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Semen Cryopreservation for Ex Situ Management of Genetic Diversity in Chicken: Creation of the French Avian Cryobank[1]

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ABSTRACT The need for semen preservation in domestic birds is a result of the reduction in genetic variability of domestic bird livestock and of the increasing risk of line extinction for health and safety reasons. Cryopreservation of embryos and primordial germ cells (PGC) is not routinely feasible in birds. The project therefore involved semen frozen in optimal safety and traceable conditions. Whole blood samples were also frozen to provide samples of analyses of genomes and health status. The feasibility of using ex situ conservation, i.e., collecting biological material to be stored outside the usual production area of the species (ex situ genetic stock), to preserve and manage rare breeds was tested with 4 subfertile populations: 3 rare experimental lines used for research into energy metabolism (R+), growth (Y33), and immunity (B4/B4), reared under known health status and the oldest endangered patrimonial French breed, the Gauloise dorée with an unknown health status. A general infrastructure was set up for the health screening and remediation of diseases, collection and storage of frozen cells and 2 sites were created for the storage of frozen samples. The screening and remediation of diseases of the Gauloise dorée, which was contaminated with various *Salmonella* and Mycoplasma strains, was achieved by successive treatment of parents, incubated eggs and young chicks with Baytril followed by Tiamulin. For each line, 474 to 994 semen straws have been frozen, thawed, and the semen evaluated. Insemination of frozen-thawed semen into females of the same genetic origin or of an egg-type commercial breed produced chicks in every case. For the most subfertile lines, insemination with egg-type females significantly increased the reproductive success. In conclusion, we report on the benefits of a semen and blood cryobanking complex for the management of endangered lines and strains of domestic birds. Current stocks made possible the restoration of more than 96% of the initial genome. This project also provided technical solutions to resolve some of the health problems frequently encountered for gene preservation in poultry.

Key words: cryobank, ex situ management of genetic diversity, chicken, frozen semen

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INTRODUCTION

Conservation of genetic variability in domestic animal species is a challenge for the sustainable production of human food resources, for land management, and more widely for the preservation of biodiversity. Half of the domestic bird breeds are currently considered to be endangered (Dohner, 2001) for various reasons, mainly related to restructuring of rural areas and human agriculture, commercial needs, and exposure to health risks. A total of 154 chicken breeds and lines have been described in France (Tixier-Boichard et al., 2001), representing a wide diversity of populations managed by amateurs, research institutes, and commercial breeders. They are exposed to risks of epidemics (e.g., avian influenza). Small populations are also exposed to management failure and deleterious inbreeding. These resources could be saved by conservation programs (Pisenti et al., 1999; Alderson et al., 2003; Blackburn, 2004, 2006; Woelders et al., 2006).

In situ management of genetic resources is a priority in every conservation program involving animal species. In addition, ex situ conservation is a strategic tool to secure genetic diversity, particularly considering the risks of epidemic diseases. This is the main reason why ex situ conservation should be associated with known health status of donors, to provide safe biological material for further population management. Cryopreservation repre-

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sents excellent complementary support to in situ conservation and may also be used in selection programs (Alderson et al., 2003; Danchin-Burge and Hiemstra, 2003).

The most feasible method for ex situ management of genetic resources in birds is semen cryopreservation (Gee, 1995; Hammerstedt, 1995; Reedy et al., 1995). However, only the male genome is conserved because in birds the female is the heterogametic sex. Therefore, in backcrossing, it would take 1 to 6 generations (depending on the genotype and need) to return to the desired genotype after semen cryopreservation. Cryopreservation of embryos and oocytes would make it possible to retain the W chromosome, but such techniques cannot be used because of the characteristics of the megalecithal egg (Blesbois and Labbé, 2003; Massip et al., 2004). The cryopreservation of blastoderm cells or primordial germ cells followed by their reimplantation in recipient embryos may provide interesting alternatives (Van de Lavoir et al., 2006). Such embryos may result in chimeric birds able to generate progeny of the donor genotype with a very low germ-line transmission rate (Tajima, 2002)). However, this method is not sufficiently effective and is too costly for large programs of genetic conservation (Petite, 2006).

The only method currently feasible for ex situ management of bird populations is semen cryopreservation, which has mainly been studied in the chicken. Some successful experiments have been performed in other domestic and wild bird species, with very wide differences in effectiveness according to species and breeding conditions (Massip et al., 2004; Blesbois et al., 2005). However, even in chickens, semen cryopreservation is not yet widely used in conservation programs because its success is highly dependent on the fertility of the breed, and there is also high individual within-breed variability.

