PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia biologica (Kraków), vol. 59 (2011), No 1-2
doi:10.3409/fb59_1-2.01-05


## Review

# Cryoconservation of Embryonic Cells and Gametes as a Poultry Biodiversity Preservation Method*

Dorota SAWICKA, Jadwiga BRZEZIŃSKA and Marek BEDNARCZYK

*Accepted October 05, 2010*

SAWICKA D., BRZEZIŃSKA J., BEDNARCZYK M. 2011. Cryoconservation of embryonic cells and gametes as a poultry biodiversity preservation method. Folia biologica (Kraków) **59**: 1-5.

A report of the Food and Agriculture Organization of the United Nations (FAO) from 2000 claims that 9% of the global farm animal population is in a critical condition and 39% is threatened with extinction. Production efficiency, exploitation and conservation of animal genetic resources are crucial not only for the global economy, but also for the environment. As many as 30% of poultry breeds are threatened with extinction and 9% have already gone extinct. To preserve the genetic resources *in situ* methods are used, however, they need to be supported by an *ex situ* strategy. This includes the storage of genetic material in liquid nitrogen under a deep freeze. This process can be performed by using electronically-controlled programs or vitrification. Data shows that usage of electronically-controlled programs leads increases cell viability. A good technique of cell culture and freezing methods will give a broad perspective for unlimited storage of genetic recourses, which in the future can be useful for the restoration of extinct species/breeds.

Key words: Biodiversity, cryoconservation techniques, blastodermal cells, primordial germ cells, chimeras.

*Dorota SAWICKA, Jadwiga BRZEZIŃSKA, Marek BEDNARCZYK, Department of Animal Biotechnology, University of Technology and Life Sciences, Mazowiecka 28, 85-225 Bydgoszcz., Poland.*
*dsawicka@utp.edu.pl*

The Convention on Biological Diversity has defined biodiversity as the diversity of all living organisms present on the Earth, in terrestrial, marine and other water ecosystems, and also in ecological groups which are a part of it (JOURNAL OF LOW 2002). Biodiversity relates to diversity within species, between species and ecosystems. The Convention was the first global agreement with reference to all aspects of biological diversity. The Convention claims that biodiversity conservation is a common concern for all humanity and is an integral part of the development of the world.

Global databases, such as Domestic Animal Diversity Information System (DAD-IS), include information from 182 countries about 35 species of birds and mammals. For poultry, chickens (*Gallus gallus*) represent the largest number of breeds (63%), then ducks (11%), geese (9%) and turkeys (5%). Pigeons and quail present 5%, and 6%, for other species (FAO 2007). A report of the United Nations Food and Agriculture Organization (FAO) from 2000 informs that 9% of the domestic animal population is in critical condition and as many as 39% are threatened with extinction.

Utilization, animal production efficiency and conservation of animal genetic resources are crucial not only for the global economy, but also for the environment (O'BRIEN 1994; CAST 1997). Intensification of industry may lead to changes in ecosystems, even to the extinction of species, which greatly contributes to reducing global biodiversity. Also, rapid structural changes and intensification of poultry production are a major threat to genetic resources, it should be mentioned that up to 30% of the breeds are threatened with extinction and 9% have already gone extinct (HOFFMANN 2009). Most of the extinct breeds originated from Europe. Regions with the largest number of endan-

*Partially supported by the Polish Ministry of Science and Higher Education, No. N N311 310236.

gered poultry breeds are North America (79%), Europe and the Caucasus (49%). They are characterized by high specialization in industrial livestock production. The endangered breeds of chicken and turkey are 6%, 31% breeds of geese, and 25% of ducks. Increase of extinction risk affects both wild (IUCN RED LIST 2006) and domestic species (HODGES 2006). Strategies for genetic resource conservation are different and are used depending on the species. In the case of wild species the most often used strategy is the protection of their natural environment (PISENTI *et al.* 1999).

Country Reports indicates that 26 countries (15%), including 8 developing countries, have poultry stock protection programs (*in situ* and *ex situ*). Twenty four out of 26 countries have chicken preservation programs, 7 for ducks and 2 countries for geese and turkey. Half of the mentioned programs are funded by governments and half by private sector groups, researchers and non-governmental organizations (NGOs). According to the world databases (Global Databank), these programs encompass about 10% of poultry breeds, including: chicken (77%), duck (9%), geese (9%) and turkey (3%). (WĘŻYK 2009).

