Excerpt from "Avian Genetic Resources at Risk: An Assessment and Proposal for Conservation of Genetic Stocks in the USA and Canada". 1999. J.M. Pisenti, M.E. Delany, R.L. Taylor, Jr., U.K. Abbott, H. Abplanalp, J.A. Arthur, M.R. Bakst, C. Baxter-Jones, J.J. Bitgood, F.A. Bradley, K.M. Cheng, R.R. Dietert, J.B. Dodgson, A.M. Donoghue, A.B. Emsley, R.J. Etches, R.R. Frahm, R.J. Gerrits, P.F. Goetinck, A.A. Grunder, D.E. Harry, S.J. Lamont, G.R. Martin, P.E. McGuire, G.P. Moberg, L.J. Pierro, C.O. Qualset, M.A. Qureshi, F.T. Shultz, and B.W. Wilson. Report No. 20. University of California Division of Agriculture and Natural Resources, Genetic Resources Conservation Program, Davis CA USA. 120 p.

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Conserving avian genetic resources

CONSERVATION OF AVIAN WILDLIFE species receives global attention because of the large numbers of species presently in decline, and because birds provide a focus for recreational activities throughout the world. Less known are the needs for conservation of progenitor, landrace, and feral populations of domesticated birds. Even less well publicized is the critical situation of many of the specialized stocks used in research, as demonstrated in Chapter 5.

Safeguarding existing biodiversity is a common theme in the conservation of all types of avian genetic resources, but the strategies used to meet this goal can be quite different, depending on the speicies. Wild species are generally conserved through natural habitat protection and take regulations (i.e., hunting or harvesting limits for a given species), with captive breeding used in only the most severe cases of species decline. Measuring the genetic diversity in wild species is being addressed in a relatively few cases with DNA markers. Such data contribute to the development of rational schemes for conserving genetic diversity. Wild progenitors of domesticated birds, such as the Red Jungle Fowl are treated similarly, but wild turkey conservation, as another example, is enhanced by restocking and translocation of individuals. This activity is motivated by the need for conservation of the species and its diversity, but the fact that turkey is a recreational game bird is perhaps a stronger motivation for conservation.

The goal of genetic resource conservation is the maintenance of genetic integrity of a species or populations within a species. For populations in nature, natural evolutionary processes remain active, thus changes in gene frequency, population sizes, and geographic distribution are to be expected. Human interventions impact species composition through disruption of habitat and harvesting. In such cases, proactive conservation strategies must be applied. The situation is similar for domesticated breeds of birds, but there is more concern for conservation of specific combinations of traits and genes, with minimal changes in gene frequency. Even more restrictive is conservation of genetic stocks where specific genes are targeted. For these genetic entities, no change in gene or genotype frequency can be accepted. Thus, we recognize a distinction between conservation and preservation. The former allows utilization with minimal genetic changes over time caused by human activity and the latter requires that no genetic changes occur, exemplified by nonliving materials, quiescent (e.g., cryopreserved) specimens of gametes and embryos, or closely bred living genetic stocks.

Assays for DNA polymorphisms can be done with nonliving biological materials, providing the opportunity for assessment of genetic diversity in historical times from museum specimens or preserved tissues, including blood serum. This emphasizes the value of preserved biological materials as a component of a conservation strategy for birds. Cloned genomic or cDNA libraries can be preserved indefinitely at low temperatures. This includes DNA clones used for preparing molecular linkage maps and for discovery of functional genes. Databases of DNA nucleotide sequences of genes or expressed sequence tags are extremely rich sources of information because sequences from many species are available for screening and comparisons with unknown sequences.

This report focuses on the conservation of genetic stocks, as defined earlier. However, a holistic view of avian conservation is appropriate because the methods used for preserving genetic stocks and breeding populations, especially semen cryopreservation, can be adopted or modified for other species, including threatened or endangered wild species. Conservation methods

need to be adopted which meet the specific requirements of the species or the trait (genes) being conserved. An avian genetic resource conservation system includes several components (acquisition, maintenance, utilization, distribution, documentation, and health and quarantine) which can provide broader service than the conservation and distribution of genetic stocks. This is elaborated in Chapter 6.