The aim of the present study was to examine the feasibility of the creation of an ex situ management program for the conservation of chicken genetic diversity using rare lines or strains of chicken. These lines or strains were characterized by different phenotypes and by a large variability in their reproductive efficiency, their health status, and their regular breeding program. It included 3 bility of the creation of an ex situ management program types of metabolic and immune research (Plachy et al., 1989; Gabarrou et al., 1997; Morisson et al., 1997; Le Bihan-Duval et al., 1998; Berri et al., 2001; Guernec et al., 2003; Miller et al., 2004). It also included a traditional breed, the Gauloise dorée (coq gaulois) chosen for its high patrimonial interest (the oldest French breed and also a patriotic symbol) and also because its introduction into the cryobank represented a pilot study for a breed that is 1) difficult to collect; 2) not adapted to standardized breeding conditions; and 3) of unknown health status with regard to the risk of disease transmission by semen.

MATERIALS AND METHODS

Animals, Breeding, and Initial Health Status

Experimental Lines of INRA. The 3 lines Y33, R+, and B4/B4, were raised in the INRA Research Center of Tours-Nouzilly (France). They represent extreme genotypes selected for different traits chosen for their importance in poultry production and for their usefulness in basic research. Their main features, including inbreeding and fertility, are summarized in Table 1. Line Y33 has been used for various research programs on metabolism and muscle development (Ricard et al., 1994; Le Bihan-Duval et al., 1998; Berri et al., 2001; Guernec et al., 2003). Line R+ is a model for the study of feed intake and metabolic efficiency (Gabarrou et al., 1997; Bordas and Minvielle, 1999) that exhibits defective mitochondrial metabolism affecting the spermatozoa (Morisson et al., 1997). Line B4/B4 forms part of a series of 12 lines containing different haplotypes of the chicken major histocompatibility complex and is used as a model for research into diseases, including sarcomas and coccidiosis (Plachy et al., 1989; Miller et al., 2004).

The Y33 and R+ lines have been part of a vaccination program for Marek's disease, infectious bursal disease, encephalomyelitis, coronaviruses, and Newcastle disease. In addition, these lines have been subjected to control measures to eradicate *Salmonella* Gallinarum Pullorum, *Salmonella enteritidis*, *Salmonella* Typhimurim, *Mycoplasma* Gallisepticum, and *Mycoplasma* Synoviae. The B4/B4 line is reared in a specified pathogen free environment.

The Gauloise Dorée Breed. This breed is one of the oldest free-range chickens in France and was very common on farms for many centuries for the production of meat and eggs. It is an ancestral type of Mediterranean chicken. It is a very active strain, with white eggs and a well-defined standard phenotype similar to the wild-type phenotype of the Red Jungle Fowl, with bright orange eyes. It is an endangered breed, since the number of Gauloise dorée chickens had decreased to approximately 200 individuals in 1995. Generally, fancy breeders establish 1 or 2 pens, each of them with 1 male and 5 to 10 females, to reproduce their stock. They exchange males or females from time to time. Gauloise dorée chickens are robust and reared in very small units with little or no vaccination. In the present study, the breeding conditions necessitated a health screening and disease remediation program before sampling the males for the cryobank (see Method of Screening and Remediation of Diseases).

For all genotypes, chicks were produced in 1 or 2 hatches and were reared in floor pens up to 16 wk of age. After this age, the healthy males were housed in individual battery cages under a 14L:10D photoperiod and fed a standard diet of 12.5 MJ/d.

Construction of a General Infrastructure for the Health Screening and Remediation of Diseases, Collection, and Storage of Frozen Cells

The work presented here was part of a major project entitled French National Cryobank of Domestic Animals (Danchin-Burge and Hiemstra, 2003). Before the present study, this cryobank contained only mammalian semen, mostly obtained from species in which artificial insemina-

Table 1. Main features of the populations sampled for the creation of the French Avian Cryobank

Name	Y33	R+	B4/B4	Gauloise dorée
Status	Closed experimental lines			Old breed
Owner	INRA			Fancy breeders
Selected trait	Growth and muscle development	Residual food intake	Major histocompatibility complex	No selection, open air, no vaccination
Number of generations	18	26	28	Not relevant
Mean inbreeding rate at sampling, %	14	50	76	
Mean number of alleles at microsatellite markers	Unknown	2.7	Unknown	3.5
Mean fertility with fresh semen and artificial insemination, %	75	65	50	Not known

tion is routinely used in insemination centers. This is not the case for chickens because semen cryopreservation is rarely used in this species.