In the early 70s of the twentieth century in Britain, the Rare Breeds Survival Trust organization took the first action to preserve old poultry breeds. In a short time, similar organizations were also established in the Netherlands, France and Bulgaria. In 1972 a group of Polish scientists led by Professor Wężyk, from the National Institute of Animal Production in Kraków, set up a collection of 7 domestic breeds of hens and the first conservative flocks farm in Poland. At the same time, in the Department of Water Poultry Breeding in Dworzyska Professor Mazanowski, from the Central Laboratory of Poultry Science in Poznań, assembled 12 indigenous breeds of geese and a collection of 6 breeds of duck of different domestic and foreign origin. Wężyk and Mazanowski jointly have worked out a conservation program for the native Polish chicken, duck and geese breeds (WĘŻYK 2009). Preservation has involved Green-Legged Partridge, Yellow-Legged Partridge, Rhode Island Red, Leghorn, Sussex and Polbar chicken (10), moreover, 12 breeds of geese and 11 breeds of ducks (CYWA-BENKO 2002).

At present, numerous organizations across the world have taken up the biodiversity conservation issue. These include FAO, United Nations Educational, Scientific and Cultural Organization (UNESCO) or Farm Animal Genetic Resources (FAnGR). These organizations carry out international programs and cooperate with various countries, combining their goals and knowledge, collected from many different fields, in order to obtain an overview of the current situation and to highlight the risks and prospects. FAO publishes reports about state of the world's genetic resources and also inter alia deals with legal bases of biodiversity management and conservation. UNESCO, by the popularization of knowledge on biodiversity, wants to engage the public opinion in reducing the rate of extinction of genetic resources in order to be able to take effective action in the future, especially in the context of food production. Also the FAnGR diversity takes up the domestic animal genetic resources conservation issue, by *in situ* and *ex situ* methods. The GLOBALDIV project emerged within international co-operation in 2007. This project gathers many experts in different fields from Europe, Asia, Africa and South America. The Netherlands, Germany, Italy, Switzerland, Kenya and Brazil have also joined the cooperation in order to spread knowledge and skills of experts through seminars, workshops and summer schools. There is also a published bulletin which comprises information about new technologies used to preserve biodiversity resources.

The importance of the actions and the international cooperation is shown by United Nations General Assembly has declared 2010 as the International Year of Biodiversity.

## Biodiversity conservation strategies for poultry

At present, two strategies are used for poultry biodiversity conservation: *in situ* and *ex situ*. The *in situ* method is based on maintaining flocks of birds in the traditional region of their occurrence. Maintaining live birds requires considerable financial investment, furthermore the flocks are exposed to loss of genetic variability as a result of selection and genetic drift. Therefore, the *in situ* method should be supported by the *ex situ* conservation strategy. The latter includes the storage of genetic material under a deep-freeze in liquid nitrogen. Materials used for cryoconservation are fragments of DNA, tissues, blood, semen, oocytes, embryonic stem cells and embryos. Cryopreserved biological material can be used to regain the lost variation within breeds and also to restore breeds which have become extinct as a result of an epidemic or the destruction of their natural habitat. The cost of maintaining the genetic material under a deep-freeze is much smaller compared to maintaining a population of live birds. Moreover, the transport of frozen material is much easier than the transport of live birds or fertilized eggs (HAMMERSTEDT 1995).

The following text concerns cryoconservation methods of bird gametes, blastodermal cells and primordial germ cells.

## Cryoconservation of gametes

Cryopreservation of chicken gametes concerns mainly semen. In 1941 it was shown that cryopreserved semen is able to fertilize an egg (SCHAFFNER *et al.* 1941). The crucial event which has contributed to the development of the research on cryopreservation of male gametes was the discovery by Polge that glycerol plays a role as a very good cell protective agent during the freezing process (POLGE *et al.* 1949). The first detailed freezing techniques of poultry semen were developed by LAKE and STEWART (1978) and SEXTON (1980,1981). The results of these studies are currently in use in the latest freezing technologies. The most effective method of freezing, by using dimethylformamide (DMF), was described by ARTEMENKO *et al.* (1990). In the study of SEIGNEURIN and BLESBOIS (2005), poultry semen diluted in mioinozytol was cryopreserved with the addition of 6% DMF. The rate of temperature decrease was 15 °C/min, and the obtained result was 50% cell viability. Similar studies were conducted by ŁUKASZEWICZ *et al.* (2002); however, the rate of temperature decrease was 60 °C/min. An electronically controlled freezing program was used and after thawing (by use of a water bath at 60°C), cell viability in this experiment was 68.4%.

