration. Best post-thaw sperm quality was obtained when combining 0.6 mol of dimethylacetamide/L with a cooling rate of ± 200°C/min. Inseminations twice per week with 0.3 billion sperm per insemination resulted in 97 and 88% fertilized eggs with fresh and frozen semen, respectively. In 2005, CGN has used this straw freezing method to extend the collection of poultry semen in the Dutch gene bank.

Key words: farm animal genetic diversity, poultry, Europe, cryopreservation, semen

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INTRODUCTION

Animal production has significantly increased during the last couple of decades. Developments in reproductive technology, application of modern genetic tools in breeding programs, and improved global logistics have enabled rapid genetic progress in production traits. However, the downside is that increased global use of highly productive breeds has been coupled to loss of genetic diversity in most species of farm animals.

Both genetic diversity within breeds and genetic diversity between breeds are under pressure. Original indigenous breeds are often replaced by globally used high productive breeds. The less popular breeds are often maintained only locally and in small populations. Consequently, these breeds are at risk for becoming extinct or may suffer from inbreeding and genetic drift.

Although the commercial breeds are represented in large numbers of animals, the genetic diversity of these breeds, or the so-called effective population size, may also be quite small, because a small number of sires are selected to have a multitude of progeny.

The decline in the genetic diversity of farm animal genetic resources (**AnGR**) is now widely recognized. Many countries have signed the Convention on Biological Diversity (CBD, 1992) and have since established policies toward conservation and sustainable use of animal and plant genetic resources. European countries have also recently issued a national strategic policy report on AnGR in the framework of the Food and Agricultural Organization of the United Nations (FAO) State of the World's Animal Genetic Resources process. These reports provide current data and developments and trends in national animal production as well as data on the breeds that are being used in that country and the trends in animal genetic diversity. Also, these reports provide details on national policy, stakeholders, organizations, and specific priorities and actions with regard to conservation, development, and use of AnGR.

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In this paper, we briefly discuss Dutch and European policies and strategies regarding AnGR conservation (ex situ and in situ) with a focus on poultry. Second, we address in more detail our recent research on cryopreservation of poultry semen.

CONSERVATION OF AnGR

There are several reasons for conservation of genetic diversity in farm animals. One reason for maintaining rare or local breeds is because these breeds may fulfill specific requirements with respect to local terrain or climate or may produce typical regional products. Also, local breeds are viewed as cultural heritage. However, with respect to the more widely used breeds, it is necessary to preserve genetic diversity. Generally, inbreeding is associated with increased frequency of heritable diseases, malformations, or dysfunctions. Furthermore, we need genetic diversity as a toolbox for continued breeding. This is especially true in the situation where future breeding goals are different from those of today.

European Strategies and Policy Matters

General. The European Union (**EU**), individual EU member states, and subregions within Europe (e.g., the Nordic countries) have developed policies in the area of conservation and sustainable use of AnGR. The Nordic Council of Ministers, representing Finland, Sweden, Norway, Denmark, and Iceland, established the Nordic Gene Bank for Farm Animals as a permanent agency to promote conservation of rare breeds of farm animals in the Nordic countries. In addition, in several European countries nongovernmental organizations (**NGO**) have become very active in conservation of AnGR.

Efforts to conserve genetic diversity of farm animals include measures to stimulate use of indigenous, rare breeds by farmers, in nature reserves for landscape management purposes, or in noncommercial farms (children farms, care farms, hobby farming). In addition to in vivo (in situ or ex situ) conservation, a number of countries have set up in vitro gene banks to preserve germplasm of rare breeds as well as the more widely used commercial breeds (ex situ conservation). As for poultry, there are only limited stocks of frozen semen in European gene banks.

European Union regulations provide a framework for national support actions and subsidies to conserve rare breeds. Most of the European countries stimulate conservation and sustainable use of rare breeds. One way to achieve this is that farmers may receive subsidies for rearing animals of rare breeds. Subsidy measures are possible under the EU rural development regulation (EU 1257/99).