Four complementary units were established on the INRA research center of Tours: 1) an experimental unit (Unité Expérimentale-Physologie Aviaire et Parasitologie, specializing in avian disease) that organizes the screening and remediation of diseases of strains of unknown health status; 2) an experimental unit [Unité Expérimentale-Station de Recherches Avicoles (**UE SRA**), specializing in avian breeding] that is the site of semen production and treatment; 3) a research unit (UR SRA, specializing in avian biology) that manages the technical procedures and ensures the freezing of samples and transitory storage; and 4) an experimental unit (Unité Expérimentale-Génétique Factorielle Avicole, involved in conservation of genetic diversity) that maintains a female flock of the Gauloise dorée breed for further characterization and fertility test.

Two geographically separate locations were established for the long-term storage of frozen semen to limit the possibility of destruction of the frozen samples. The first is close to the site of semen collection and located at the EFS (French Blood Institute) of Tours. The second storage site is near Paris at the primary site of the French National Cryobank of Domestic Animals (ACSEDIATE, Maisons-Alfort). In each case semen and blood samples were stored in liquid nitrogen tanks, under optimal quality and traceability conditions.

Method of Health Screening and Remediation of Diseases of the Gauloise Dorée Breed

The Gauloise dorée breed was of unknown health status and gave us the opportunity to test a system of health screening and remediation of diseases on animals originating from 10 different breeders scattered throughout France. To minimize disease risks, only eggs from the Gauloise dorée breed were taken to the cleaning site prior to incubation. Parental flocks were submitted to serological analyses using specific antigens (*Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella gallinarum pullorum*) provided by Intervet International (Booxmeer, Holland) or the Laboratoire Départemental d'Armor (Ploufragan, France). In case of positive result of serological analyses, the whole parental flock was treated by distribution of Baytril (Bayer Pharma, Puteaux, France) in the drinking water (1 mL/liter).

Because of the possible vertical transmission of infection, incubated eggs were first disinfected by 5 min of dipping in Divosan (1%, Johnson Diversey, Fontenay sous Bois, France) and also treated with Tiamulin at d 8 of incubation (Tiamutine, Bayer Pharma, Puteaux, France). The dose was selected following toxicity assays of the inoculation of 3 concentrations of Tiamulin (dose 0: saline solution, 10, 20, or 40 mg/kg) in the yolk sac of fertile eggs from a laying strain. After hatching, the chicks were reared in adapted isolators for 3 mo before their transfer to the UE SRA for the end of rearing. Tiamulin treatment was repeated during these 3 mo (3 successive days of inoculation every 4 wk), and absence of infection was tested by PCR on tracheal swabs (Laboratoire Départemental d'Armor, Ploufragan, France).

Further growth of Gauloise dorée chickens was generally slow (mean weight at 17 wk was 1,100 g for the males and 850 g for the females).

Semen Collection and Quality Analyses

When the males had reached sexual maturity (from 24 to 35 wk of age, depending on the line), 30 males from each experimental line and 48 Gauloise dorée males were selected for semen collection, evaluation, and cryopreservation. Semen was routinely collected twice a week by massage (Burrows and Quinn, 1937), and sperm concentration, motility, and viability (see Blesbois et al., 2002) were assessed. Sperm concentration was determined by light absorption of semen with a photometer (IMV, L'Aigle, France) at a wavelength of 545 nm (Brillard and Mc Daniel, 1985). Mass motility (subjective evaluation of the speed of the movements of a group of sperm in 20 μL of semen), ranging from 0 to 9, was measured by subjective observation under a light microscope (250×), giving a general view of the type and intensity of spermatozoa movement and the impact of movement on the number and size of agglutinations. As an example, the highest notation, 9, represented samples with rapid swirls of sperm, and the notation 1 represented sperm mostly agglutinated, very few of them moving. Semen viability was measured after staining with SyBr14/IP fluorescent dyes (Chalah and Brillard, 1998) or with eosin-nigrosin smears (Blom, 1950). Proportions of viable spermatozoa were then observed under the microscope. The 20 best

donors for each line were kept for further semen freezing. For this choice, we retained only the males that gave ejaculates with a minimum concentration of 3×10^9 spermatozoa/mL and a minimum volume of 250 μL.

Then, the 2 criteria, mass motility, and viability were combined (with the same weight for each criteria) to select the donors with the highest motility and viability (best donors). The measures were assessed on 3 ejaculates per male.

Methods of Semen Freezing

Considering the usual negative effects of freezing on semen fertilizing ability, 2 methods of semen freezing-thawing were first tested on one of the less fertile lines, the R+ line. One method (Seigneurin et Blesbois, 1995) used glycerol as the cryoprotectant and French straw packaging for semen (IMV, L'Aigle, France). The other method (Blesbois et al., 2002), which was adapted from Tselutin et al. (1999) used dimethyl acetamide (**DMA**) as the cryoprotectant and included rapid freezing (50°C/min) and packaging in the French straws. More precisely, the DMA straws method that has been less described in previous papers than the glycerol's method was characterized by the following procedure: 1) dilution of sperm at 20°C in a saline diluent (FEB, Tselutin et al., 1999) up to a mean of 2×10^9 cells/mL; 2) cooling to 4°C (20 min); 3) addition of 6% DMA and 1 min later packaging in straws (0.5 mL French straws, IMV); 4) freezing at the rate 50°C/min up to −140°C; 5) plunging and storage in liquid nitrogen; 6) for thawing, the straws were plunged for 5 s in a water bath at 50°C. The method yielding the best percentage of fertility after insemination was then used to prepare the straws for semen storage in the cryobank.