In birds, sex is determined by the arrangement of ZZ and ZW chromosomes. Unlike mammals, the female is heterogametic in birds. Absence of a chromosome W in male gametes makes semen poor biological material for the protection of genetic resources. The best strategy for maintaining bird genetic material would be the cryopreservation of female gametes, but in this case the current technical possibilities are the obstacle.

## Cryoconservation of chicken germ cells

In birds, due to the large size and structure of female gametes the cryopreservation technique is not applied. Germ cells used for cryoconservation include blastodermal cells (BCs), and primordial germ cells (PGCs). This process can be carried out via an electronically controlled program (VARKONYI *et al.* 2007) or vitrification (KOHARA *et al.* 2008). Vitrification is a process based on the progressive increase in the viscosity of liquid, which occurs during a rapid decrease of temperature. Vitrification solutions should be characterized by a maximum ease of glaze formation and its stability and also minimal toxicity in relation to the cells (PAPIS 1997). Vitrification is a simple and inexpensive method that can be used for cryopreservation of genetic material in poultry (KOHARA *et al.* 2008). In the published literature, as of so far, there is no data on chick blastodermal cell survival rate after vitrification. It has been proved that BCs (Fig. 1) tolerate deep-freezing well and after thawing are viable and show a potential ability to produce chimeras (NAITO *et al.* 1992). A chimera is an organism made up of cells from two or more donors that differ in their genetic background. Chicken chimeras can be used inter alia, to restore breeds threatened with extinction. In practice injection of BCs or PGCs into recipient embryos allows obtaining sex chimeras, and through appropriate mating enables the restoration of the genotype of the donor. For this purpose either fresh or thawed BCs or PGCs can be used (KINO *et al.* 1997; NAITO *et al.* 1994). Studies have shown that after thawing cells have correct structure and after injection into the recipient embryo show the ability of further growth and development. Injected donor blastodermal cells incorporate and differentiate in the somatic tissues of

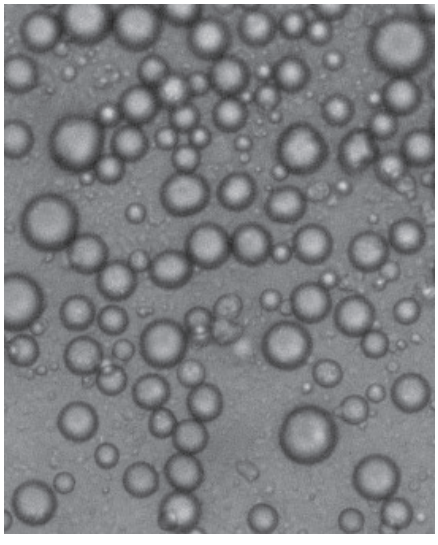

Fig. 1. Living chicken BCs isolated from blastodisc in X stage of development (according to Hamburger–Hamilton) (200×).

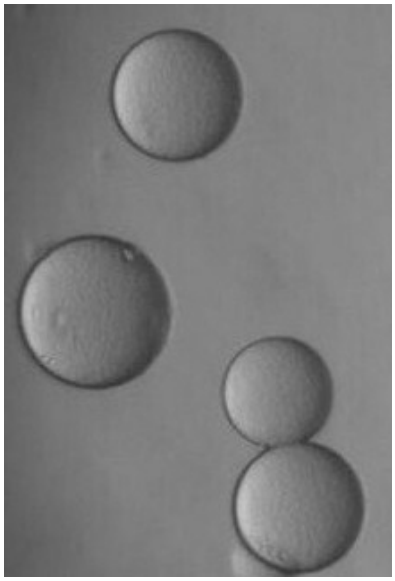

Fig. 2. PCGs isolated from chicken embryos in XIII-XVI stage of development (according to Hamburger–Hamilton) (400×).

recipient embryos (CARCIENCE *et al.* 1993; NAITO *et al.* 1992), while primordial germ cells (Fig. 2) demonstrate the ability to develop and migrate to the recipient gonads. In the gonads, these cells retain the capacity to differentiate and produce functional gametes (KAGAMI *et al.* 1995).