Between European countries, there can be important differences in the strategies or approach toward conservation of genetic diversity. For instance, the government of The Netherlands is highly in favor of a market-driven approach for in vivo or in situ conservation of live animals of rare breeds of Dutch origin, such that these breeds will no longer be endangered, without using structural subsidy measures. However, in other countries, more weight is put on conservation of AnGR in combination with specific support measures (subsidies) within the framework of the EU rural development regulation. In addition, the extent to which subsidy measures are used also varies between animal species. Although it is allowed to support the in vivo or in situ conservation of rare poultry breeds under the EU rural development regulation, relatively little emphasis is put on financial support for poultry compared with other (larger) species such as cattle, horses, or sheep. Partly, this is due to the fact that the genetic difference between the mainstream commercial poultry breeds and the rare and fancy poultry breeds is so big that the rare breeds will not be considered for commercial egg or broiler production, with or without subsidy. Also, the fact that hobby poultry breeders want to maintain rare and fancy breeds just as a hobby will be little affected by subsidy measures. On the other hand, appropriate subsidy measures can help commercial cattle farmers to experiment with rare cattle breeds on a commercial basis, for example.

There is a tendency that the EU will become even more important in setting a framework for measures to promote conservation and sustainable use of farm AnGR. For the new member states of the EU, European policies and legislation also become relevant. There is an increased interest in sharing information and research cooperation on a supranational level. One initiative in this respect is that the European National Coordinators for AnGR have set up the European Regional Focal Point intended as a forum for exchange of knowledge, ideas, and expertise on AnGR conservation and management. Also, through the European Regional Focal Point, the National Coordinators can influence policies regarding conservation of genetic diversity on a supranational (European) level.

National Agencies. Several European countries have instituted or are planning to establish national organizations for the conservation and sustainable use of plant and AnGR. In The Netherlands, the Center for Genetic Resources, The Netherlands (**CGN**) was established for this purpose (Table 1). The aims of CGN and similar organizations with respect to AnGR may include the following.

- Monitoring and documentation of indigenous, locally adapted, and foreign breeds on a national level
- Stimulate, coordinate, and support actions of NGO and private sector in conservation and sustainable use of AnGR
- Increase public awareness regarding the importance of conservation and sustainable use of farm AnGR
- To give advice to (national) governments on policies related to AnGR conservation and use
- Establishment and management of in situ and ex situ collections of farm AnGR
- Advice on genetic management of small populations
- Perform, instigate, or coordinate research with respect to conservation and sustainable use of AnGR (i.e., genetics and cryobiology)

Table 1. Present Dutch gene bank collections for various livestock species

	Breeds per lines	Males per breed	Doses per breed
	(n)		
Cattle	8	2 to 2,000	400 to 50,000
Sheep	5	12 to 27	1,100 to 3,000
Pig	16	6 to 34	200 to 800
Poultry	11	10	500 to 700
Horse	3	4 to 8	60 to 100

NGO. There are various NGO taking responsibility for the conservation of rare breeds (e.g., rare breed trusts, herd books, breeding associations, and other unions of livestock keepers or hobby farmers). These organizations play a very important role in breed conservation and development, monitoring, and stimulation of sustainable use and in situ conservation, as well as stimulation of public awareness. Some organizations and societies actually involve the citizen and ask for donations for funding of conservation efforts for specific rare breeds.

Hobby breeders and farmers and commercial farmers who want to keep rare breeds are very important for maintaining live populations of rare breeds. Also organizations such as children farms and nature and landscape organizations contribute to the conservation of genetic diversity, both by raising public awareness and by maintaining populations of rare breeds. An example is that Aurochs-type cattle (Heck cattle) and Scottish Highland cattle or Konik horses, as well as heath sheep breeds, are used in nature reserves in which grazing is necessary to maintain open and varied grassland with a high species diversity. For rare (fancy) poultry breeds, hobby breeders are most important for conservation of specific varieties or breeds.

Poultry Genetic Diversity

Originally, each country had its own domestic poultry breeding using both native and foreign breeds. In the last decades, commercial poultry production has completely come in the hands of a few international breeding companies for chicken and turkeys. Governmental organizations or NGO involved in conservation of genetic diversity have little or no influence on the breeding and genetic diversity policy of these commercial breeding companies.