Blood Freezing

For each male suitable for semen freezing, 15 straws of blood were also stored in the cryobank for potential further study of diseases that are as yet unknown, or other complementary genetic analyses on genomic DNA. Twenty percent glycerol was added to the whole blood sample before freezing in CBS straws (CBS-IMV).

Semen and Blood Storage

Semen and blood samples were stored in liquid nitrogen in Arpège containers (Azote Liquide, Paris, France) combined with automatic liquid nitrogen adjustment. Each straw was individually identified by a series of 16 letters and numbers indicating the nature of the sample (1 letter for semen or blood), the species (3 letters), the line or breed (3 digits), the year of birth (2 digits), the generation number for the experimental lines (3 digits), and the identification number of the male (4 digits).

Fertility and Hatchability Tests

Fertility and hatchability were measured during incubation and after hatching, respectively. Fertility (% fertile/incubated eggs) and early embryonic mortality (% dead/incubated eggs) were measured by candling the eggs at d 8 of incubation. At this stage, very early embryonic mortality was mixed up with infertile eggs. A second candling was performed at 15 d for the Tiamulin toxicity test (see Method of Health Screening and Remediation of Diseases) to determine the proportion of viable embryos at this stage. Hatchability (% viable/fertile eggs) was measured after 21 d of incubation.

To test the efficacy of the frozen/thawed semen in restoring the genetic diversity of semen stored in the cryobank, a fertility test was set up. A subset of 4 to 8 males representing each line was used to individually inseminate 3 to 4 females per male every 4 d over 2 consecutive weeks (3 inseminations per hen). The insemination dose was 600 million spermatozoa/artificial insemination/female. The experiment was performed with females of the same genetic origin (so-called homologous) or Isabrown commercial egg type females (heterologous; ISA, Chateaubourg, France). Eggs were examined at 2 to 9 d postinsemination. Because the differences in fertility obtained between breeds with frozen semen were high and embryo mortality remained low (4 to 5% as previously described, Seigneurin and Blesbois, 1995), only fertility rates are reported in the Results section.

Testing the Efficacy of the Cryobank to Restore a Line

The fertility rate values obtained with frozen-thawed semen were used to simulate a backcross design aimed at restoring a line. In such a case, heterologous females are inseminated with frozen semen of the line to be restored. This produces F_1 birds. The F_1 females are then inseminated with frozen semen of the pure donor genotype to produce backcross 1 birds. Different males are used at each generation to avoid close inbreeding. Repeating this backcrossing of crossbred females with frozen semen of the pure donor genotype should restore 96.875% of the donor genome at the fourth backcross and 98.43% at the fifth backcross. As long as the percentage of donor genome increases, the fertility rate becomes closer and closer to the values obtained with homologous females.

Statistical Analyses

The results of Tiamutin toxicity on embryos were submitted to 1-way ANOVA. Fertility results were compared by Pearson's chi-squared test (Statview Software, Abacus Concepts Inc., Berkeley, CA).

RESULTS

Health Screening and Remediation of Diseases of the Gauloise Dorée Breed

The experimental lines of INRA were reared with standard disease prevention programs and were free of dis-

Table 2. Effects of Tiamuline[1] on embryo viability

Embryo	Number fertile/ incubated (8 d)	Number viable/ incubated (15 d)	Number hatched/ incubated	Hatched/ incubated (%)
Untreated	35/38	35/38	35/38	91[a]
Inoculated ρ serum[2]	35/38	32/38	23/38	60[bcd]
Inoculated Tiamuline, 10 mg/kg	36/38	30/38	27/38	69[b]
Inoculated Tiamuline, 20 mg/kg	37/39	30/39	21/39	54[d]
Inoculated Tiamuline, 40 mg/kg	37/39	22/39	17/39	39[e]

[a–f]Indicates significant differences ($P < 0.05$).

[1]Bayer Pharma, Puteaux, France.