## The procedures of chicken blastodermal cell cryopreservation

The survival of cells after thawing depends, inter alia, on the type and concentration of applied cryoprotectants and also on the rate of temperature decrease during freezing, which is associated with exposing the cells to damage during the processes of dehydration and hydration (MAZUR 1963). TERESHCHENKO *et al*. (1994) studied the impact of cryoprotectants and cooling rate on the survival of chicken BCs. Application of a cooling rate of 1°C/min. and 10% dimethylsulfoxide (DMSO) resulted in 80 to 90% of viable cells (TERESHCHENKO *et al.* 1994). CHEŁMOŃSKA *et al.* (1997) suspended BCs in 10% DMSO equilibrated for 10 min and frozen at a cooling rate of 1°C/min by means of an electronically controlled program (Kriomedpol, Warsaw, Poland). The frozen-thawed blastodermal cell viability was determined by trypan blue dye. Survival of BCs in this experiment was 83% (CHEŁMOŃSKA *et al.* 1997). Experiments show that the application of 10% DMSO and a cooling rate of 1°C/min results in the greatest number of live chicken BCs (TERESHCHENKO *et al.* 1994; CHEŁMOŃSKA *et al*. 1997). In our study (unpublished), chicken blastodermal cells were frozen by means of an electronically controlled program (freeze control CL- 8800 system, CryoLogic, Australia), with the addition of different cryoprotectants: 10% DMSO and a commercial cryoprotectant. For 10% DMSO, 83 % survival of BCs was obtained, similar to CHEŁMOŃSKA *et al.* (1997). However the best survival rate was obtained by using a commercial cryoprotectant. Regardless of the thawing technique, the viability of BCs was from 91 to 94%. NAITO *et al.* (1992) conducted an experiment on chicken and quail BCs. They used 10% DMSO and rate of temperature decrease of 1°C/min, until a final temperature -80°C. For quail, the survival of cells after thawing was 62.7%, despite using the same procedure as in the case of chicken BCs. This departure from the results of TERESHENKO *et al.* (1994) and CHEŁMOŃSKA *et al.* (1997) may be caused by interspecific differences.

## The procedures of chicken primordial germ cell cryopreservation

In 1994 NAITO *et al*. froze chicken primordial germ cells in the presence of 10% DMSO. In these studies a freezing vessel was used (Bicell; Nihon Freezer Co., Tokyo, Japan) and a 1°C/min cooling rate. PGCs were stored in a haze of liquid nitrogen in a deep-freeze and then thawed in water at 4°C (5 min) which resulted in 94.2% viable cells. SETIOKO *et al*. applied 10% DMSO as a cryoprotectant for chicken PGCs and decreased the temperature by 1°C/min. using a NALGENE Cryo 1°C Freezing Container. After freezing, cells were stored in liquid nitrogen. To determine the survival rate, the samples were thawed in a water bath (39°C); the percent of live PGCs was 83.5 (SETIOKO *et al.* 2007). Further studies which dealt with this issue were conducted by KOHARA *et al.* (2008). KOHARA *et al.* used the modified methodology of TAJIMA *et al.* (1998). Cells were suspended in Minimum Essential Medium (MEM) with 10% DMSO and were incubated in ice water for 5 minutes. Then the samples were frozen by using a freezing container (Bicell). The PGC survival rate after thawing at 37.8°C was 91.2 percent.

The procedures mentioned above are related to chicken PGC cryopreservation by using electronically controlled programs. However, this can also be done be using the vitrification process, as applied by KOHARA *et al.* (2008). Using the modified methodology of NAKAO *et al.* (1997), 85.8% living cells were obtained. From the studies mentioned above we can infer that the usage of electronically controlled programs leads to a better primordial germ cell survival rate. The survival rate in all presented experiments was determined by using trypan blue dye.