Apart from the commercial breeding of poultry, the various countries have large numbers of noncommercial breeds, which may be indigenous, locally adapted, or foreign breeds. Because these breeds are not used for large- or medium-scale production, for some indigenous breeds, the number of animals is low. The Dutch country report on AnGR (LNV, 2002) lists 32 rare poultry breeds or varieties of which 28 breeds had the status "endangered" or "critical." Maintaining in situ populations of the noncommercial (fancy) breeds largely relies on part-time, leisure-time, and hobby breeders and farmers.

Presently, in The Netherlands, actions are being taken to increase the number of rare poultry breeds that are safeguarded in gene bank stocks. The present methodology for cryopreservation of fowl semen is considered to be suboptimal for efficient safeguarding and future use of frozen semen stocks. Therefore, research is ongoing to improve these methods.

Gene Banking: Ex Situ Conservation

Purposes of Ex Situ Genetic Resources. Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Mazur 1985) but probably much longer. This means that we can preserve the present wealth of genetic diversity in long-time storage in a biological "safe deposit vault." A germplasm repository may serve a number of different purposes (Woelders et al., 2004). One purpose of a germplasm repository is to provide the possibility of recreating breeds or breeding lines in case they are lost (as a consequence of a calamity). For example, culling measures that were taken to contain a recent outbreak of avian flu several years ago threatened to diminish or wipe out stocks of rare poultry breeds. If a breed would become extinct, re-creation of that breed through repeated backcrossing would be possible provided that germplasm from an adequate sample of animals is cryopreserved. Storage of germplasm for this purpose would typically be long-term storage without fre-

Table 2. Poultry breeds in the Dutch Center for Genetic gene bank collections

Poultry breed	Males	Ejaculates	Doses
		(n)	
Barnevelder	10	91	499
Drente Fowl	10	95	454
Dutch Bantam	19	185	342
Twente Fowl	10	96	614
Dutch Uilenbaard (Dutch Owlbeard)	11	113	839
Welsumer	10	93	647
Brabanter	9	151	1,059
Fries Hoen (Frisian Fowl)	12	181	722
Kraaikop (Breda Fowl)	11	157	992
Lakenvelder	8	122	742
Dutch Baardkuifhoen (Dutch Bearded Poland)	11	169	852
Total	121	1,453	7,762

Table 3. Results of insemination with fresh semen or semen frozen in straws or pellets[1]

Treatment	Fertilized eggs	Eggs with embryos	Decline in fertility after day[2]	Mean of last day fertilized egg[3]
	(%)			
Fresh semen in ASG[4] medium	96.6[a]	90.5[a]	12	16.4[a]
Frozen in straws in ASG medium with DMA[5]	87.6[b]	80.4[b]	9	12.7[b]
Frozen in straws in Lake's medium[6] with DMA	78.1[b]	68.9[b]	7	9.9[c]
Frozen in pellets in Lake's medium with DMA	85.9[b]	77.8[b]	8	12.3[b]

[1]Split-sample approach with 4 replicates with 3- or 4-d intervals. Mean results of 23 hens per group. Means or percentages within a column lacking a common superscript differ ($P < 0.05$).

[2]Number of days after the last insemination during which there was no appreciable decline in the percentage of fertilized eggs per group.

[3]Mean day number of the last fertilized egg per hen (the day of the last insemination was taken as d 0).

[4]ASG = Animal Sciences Group (Wageningen University, Lelystad, The Netherlands).

[5]DMA = dimethylacetamide.

[6]Lake's freezing medium (Lake, 1968).

quent use of the stored material and without the need for regular update of the collection.

A second way to make use of gene bank resources is to support in situ conservation. Frozen semen and embryos can be used to minimize inbreeding and genetic drift in small, managed populations, and the combination of live animals and cryopreserved germplasm can be a powerful tool in conservation of small populations (Meuwissen, 1999). Sonesson et al. (2002) proposed a scheme in which semen is collected from the first 2 generations and used alternatively on dams, allowing a reduction of the rate of inbreeding.