[2]ρ serum = physiologic serum.

eases known to be potentially transmitted by semen. Alternatively, the adult Gauloise dorée chickens were initially positive to different pathogenic bacteria, especially *Salmonella* Gallinarum, *Mycoplasma gallisepticum*, and *Mycoplasma synoviae*. The on-farm treatment of the parents with Baytril in the drinking water (10 different breeders) followed by the choice of eggs originating from parents negative to *Salmonella* Gallinarum, and by inoculation of the incubated eggs with Tiamulin (10 mg/kg) resulted in the lack of *Salmonella* Gallinarum and *Mycoplasma gallisepticum* at hatching. However, inoculation of incubated eggs resulted in a 30% decrease in embryo viability (Table 2). In addition, all the eggs collected from 2 farms were infertile. Consequently, of the 549 initially incubated eggs, 338 were fertile and 177 chicks were obtained with a balanced sex ratio. Hatching rates varied from 11 to 62% for the 8 breeders that provided fertile eggs (Table 3). Maintenance of negativity to *Mycoplasma synoviae* required further Tiamulin inoculations during the rearing period (including the 3 first months in isolators) checked by PCR on tracheal swabs.

Choice of Semen Freezing Method

The method using glycerol as the cryoprotectant gave significantly higher fertility rates than the method with DMA ($P \leq 0.05$; 30.4 vs. 2.8% fertility, Table 4) in the preliminary test set up with line R+. This method was then applied for all the lines.

Sampling of Males to Produce Frozen Semen

Gauloise Dorée Breed. Fifty-four 4-mo-old Gauloise dorée males hatched at INRA and free of transmissible disease were selected based on phenotype and different breeders' origins (Table 3). However, only 7 breeders were represented at this stage. The sexual maturity of the males was quite late (30 to 35 wk of age) and the persistence of semen production quite low (2 to 3 mo). Semen production was suitable, in quantity and quality, for freezing in individual straws at the adult stage for only 7 males obtained from 2 of the 10 breeders who initially provided eggs (Table 3). For the other males, semen had to be pooled to be frozen. This was done for 2 pairs of brothers originating from 2 other breeders and also for sets of 4 or 5 brothers originating from the same breeders already represented by individual straws. Thus, only 4 of the 10 initial breeders were the origin of the 491 straws stored in the cryobank for the Gauloise dorée breed (Table 5).

Experimental Lines. Similar numbers of adult males providing semen (20 to 22) were retained for each line. All the males retained were able to produce semen, but

Table 3. Summary of the results and representativeness of the samples stored in the cryobank for the Gauloise dorée breed; from the number of eggs collected to the number of donor males and the number of straws stored in the cryobank

Breeder's origin	Incubated eggs	Fertile eggs	Hatched eggs	Hatched/ incubated (%)	Adult males	Donor males	Number of straws per male or pool
V	133	121	69	51	26	3 + pool of 4 brothers	38 to 70 70
G	102	63	32	62	7	Pool of 2 brothers	53
B	60	41	31	50	14	4 + pool of 5 brothers	8 to 39
P	50	0	0	0	0	0	0
Me	40	16	9	22.5	1	0	0
D	37	27	4	11	0	0	0
A	35	0	0	0	0	0	0
Gr	32	28	10	31	1	0	0
W	31	27	10	32	3	0	0
Ma	29	24	12	41	2	Pool of 2 brothers	33

Table 4. Fertility obtained with R+ semen frozen with glycerol or dimethyl acetamide (DMA) cryoprotectants

Item	Semen frozen-thawed with glycerol	Semen frozen-thawed with DMA
% of fertile eggs	2.8[b]	30.4[a]
Number fertile/incubated eggs	9/321	65/241

[a,b]Indicates significant differences ($P < 0.05$).

wide differences in efficacy were observed between males and lines (Table 5). The corresponding number of semen straws stored in the cryobank of the B4/B4 line was half that of the 2 other lines.

Fertility Obtained with Frozen-Thawed Semen Inseminated in Females of the Same Genetic Origin or in Commercial Egg Type Females

In all cases, even with subfertile lines, frozen-thawed semen yielded fertile eggs (Table 6) and thereafter chicks. However, there were wide differences between lines and breeds. The Y33 line gave the highest fertility results after insemination in both Y33 and Isabrown females. For all the other genetic origins tested, fertility was significantly lower when insemination was performed with homologous females compared with commercial females. The greatest contrast was obtained with the B4/B4 line that gave very low fertility when B4/B4 females were used (7% fertile eggs). In this case, insemination in commercial egg type females was much more successful in terms of fertility (43% fertile eggs). Similar results were obtained for the R+ line and the Gauloise dorée breed, but with lower orders of magnitude, the fertility rate with heterologous females being almost twice that with homologous females. The fertility rate of the Gauloise dorée breed was higher than that of the R+ line.