## Summarizing

For the preservation of global biodiversity, important are not only detailed knowledge or advanced methods, but the strategic point is above all cooperation and exchange of information between farmers, countries, scientists and organizations. The quantity and quality of national and global farm animal genetic resources is the capital in the development of animal production, in the production of healthy food and development of agriculture. Current agriculture is based on the rearing and breeding of a very small number of species and breeds. Local breeds and their natural environment are not taken into consideration. This in a short time can cause serious problems in a very important economic sector. Animals naturally present locally and not used for agricultural purposes are of great importance for a country's economy. These animals are adapted to variable environmental conditions, disease resistant and are not under selection pressure exerted by man. Foods produced from these animals have a different taste and nutritional values.

The presented reasons for biodiversity conservation and the methods of preservation are of great importance not only in poultry production. Development of an appropriate method of cell culture and freezing techniques gives a broad perspective of virtually unlimited storage of genetic material, which may be used in the future to restore extinct species of animals (BEDNARCZYK *et al.* 2002) or to increase genetic variability within a breed.

In this work, *in vitro* studies were presented. However, the evaluation of the efficiency of freezing techniques should also be conducted *in vivo*, for example, after injection of BCs or PGCs to the recipient and assessment of offspring (phenotypic and sex chimeras), but unfortunately these studies have so far been conducted in a very limited scope.

## References


ARTEMENKO A. B., TERESHCHENKO A. V., NOVIKOV A. N., LINNIK T. P. 1990. Cryoconservation of fowl sperm. Ptitsevodstvo (Kyiv) **43**: 14-17.

BEDNARCZYK M., LAKOTA P., SLOMSKI R., PLAWSKI A., LIPINSKI D., SIEMIENIANKO B., CZEKALSKI P., GRAJEWSKI B., DLUZNIEWSKA P. 2002. Reconstitution of a chicken breed by inter se mating of germline chimeric birds. Poult. Sci. **81**: 1347-1353.

CARCIENCE R. S., CLARK M. E., VERRINDER GIBBINS A. M., ETCHES R. J. 1993. Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Development **117**: 669-675.

CAST 1999. Benefits of biodiversity. Report No.133, Council of agricultural Science and technology. Ames, Iowa, 33pp. www.cast-science.org

CHEŁMOŃSKA B., POKORNY P., WOJCIECHOWSKI A. 1997. Cryopreservation of chicken blastoderm cells. Anim. Sci. Pap. Rep. **15**: 73-83.

CYWA-BENKO K. 2002. Genetic and phenotypic characterization of native strains of hens under the biodiversity conservation program. Habilitation Thesis. Institute of Animal Production, Cracow. (In Polish).

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATION. 2007. The State of the World's Animal Genetic Resources for Food and Agriculture.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATION. 2000. World Watch List for Domestic Animal Diversity. 3rd eds. Rome.

HAMMERSTEDT R. H. 1995. Cryopreservation of poultry semen-current status and economics. Pages 229-250 in Proc. 1st Int. Symp. Artif. Insem. Poultry M. R. Bakst and G. J. Wishart, ed. Poult. Sci. Assoc. Champaign, IL.

HODGES J. 2006. Conservation of genes and culture: historical and contemporary issues. Poul. Sci. **85**: 200-209.

HOFFMANN I. 2009. Open questions on poultry genetic diversity. Proc. 6th European Poultry Genetic Symposium in Poland: 61-73.

IUCN RED LIST 2006. http://www.redlist.org/

JOURNAL OF LAW. 2002 No.184 pos.1532.

KAGAMI H., CLARK M. E., VERRINDER GIBBINS A. M., ETCHES R. J. 1995. Sexual differentiation of chimeric chickens containing ZZ and WW cells in the germline. Mol. Reprod. Develop. **42**: 379-388.

KINO K., PAIN B., LEIBO S. B., COCHRAN M., CLARK M. E., ETCHES R. J. 1997. Production of chicken chimeras from injection of frozen-thawed blastodermal cells. Poult. Sci. **76**: 753-760.

KOHARA Y., KANAI Y., TAJIMA A. 2008. Cryopreservation of Gonadal Germ Cells (GGCs) from the Domestic Chicken Using Vitrification. Poult. Sci. **45**: 57-61.

LAKE P. E., STEWART J. M. 1978. Preservation of fowl semen in liquid nitrogen – an improved method. British Pouly. Sci. **19**: 187-194.