Additionally, gene bank resources may be used as a back-up in case genetic problems occur. A decrease in effective population size and the resulting high level of inbreeding can lead to an increased relative frequency of deleterious alleles that were not apparent in a larger population. This happens not only in rare breeds but also in large commercial breeds (e.g., when a very small number of sires is responsible for a very high number of offspring). In such cases, the effective gene pool size is still very small. Gene bank resources may be needed to remove deleterious genes from the population by introducing new genotypes (e.g., semen doses) from the original (larger) population.

A fourth important use of the cryopreserved genetic resources is to allow development of new lines or breeds or to quickly modify or reorient the evolution or selection of the population. For instance, storage of original or extreme genotypes can be of use to quickly modify or reorient the evolution or selection of a selected population. It has been suggested (Verrier et al., 2003) to store original and extreme genotypes identified as having extreme breeding values for specific traits, or that carry rare alleles, or that represent specific founders or pedigree lines.

Gene-Banking of Poultry Germplasm in Europe. There are very limited stocks of semen of rare poultry breeds in Europe. In most countries, the emphasis has been on in situ conservation, largely relying on the dedication of hobby breeders. In at least 2 countries, France and The Netherlands, there has been a systematic effort to

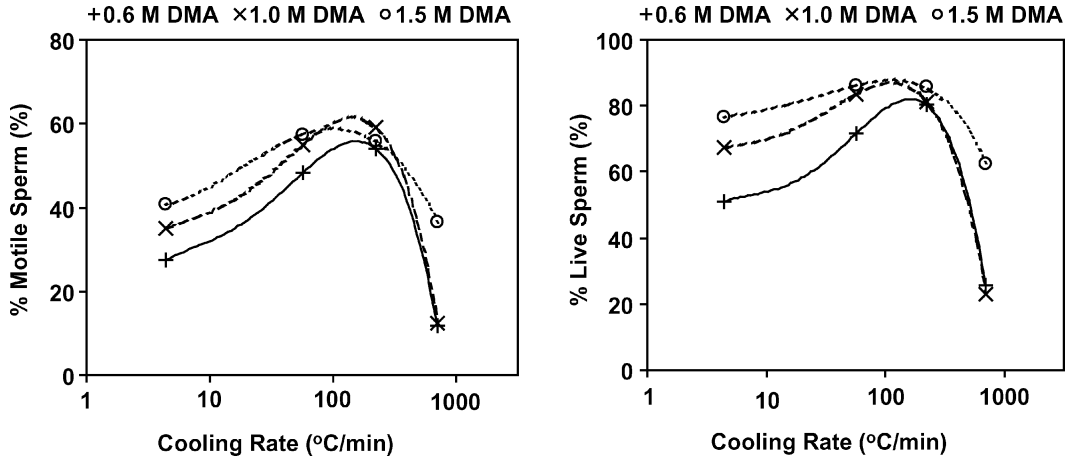

Figure 1. Post-thaw percentage of motile and live cock spermatozoa as function of cooling rate and dimethylacetamide (DMA) concentration. Three straws per combination were thawed and evaluated. The percentage motile sperm (left panel) was judged subjectively in wet mounts using phase-contrast microscopy. The percentage of live spermatozoa (right panel) was determined in wet mounts after staining by 4′-6-Diamidino-2-phenylindole (DAPI) and fixing the cells with glutaraldehyde. Two hundred cells were counted per sample.

collect, freeze, and store semen of rare indigenous breeds, albeit at present, the number of breeds of which semen stocks are available is still limited. In France, semen of 3 breeds is stored in the National Cryobank (Danchin-Burge, 2005). In The Netherlands, until 2003 only very small stocks of semen of a number of poultry breeds were kept by the Dutch CGN. Since 2003, CGN has started with a more systematic effort to collect, freeze, and store semen of indigenous Dutch poultry breeds. At present, the CGN gene bank contains semen of 11 Dutch rare poultry breeds, including semen of 10 males per breed and 50 to 100 insemination doses per male (Table 2).