Forecast of Line Restoration with Frozen-Thawed Semen

A tentative design is described in Table 7. Subsets of 4 to 7 different males were used from F_1 to backcross 3 generations to represent the entire genetic basis of the line. Groups of 3 to 4 females were inseminated per male with 2 to 9 inseminations until backcross 3. Depending on the total number of doses and on the decision to use all the stored material or not, backcrossing should be continued until backcross 4 or 5. The number of straws used increased in subfertile lines with the percentage of the donor genome, and this was taken into account from backcross 2. For the last backcross generation, a higher number of males (8 to 10) was used to produce a higher number of birds representing all the family origins of the restored line. Because the fertility test yielded numbers of fertile eggs but not numbers of chicks, a 10% embryo mortality rate was assumed to derive a number of chicks from numbers of fertile eggs. Then a 50% sex ratio was assumed, and the proportion of female chicks becoming fertile adult females was set at 75%, which is a rather conservative hypothesis. According to these options, the total stock of frozen semen would be used for the B4 line and the Gauloise dorée breed to restore a line with 96.875% identity to the original population. A higher number of stored doses made it possible to continue until backcross 5 in the R+ line, with 98.43% recovery of the original genome. The same recovery rate was obtained for the Y33 line but required only 55% of the stored straws, so that it would still be possible to add a further backcross generation to reach 99.21% of the initial genome.

Table 5. Number and origin of the straws stored in the cryobank

Line or strain	Number of males	Number of straws/male	Number of straws/line
Gauloise dorée	20	8 to 40	491
R+	22	30 to 55	994
Y33	22	21 to 61	982
B4	20	7 to 41	474

Table 6. Fertility obtained with frozen-thawed semen, according to male origin and female origin[1]

Origin	Female of the same line as the males	ISA Brown females commercial brown-egg layer
Strain Gauloise dorée, 8 males		
% Fertility	21[de]	39[c]
Number fertile/incubated eggs	22/103	108/278
Line R+ 8 males		
% Fertility	14[ef]	25[d]
Number fertile/incubated eggs	27/195	64/257
Line Y33 8 males		
% Fertility	68[a]	59[ab]
Number fertile/incubated eggs	107/158	133/226
Line B4 4 males		
% Fertility	7[f]	43[bc]
Number fertile/incubated eggs	9/128	43/100

[a–f]Indicates significant differences ($P < 0.05$).

[1]There were 4 to 8 males per line, 4 females per male and 3 straws used for each female.

DISCUSSION

We report here the construction and utilization of an avian cryobank for the ex situ management of rare and endangered domestic lines and breeds of the species *Gallus gallus*. We also showed technical solutions to resolve some of the most frequent health problems encountered in poultry for gene preservation. In addition, even subfertile lines were able to yield chicks after semen cryopreservation. As a consequence, strategies may be suggested to restore the original lines.

The 2 main aspects to be discussed are the parameters of a successful cryobanking project, and the potential uses of the stock of frozen semen for the ex situ manage-

Table 7. Results of a simulated backcrossing design to restore lines from the frozen semen of the cryobank; from the production of F_1 birds after insemination of commercial layers with frozen-thawed semen[1]

Line	Stage	Males, n	Dams, n	Artificial inseminations per dam, n	Fertile eggs, n	Chicks, n	Females, n	Layers, n	Straws, n	Donor genome, %
R+	F_1	5	20	4	53	48	24	18	80	50
	BC1	5	18	5	60	54	27	20	90	75
	BC2	5	20	6	64	58	29	22	120	87.5
	BC3	5	21	9	76	68	34	26	189	93.75
	BC4	10	26	9	78	70	35	26	234	96.87
	BC5	10	26	10	87	78	39	29	260	98.4
Total									973	
Gauloise dorée	F_1	5	20	2	45	41	20	15	40	50
	BC1	5	15	3	51	46	23	17	45	75
	BC2	5	17	4	61	55	28	21	68	87.5
	BC3	5	21	6	85	77	38	29	126	93.75
	BC4	8	29	7	114	103	51	39	203	96.87
Total									492	
Y33	F_1	5	20	3	83	75	37	28	60	50
	BC1	5	26	2	72	65	32	24	52	75
	BC2	5	23	2	64	57	29	22	46	87.5
	BC3	7	20	4	111	100	50	37	80	93.75
	BC4	10	34	4	188	170	85	64	136	96.87
	BC5	10	40	4	222	200	100	75	160	98.4
Total									536	
B4	F_1	5	20	3	54	48	24	18	60	50
	BC1	5	18	3	48	44	22	16	54	75
	BC2	5	16	4	46	41	21	15	64	87.5
	BC3	5	15	9	60	54	27	20	135	93.75
	BC4	10	20	8	29	26	13	10	160	96.87
Total									473	

[1]BC 1, 2, 3, 4 = successive backcrossing with frozen-thawed semen from a line. Changes in fertility rates with backcrossing generations: BC2, 80% of F_1 for B4, R+ and GLD lines. BC_3, 60% of F_1 for R+ and GLD lines and 50% of F_1 for B4. BC4 and after, 50% of F_1 for R+ and GLD lines and 20% of F_1 for B4. Fertility rate decreased with an increasing percentage of donor genome for the R+, Gauloise dorée breed, and B4 lines. Different males were used to produce F_1, BC1, BC2, and BC3 females.

ment of rare and endangered lines or breeds of the species *Gallus gallus*.