ŁUKASZEWICZ E. 2002. An effective method for freezing White Italian gander semen. Theriogenology **58**: 19-27.

MAZUR P. 1963. Kinetics of water loss from cells at sub-zero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. **47**: 347-369.

NAITO M., NIRASAWA K., OISHI T. 1992. Preservation of quail blastoderm cells in liquid nitrogen. British Poul. Sci. **33**: 449-453.

NAITO M., TAJIMA A., TAGAMI T., YASUDA Y., KUWANA T. 1994. Preservation of chick primordial germ cells in liquid nitrogen and subsequent production of viable offspring. J. Reprod. Fertil. **102**: 321-325.

NAKAO K., NAKAGATA N., KATAUKI M. 1997. Simple and efficient vitrification procedure for cryopreservation of mouse embryos. Experimental Animals **46**: 231-234.

O'BRIEN S. 1994. A role of molecular genetics in biological conservation. Proc. Nat. Acad. Sci. USA **91**: 5748-5755.

PAPIS K. 1997. Methods of cryoconservation of mammalian embryos. Biotechnology of Animals. PWN, Warsaw, 472-499.

PISENTI J. M., DELANY M. E., TAYLOR R. L., ABBOTT Jr. U. K., ABPLANALP H., ARTHUR J. A., BAKST M. R., BAXTER-JONES C., BITGOOD J. J., BRADLEY F. A., CHENG K. M., DIETERT R. R., DODGSON J. B., DONOGHUE A. M., EMSLEY A. B., ETCHES R. J., FRAHM R. R., GERRITS R. J., GOETINCK P. F., GRUNDER A. A., HARRY D. E., LAMONT S. J., MARTIN G. R., MCGUIRE P. E., MOBERG G. P., PIERRO L. J., QUALSET C. O., QURESHI M. A., SHULTZ F. T., WILSON B. W. 1999. Avian Genetic Resources at Risk: An Assessment and Proposal for Conservation of Genetic Stocks in the USA and Canada. University of California Division of Agriculture and Natural Resources, Genetic Resources Conservation.Program, Davis CA USA 120p.

POLGE C., SMITCH A. V., PARKERS A. S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature **164**: 666.

SCHAFFNER C. S., HENDERSON E. W., CARD C. G. 1941. Viability of spermatozoa of the chicken under various environmental conditions. Poultry Science **20**: 259-265.

SEIGNEURIN F., BLESBOIS E. 2005. The first method of cryopreservation of Guinea Fowl semen. Sysaaf, Ur Sra, Sra-Inra 37380 Nouzilly, France.

SETIOKO R., TAGAMI T., TASE H., NAKAMURA Y., TAKEDA K., NIRASAWA K. 2007. Cryopreservation of Primordial Germ Cells (PGCs) from White Leghorn Embryos Using Commercial Cryoprotectants. J. Poul. Sci. **44**: 73-77.

SEXTON T. J. 1980. Optimal rate a commercial method for cooling chicken semen from +5°C to -196°C. Poul. Sci. **59**: 2765-2770.

SEXTON T. J. 1981. Development of a commercial method for freezing turkey semen. Effect of prefreeze techniques on the fertility of processed unfrozen and frozen-thawed semen. Poult. Sci. **60**: 1567-1573.

TAJIMA A., NAITO M., YASUDA Y., KUWANA T. Production of germ-line chimeras by transfer of cryopreserved gonadal primordial germ cells ( PGCs) in chicken. J. Exp. Zool. **260**: 265-267.

TERESHCHENKO A. V, ARTEMENKO A. B., TAGIROV M. T., SAKHATSY N. T. 1994. Freezing and applying blastodermal cells in production of chimer of hens. Abstract of reports Intern. Symp. Poultry WPSA in Bydgoszcz, Pieczyska: 91-92.

VARKONYI E., VEGI B., VARADI E., LIPTOI K., BARNA J. 2007. Preliminary results of cryopreservation of early embryonic cells of goose. Proceedings of the 5th Vietnamese-Hungarian International Conf. on "Animal Production and Aquaculture for sustainable Farming": 82-85.

WĘŻYK S. 2009. The poultry gene resources preservation program in Poland- reason and aims. Proc. 6th Europ. Poultry Genetic Symposium in Poland: 13-25.