RESEARCH ON CRYOPRESERVATION OF POULTRY SEMEN

In the case of birds, it is not possible to cryopreserve embryos or oocytes, largely because of their large size, the high lipid content, and the polar organization (vegetal and animal pole). Cryopreservation of isolated embryonic primordial germ cells could be an option. Frozen-thawed primordial germ cells can be injected into recipient embryos and populate the recipient gonads. The resulting chimeric embryos can be used to produce future progeny of the donor genotype (Song et al., 2005). Semen of various fowl species (chicken, goose, duck, turkey) can be cryopreserved successfully (Hiemstra et al., 2005). Because the collection, freezing, and use of semen (artificial insemination) are practical state-of-the-art methods, present poultry gene bank collections exclusively contain semen.

Semen-freezing techniques for chicken, turkey, goose, and duck render a fair post-thaw sperm survival of up to 60% live spermatozoa. Reasonable insemination results with frozen-thawed semen have been reported for the major poultry species (Hammerstedt and Graham, 1992; Blesbois and Labbé, 2003). However, there is a striking variation between studies in the reported percentages of fertilized eggs, as listed in Hammerstedt and Graham (1992), ranging from 9 to 91%. Moreover, the number of spermatozoa that gives maximal fertilization levels in chickens is much higher for frozen-thawed semen compared with fresh semen (Wishart, 1985).

Glycerol is widely used as a suitable cryoprotective agent (**CPA**) for semen in mammalian, bird, and fish species. However, in fowl, it was found that glycerol is a contraceptive, i.e., the semen must be washed free of glycerol after thawing (Hammerstedt and Graham, 1992). A number of alternative CPA have been tried for cryopreserving poultry semen, most notably dimethylsulfoxide (**DMSO**) (Bakst and Sexton, 1979; Van Voorst and Leenstra, 1995) and dimethylacetamide (**DMA**). Tselutin et al. (1999) and Chalah et al. (1999) have reached a very high level of fertility with cock semen frozen with DMA by a very rapid cooling technique that involves plunging 50-μL semen droplets straight into liquid nitrogen. However, Tselutin et al. (1999) obtained much lower fertility when the semen was frozen in straws in a programmable freezer, using the same extender and cryoprotectant solution, compared with the pellet freezing method (26.7 vs. 84.7%). Therefore, we have investigated the interaction between type of cryoprotectant (glycerol, DMA, DMSO, and ethanediol (**ED**), cryoprotectant concentration, and cooling rate, in a factorial design. Furthermore, we have experimented with 2 different carrier medium compositions. Finally, fresh semen and semen frozen with a number of combinations of freezing method and carrier medium was used to study the fertilizing ability of that semen in a split sample insemination experiment.

In Vitro Study

In 2003 and 2004, CGN carried out an in vitro study comparing different media and cryoprotectants (glycerol, DMSO, ED, and DMA). A full account of this series of experiments will be published elsewhere. Pooled broiler breeder semen was diluted with an equal volume of poultry semen extender and was then cooled to 5°C. The semen was then mixed with the same extender, but containing various concentrations of glycerol, DMSO, ED, and DMA at 0.6, 1.0, or 1.5 mol/L, packed in straws and frozen in a programmable freezer and using a wide range of cooling rates from 5 to 600°C/min. Alternatively, the semen was frozen by plunging 50-μL droplets in liquid nitrogen. The latter technique had an average cooling rate between 5 and –200°C of approximately 600°C/min. Post-thaw motility and viability were significantly and considerably lower with ED and DMSO than with glycerol and DMA.

Glycerol was not compatible with freezing at very high rates, i.e., by plunging droplets in liquid nitrogen or by straw-freezing at a cooling rate of 600°C/min. As was found by Tselutin et al. (1999), cock semen could cope with extremely high cooling rates (plunging) when DMA was used as cryoprotectant. In our study, we could get similar results with DMA when the semen was frozen at very high cooling rates in straws (600°C/min). However, we found that better results with DMA were obtained with straw freezing at intermediate cooling rates of about 200°C/min (Figure 1).

Generally, the post-thaw sperm motility and viability was related to the CPA concentration, i.e., best results were obtained with 1.5 mol of CPA/L. For DMA, the dependence of post-thaw sperm viability on CPA concentration was stronger than that of glycerol. Conversely, there were clear indications that the high DMA concentrations had very strong toxic effects on the spermatozoa. Therefore, while the percentage of live spermatozoa directly after thawing was higher at 1.0 and 1.5 mol of DMA/L, the spermatozoa deteriorated much faster after freezing/thawing with 1.5 mol/L than with 0.6 mol of DMA/L. Such a clear disadvantage of a high CPA concentration was not observed for glycerol.