Cryobanking Issue

The construction of an avian cryobank in France has been in preparation for many years. Three main difficulties have been encountered: 1) the availability of an effective semen freezing procedure suitable for subfertile sires, 2) the heterogeneity of the health status of chicken populations and the possible risks of disease transmission by semen, and 3) financial support.

The first point was solved by the availability of different freezing methods for chicken semen in our laboratory (Seigneurin and Blesbois, 1995; Chalah et al., 1999; Tselutin et al., 1999). These methods mainly use DMA or glycerol and 2 different methods of packaging of frozen semen (pellets or straws). Two of these methods were of similar effectiveness: the method using DMA and pellets and the one using glycerol and straws (Tselutin et al., 1999). The DMA method was much easier to perform because it does not need removal of the cryoprotectant at thawing in contrast to the glycerol method. However, the use of frozen semen from a cryobank requires strict identification of each sample. Pellets are not convenient in these conditions. One of the preliminary experiments of the present study was to prepare a freezing method that combined the use of DMA as cryoprotectant with straws as semen packaging. However, the fertility results of this method were lower than when the glycerol method was used (Table 4). Previous works in our laboratory (Blesbois and Grasseau, 2002; Labbé et al., 2003) indicated that straws were less efficient than pellets to restore fertility of chicken or turkey semen frozen with DMA cryoprotectant. This could be due to an effective difference in sperm freezing curve between the 2 procedures (pellets or straws) or to a deleterious interaction between DMA and the plastic of the straws. In the case of normally fertile breeds, the decrease in fertility, which is induced by the use of DMA with straws (compared with DMA with pellets), is not dramatic, and this method may be recommended because it is easier than the glycerol method. However, most of the chicken lines chosen for the present study were subfertile, and our objective was to obtain chicks in every case after semen freezing. The results presented on Table 4 showed that this objective could probably not be fulfilled by the use of DMA and straws. Although their technique requires a higher technical skill, we selected the cryopreservation method of Seigneurin and Blesbois (1995) for the continued studies of semen cryopreservation.

The Y33 line, selected on body conformation (Le Bihan-Duval et al., 1998; Berri et al., 2001; Guernec et al., 2003), gave quite good levels of fertility after semen freezing and insemination of Y33 females or of commercial egg type females. For the other populations, the fertility results were much lower with homologous females and markedly improved after insemination in heterologous

females, indicating that female fertility was also decreased in these populations. This decrease in female fertility was possibly a result of inbreeding (B4 and R+ lines), of metabolic characteristics of the line (R+; Morisson et al., 1997), or because of marked seasonality and lack of adaptation of the females to the new environment of the experimental facilities (Gauloise dorée breed).

The second major problem was to ensure the best possible health conditions for semen production, evaluation, storage, and artificial insemination. This is important to avoid transmission of infectious diseases by frozen semen and should be mandatory for the quality assurance of a cryobank. The introduction of the Gauloise dorée breed into the cryobank was very interesting in this respect because, in its usual breeding conditions, this breed was contaminated by the legally controlled pathogen bacteria encountered in poultry (*Salmonella* and mycoplasma). The screening and remediation of diseases of this strain was therefore absolutely necessary and required the treatment of 2 successive generations of animals because of the existence of unaffected carriers and of the potential vertical transmission of pathogens from the dam to the egg. The procedure consisted of repeated treatments with Baytril for the parents followed by Tiamulin for embryos and chicks. Tiamulin was chosen for the second generation because *Salmonella* and mycoplasma are highly chemosensitive to this drug (Kempf et al., 1988; Cerda et al., 2002). However, a certain degree of toxicity is possible with Tiamulin injection, particularly for the embryo. The toxicity test for Tiamulin injection in 8-d-old embryos showed a deleterious effect of the injection itself in our experimental conditions, with a mean loss of 30% of hatched eggs. Tiamulin had an additional deleterious effect at a dose of 40 mg/kg. Because of the toxicity of Tiamulin injection to the embryo, a low dose (10 mg/kg) was chosen, which did not result in total eradication of mycoplasma. Further injections and the elimination of chicks resistant to treatment were necessary to ensure total eradication. Technical solutions were thus found but at a high cost. Setting up programs to detect and eliminate disease in local breeds would be a valuable alternative in the future to improve the general health status of poultry breeds and decrease the cost of cryobanking.