Methods of conservation of genetic stocks

Avian genetic materials may be preserved in the form of live birds, as cryopreserved semen and dispersed embryonic or primordial germ line cells, as preserved tissues from which DNA can be isolated, as cloned genomic or cDNA, or as nucleotide sequence data. Of these methods, living birds and cryopreservation methods permit recovery of animals, while preserving DNA gives the potential of recovering selected genes or defined segments of chromosomes. However, it should be remembered that at best each DNA sequence represents only a very small portion of a total genotype and that, while samples of total DNA from a population may be preserved, sorting out the genes useful for a particular purpose may be a difficult task, particularly when gene interactions must be taken into consideration. Cryopreservation of semen and embryonic cells is gaining acceptance as a means of preserving endangered genetic resources, including wild species, rare breeds, and mutant or specialty lines used in research.

Ultimately, the preferred conservation method depends upon 1) the reliability of the method to yield the desired number of live animals, 2) the characteristics of the stock, and 3) the availability of technical support, instrumentation, and storage facilities. Ideally, more than one method of conservation should be used, or there should be a backup site to reduce the risk of loss due to fire, equipment failure, and human errors. Below are outlined some features of live-bird conservation and three methods of cryopreservation of semen or embryonic cells from which live birds may be recovered. Semen cryopreservation has the longest history, although not a highly efficient recovery rate. Dispersed blastodisc and primordial germ cell cryopreservation are promising emerging technologies that will require further research to improve recovery rates to more acceptable levels.

Live animals

The maintenance and periodic reproduction of live animals is the most dependable alternative available for preserving a genetic stock. This is particularly true for the inbred and long-term selected stocks, with their integrated collections of quantitative trait alleles. This is also the most reasonable way to maintain stocks that are often used in research. However, due to financial constraints, most researchers cannot justify the live maintenance of stocks that are used only sporadically, and recent events have shown (Chapter 5) that many unique lines are terminated for this reason.

The average number of birds required to maintain a given genetic stock depends upon its characteristics. Randombred and selected stocks require a larger base population (often several hundred birds) than those carrying single-gene mutations. The latter may be realistically continued with fewer than ten mutant-carrier birds if a robust outbred stock is used as one parent line each generation. Other factors that affect the number and distribution of birds needed to maintain a viable population include bird liveability, amount of inbreeding depression, endemic diseases, and the risk of natural or man-made disasters, which can literally wipe out genetic stocks in a very short period of time.

Maintaining stocks as live animals is very expensive, especially the selected lines and randombred populations. Even temporary shifts in interests of the curator or sponsoring institution can quickly orphan such genetic stocks, often leading to stock elimination and extinction of valuable genes or gene combinations. These realities are documented in Chapter 5, emphasizing the need for backup sites or a permanently dedicated conservation program.

Semen cryopreservation

The technology for semen cryopreservation (BAKST 1990; BUSS 1993; HAMMERSTEDT 1995) has been successfully adapted for the preservation and propagation of chickens and certain feral and endangered species (GEE 1995). Many different cryopreservation protocols have been evaluated (SEXTON 1979, LAKE 1986, HAMMERSTEDT and GRAHAM 1992, BUSS 1993, HAMMERSTEDT 1995). According to HAMMERSTEDT (1995) semen cryopreservation is the most cost-effective germplasm conservation strategy. Hammerstedt outlined the benefits of semen cryopreservation for the commercial poultry industry, but this preservation technique has not yet been widely

adopted. An important feature of semen cryopreservation for unique or endangered poultry lines is that semen can be collected frequently (twice weekly) from a small number of males for use when females become available or in the future establishment of founder flocks. Other benefits of semen cryopreservation are:

- Frozen semen is not easily affected by diseases or natural disasters.
- Semen cryopreservation from many birds of several lines can be accomplished at a relatively low cost
- The expense of maintaining a flock is reduced or eliminated.
- Frozen semen is more easily transported nationally or internationally than live birds or fertile eggs (HAMMERSTEDT 1995).