There was a significant interaction of cooling rate and CPA concentration. Using an intermediate-high cooling rate of ±200°C/min rendered the post-thaw viability less dependent on the DMA concentration. Thus, a relatively good post-thaw motility and viability could be obtained with only 0.6 mol of DMA/L. In another experiment, it

was found that lower DMA concentrations (0.4 and 0.2 mol/L, not shown) proved insufficient, even at a high cooling rate of 220°C/min. Thus, it was concluded that to be able to use DMA as an alternative for glycerol, the combination of 0.6 mol of DMA/L with a cooling rate of ±200°C/min would probably be the best combination, as it would combine reasonable post-thaw sperm survival with reduced toxic effects at the relatively low DMA concentration.

Insemination Experiment

The in vitro results prompted us to perform an insemination trial. The aim of the insemination trial was to compare the fertility of semen frozen with the pellet freezing method and semen frozen in straws using 0.6 mol of DMA/L and intermediate high cooling rates of ±200°C/min. A second aim was to compare 2 different freezing extenders. A full account of this insemination experiment will be published elsewhere.

In this trial, we have used 4 groups of ISA Brown hens, 23 hens per group. The groups were inseminated with fresh or frozen semen as shown in Table 3. Per insemination day, semen was collected from broiler breeder cocks, pooled, processed (fresh or frozen), and inseminated. This procedure was replicated 4 times with 3- or 4-d intervals. Per insemination day, a split-sample approach was used, i.e., the same pooled semen had been used for the fresh and frozen-thawed treatment groups. Two extenders were used: Lake's freezing medium (Lake, 1968) and a proprietary poultry extender of the Animal Sciences Group (**ASG**). The semen was frozen using 0.6 mol of DMA/L. Pellets were frozen by plunging 50-μL droplets of semen into liquid nitrogen. Straws were frozen in a controlled-rate freezer at ± 200°C/min. The hens were inseminated with 300×10^6 in 0.25-mL approximately 6 h after collection of the semen. Eggs were collected during the 2 wk in which the hens were inseminated plus the following 2 wk to determine how the percentage fertilized eggs would decline after the last insemination. After 7 d of incubation, all eggs were opened to inspect fertilization and embryo development.

The fertility of cock semen frozen with the pellet method (Treatment 4) was good, which is consistent with the results published by Tselutin et al. (1999) and Chalah et al. 1999). The same semen, frozen in the same medium, but frozen in straws at a cooling rate of ±200°C/min (Treatment 3) resulted in somewhat lower results. (The difference in duration of fertilization of the last insemination was significant.) This result shows that reasonably good results are possible also with the straw freezing method. The straw freezing method could be further improved by using the ASG medium instead of Lake's medium (Treatment 2 vs. Treatment 3). The ASG medium resulted in an almost significantly higher ($P = 0.07$) percentage of eggs with developing embryo and a significantly higher duration of fertilization of the last insemination ($P = 0.001$).

The pellet freezing method would be very appealing for cryobanking of poultry semen, as quite high fertility rates can be obtained with frozen-thawed semen. However, straw freezing has the advantage that the semen is packaged, which is more hygienic. Moreover, sample identification is more easy and reliable, as straws can be printed with the identity of the donor cock, the breed, collection date, and other data. By optimizing the cooling rate and the medium composition, good fertility can also be obtained with semen frozen in straws. Using the ASG medium with 0.6 mol of DMA/L and a relatively high cooling rate of ± 200°C/min, fertility rates with frozen-thawed semen were not much lower than those with fresh semen. Moreover, the percentage of fertilized eggs (or percentage of eggs with developing embryo) did not go down appreciably until d 9 after the last insemination. This indicates that inseminations once weekly would even be sufficient for this frozen-thawed semen. As of 2005, the CGN uses this freezing method (Treatment 2; Table 3) to extend the collection of poultry semen in the Dutch gene bank.

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