Financial support was obtained by combining different local and national public grants within the framework of the national policy for construction of Biological Resource Centres, including a National Cryobank for Domestic Animals (Danchin-Burge and Hiemstra, 2003). The need for such programs was pointed out in the international context of sensitivity to the maintenance of biodiversity. We must emphasize that a standardized procedure of ex situ preservation of strains in good technical conditions is still expensive in birds. Our estimation is a minimum cost of 20,000 Euros for a line reared in optimal health and breeding conditions, two-thirds of this cost being due to manpower. The cost reaches 30,000 Euros for a strain of unknown health status requiring a cleaning up procedure. The management of frozen semen straws is much cheaper (300 Euros/line/year).

When all other precautions were taken, between 474 and 960 straws of frozen semen were included in the cryobank per line in the present experiment. The difference in number of straws between lines (ratio of 1:2) was mainly related to the biological characteristics of the lines. The rearing conditions were the same for all the males used for semen freezing. However, the Gauloise dorée breed was unique. The adult males of this breed had an especially short reproductive period with a short duration of semen production coupled with poor semen quality. One hypothesis to explain this phenomenon is that this breed is usually reared in extensive farming conditions and may have retained pronounced characteristics of seasonality that have been lost by most other breeds. Another difficulty with the Gauloise dorée breed was the highly unbalanced contributions of the breeders to the final stock of frozen semen. This was due not only to unequal numbers of eggs but also to differences in hatchability and in semen production and quality of adult males. Successful cryobanking for a local breed should therefore involve the largest possible number of breeders because it is almost impossible to assess the on-farm fertility of animals of noncommercial populations.

Potential Uses of the Cryobank for Population Management

All the lines tested here exhibited nonoptimal to low reproductive performance for their species (mean: 50 to 75% fertility vs. more than 90% for usual commercial breeds). However the ability to obtain chicks after semen cryopreservation was very different from one strain to another. This was particularly true for fertility obtained after artificial insemination of frozen-thawed semen with females of the same strain, which ranged from 7 to 68% (Table 6).

It may be necessary to use frozen semen for homologous females for different purposes (e.g., increasing the effective number of sires to limit the loss of genetic variability in a small population, producing families from males of ancient generations to estimate genetic trends and, more generally, comparing genotypes at different stages of a population history). Blood samples could be used for molecular studies, screening for alleles of a gene of interest, and choosing to use a subset of frozen semen to study new alleles. A wide range of possibilities can be compared that would go beyond the purpose of this paper. Nevertheless, it seems that the B4 line would not benefit greatly from such possibilities because of its very low fertility as a pure line. Possible uses should also be decided without forgetting that one major objective of the cryobank is for long-term storage. Thus, specific uses should be compensated for by a new collection stage to maintain the stock of frozen semen.

It will be necessary to use frozen semen and heterologous females to restore a line or a breed after the outbreak of an epidemic and subsequent loss of a given population. The tentative design simulated in the present study showed that it was possible to restore at least 96% of the

donor genome, which is a very satisfactory rate, except maybe in the B4 congenic line. Molecular markers may be used to identify the differences in genomic regions between the restored line and the donor line. This would be possible with the high-density map of single nucleotide polymorphisms available for the chicken (International Chicken Polymorphisms Map Consortium, 2004).

In addition, even if it is possible to freeze semen and obtain chicks from thawed semen in low fertile lines, we highly recommend starting saving frozen semen before a line has reached a critically low level of fertility. We also recommend that semen be frozen regularly, particularly for inbred lines where there is no or little genetic trend, so that the sampling generation would not be a major parameter for future use.

Other designs to restore a line may of course be studied. For instance, F_1 males and females could be mated to produce an F_2 in which molecular markers could be used to select those animals with the highest percentage of the donor genome. The effectiveness of alternative designs could be tested in further simulation studies, based upon the data gathered in the present project.

To conclude, this study showed the feasibility of the construction of an avian cryobank to restore a range of genotypes, even in cases of low fertility. We must ensure the development and durability of this structure. This is in process, and 12 other experimental lines of the species *Gallus gallus* are now under study for inclusion in the cryobank. However, it must be understood that the susceptibility to cryopreservation of sperm collected from different lines and species of domestic poultry is highly variable, and will require the development of new predictors of semen suitability for cryopreservation. It will be also very important to be able to include other bird species (turkeys, muscovy and pekin ducks, gander, guinea fowl) in cryobanks, and this will first require the standardization of semen freezing procedures in each of these species.

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