There are obvious limitations to the routine use of semen cryopreservation. For some species and genetic stocks the method may not be reliable or yet suitably developed. Another consideration is that only the male genome is preserved, which means that the complex genome of highly inbred or selected stocks could not be preserved intact if only maintained as frozen semen, because, of course, none of the corresponding inbred or selected females would be available for insemination with the cryopreserved material. Thus, while semen cryopreservation permits the incorporation of preserved gene complexes into diploid animals, these gene complexes would be heterozygous in the progeny and subject to genetic recombination in subsequent intermatings of progeny. On the positive side, semen cryopreservation may prove to be the most appropriate method for preserving single-gene mutant stocks, particularly if the genetic background is not a great concern in the modification of the mutant gene expression.

Another problem with cryopreserved semen is that the quality of thawed semen is currently quite low. This is reflected in fertility levels rarely exceeding 70%, and more typically less than 50%, due to sperm destruction and deformity caused by semen cryopreservation and recovery procedures. One researcher estimated that cryopreserved semen has less than 2% of the fecundity of fresh semen (WISHART 1985). This is in spite of the fact that most of the successful cryoprotectants, diluents, and freezing techniques were developed with semen from highly productive commercial broiler chicken stocks. Unfortunately, the semen from inbred, specialty, and mutant-carrier stocks most at risk often does not respond well to cryopreservation procedures that worked reasonably well with commercial stocks. Frozen-thawed semen from these less-robust stocks often exhibit extremely high or complete infertility associated with poor motility and high numbers of freeze-damaged or killed spermatozoa. Obviously, further research is needed, not only in the development of improved semen cryopreservation procedures (improved diluents, cryoprotectants, and other supplements, and optimizing cooling and thawing rates), but also addressing the significance of species variation, male-line variation within a species, and individual male variation within a given strain. However, despite the potential utility of this procedure in preserving the germplasm of rare or endangered stocks or species, only a few researchers in Japan, England, and the US are currently conducting research on semen cryopreservation.

Blastodisc cryopreservation

Cryopreservation of the cells from an avian blastodisc (Figure 14, the embryonic cells in an unincubated fertile egg) is a new technique that can be used to preserve the intact genome of poultry genetic stocks (REEDY *et al.* 1995, Box 19). While the methods are still being perfected, frozen-thawed blastodisc cells have been successfully integrated into the blastodiscs of host chicken eggs, and developed into reproductively competent adults. The long-term viability of cryopreserved blastodisc cells is not yet known, although cryopreservation studies with semen

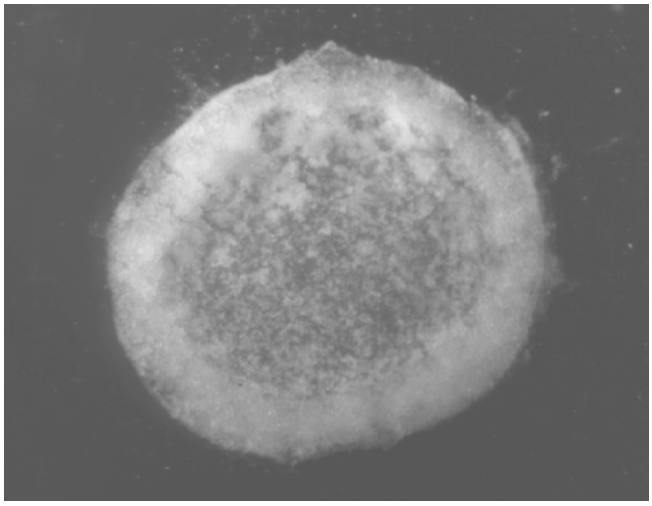

Figure 14. Stage X blastodisc (at time of oviposition) (Photo courtesy of M.E. Delany, University of California–Davis).

and other cell-types suggests that properly cryoprotected and frozen specimens will remain viable indefinitely if kept in liquid nitrogen.

The large size and complex structure of the avian embryo (already composed of 40,000 to 50,000 cells when the egg is laid) do not permit the application of techniques that have been developed for cryopreservation of mammalian embryos. A different strategy is required which uses dispersed cells isolated from the relatively undifferentiated embryonic cells present in the newly laid fertile egg (Box 19). Recent experiments have demonstrated that frozen-thawed blastodermal cells from purebred chickens of the Barred Plymouth Rock breed can successfully integrate into an irradiated White Leghorn host blastoderm to form embryos that are somatic and germline chimeras. These chimeric chickens hatch and mature normally, and, when interbred, produce some offspring that are purebred Barred Rock (KINO *et al.* 1997). While frozen-thawed blastodermal cells form germline chimeras less frequently than fresh blastodermal cells, a sufficient number of "reconstituted" birds of lines maintained as cryopreserved blastodermal cells can be obtained to use the technology to store valuable genetic material (KINO *et al.* 1997). Thus, this technique should eventually provide an inexpensive long-term solution for preserving the intact genome of stocks (particularly inbred and selected stocks) that are considered to be valuable genetic resources but are not currently required in an active research program (Box 20).

Box 19. Mosaic chickens and genetic stock preservation

A NEW TECHNIQUE HAS BEEN developed at the University of Guelph (Ontario, Canada) that can be used to reclaim both freshly dissociated and cryopreserved chicken embryonic cells (Figure 15, REEDY *et al.* 1995). Briefly, cells from the stage X embryo (EYAL-GILADI and KOCHAU 1976) can be removed, dispersed, and injected into recipient blastodiscs that are at the same stage of development. The resulting chimeras (mixture of host and donor cells), which can be either male or female, produce donor-derived gametes at rates of up to 100% (PETITTE *et al.* 1990; 1993; CARSIENCE *et al.* 1993; THORAVAL *et al.* 1994; KAGAMI *et al.* 1995). Matings of male and female chimeras that each produce donor-derived gametes yield offspring with the complete donor genotype at a rate that is the product of the frequency of the production of donor-derived gametes by eachof the chimeric parents (KINO *et al.* 1997).

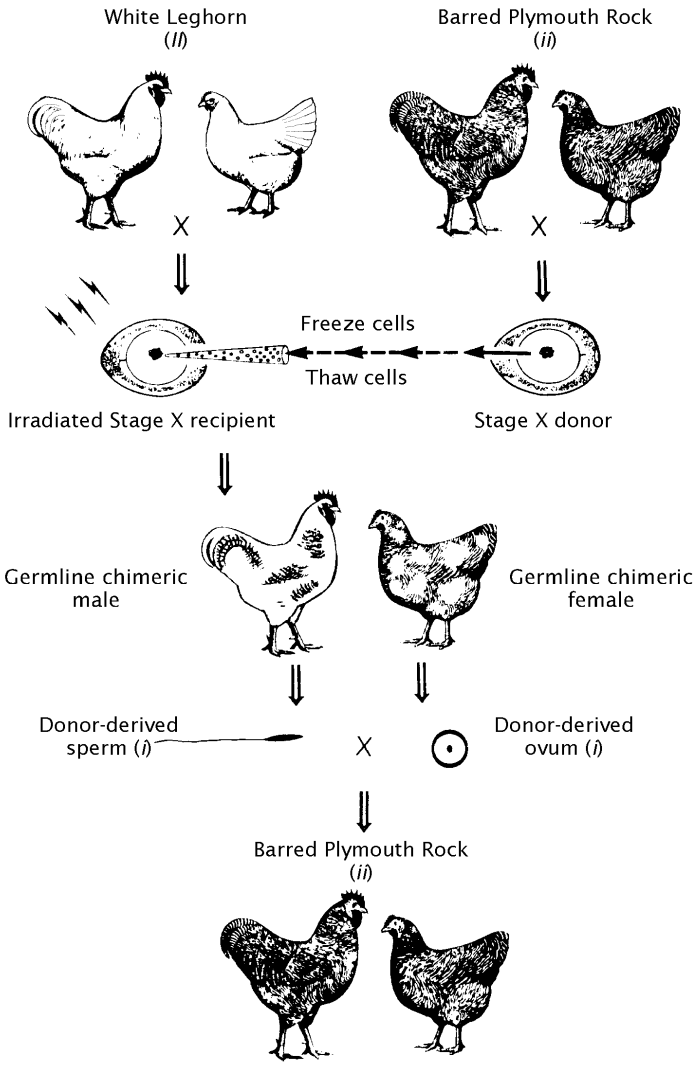

Figure 15. Diagram of avian blastodisc cryopreservation and recovery (from REEDY *et al.* 1995).

Research aimed at improving the rate of germline transmission by cryopreserved blastodermal cells could increase the effectiveness of this technology and reduce the number of blastoderms that are required to ensure that the genetic diversity within a stock is maintained in the reconstituted population. While this would be a welcome improvement in the technology, it should be emphasized that the current technology can be used to conserve genetic resources and that it is the only fiscally realistic way to maintain the genome of stocks in an unadulterated state at the present time.

Box 20. Orphaned CFAR stocks preserved as frozen embryonic cells

THE FIRST LARGE-SCALE DEPOSITION of frozen blastodermal cells was completed in 1998, preserving over 30 of the stocks once kept at the Centre for Food Animal Research (CFAR). This had been the flagship research center for poultry research for Agriculture and Agri-Foods Canada. The cryopreserved CFAR stocks were among those that could not be placed with other institutions before April 1, 1997. Blastodermal cells were harvested and frozen at the Avian Physiology and Genetics Laboratory at the University of Guelph, (Ontario, Canada). If these stocks are required in the future, trained technicians can thaw the cells and make germline chimeras (see Box 19), which can then be mated to obtain pure "reconstituted" birds from the original CFAR lines.

In the future, the range of genetic diversity may increase as transgenic stocks are produced. Many of these stocks will be developed for specific experimental uses that have short-term utility in research. It would be prudent to store these stocks as frozen blastodermal cells rather than discard the time and effort expended in their production.

Primordial germ cell cryopreservation

Cryopreservation of primordial germ cells (PGCs) isolated from the blood of early-stage chick embryos is another way of preserving the intact genome of both male and female birds (NAITO *et al.* 1994a). The PGCs are the precursors of the adult gametes (ova and spermatozoa) and form a distinct population of readily distinguishable cells even before they move from the extra-embryonic germinal crescent into the blood vessels. After a short period of time being carried through embryonic and extra-embryonic circulatory systems, these cells will migrate out of the blood vessels and selectively colonize the gonadal ridges. While in the migratory phase (mostly between HAMBURGER and HAMILTON (1951) stages 13 and 15), the PGCs can be removed from the embryo in blood samples. Primordial germ cells can be partially separated from the blood cells by density gradient centrifugation and can then be cultured (CHANG *et al.* 1995; KUWANA *et al.* 1996), cryopreserved (NAITO *et al.* 1994a), or immediately injected into a host embryo (TAJIMA *et al.* 1993; NAITO *et al.* 1994b). One of the benefits of this method of germplasm preservation is that a highly enriched population of germline cells of approximately 60% PGCs (NAITO *et al.* 1994b) can be introduced into a host embryo. Thus, even when frozen-thawed PGCs were injected, up to 25% of the gametes were shown to be from the donor germline (NAITO *et al.* 1994a). This germline recovery rate is substantially better than that obtained from frozen-thawed blastodisc cells (KINO *et al.* 1997).