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The role of the National Research Institute of Animal Production in the development of cryopreservation technology of livestock biological material and its use in *ex situ* conservation

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The protection of endangered breeds allows for the preservation of unique genotypes in breeding and their protection against extinction. The tasks related to the preservation of genetic resources of individual species and breeds are extended to ex situ conservation. It involves collecting biological material in banks in the form of gametes, embryos, somatic cells and tissues. The stored material can be used in assisted reproduction techniques or advanced procedures, e.g. for cloning. Effective cryopreservation methods have been developed in the National Research Institute of Animal Production to collect biological material and implement the objectives of the ex situ conservation program for livestock genetic resources in Poland.

Key words: biological material, cryopreservation, livestock, ex situ conservation

Environmental changes, related to the negative expansion of agricultural lands and the introduction of the species with characteristics preferred by humans into the environment are the main threats that cause a reduction in the animal biodiversity. The crucial element is therefore the conservation of genetic resources of native animal breeds, which are perfectly adapted to local, often very harsh environmental conditions and are characterized by high resistance to disease and good health. The animals can be fed on poor feed resources based on permanent grassland, which also makes it possible to manage and protect areas of high landscape values (Krupiński, 2012). According to the World Conservation Union (IUCN), 30 000 animal and plant species are threatened with extinction, which accounts for 27% of all species on Earth (https:// www.iucnredlist.org/). Their extinction may eventually lead to irreversible biodiversity loss.

Actions on the protection of animals in Poland are based on *in situ* conservation, that is maintaining animals in their natural environment with a reduced use of breeding techniques. The need to rebuild the number of endangered populations was the reason why farm animal conservation was based on *in situ* method. However, Hiemstra et al. (2014) drew attention to the fact that while planning long-term genetic resources conservation it works well to supplement *in situ* method by *ex situ* method, which consists in collecting biological material in banks among others in the form of: male and female gametes, embryos and somatic cells. This is especially important for native breeds, small populations of which are exposed to high level of inbreeding and the effect of genetic drift. In biodiversity conservation programmes, cryopreservation of genetic material can be used as an alternative method for the maintenance of protected animal populations living in the wild, reconstruction of breeds in case of their disappearance or significant decrease of the number of animals. Cryopreservation offers opportunities both to collect and store biological material derived from animals with unique performance and adaptive traits for an indefinite time, it also enables rational controlled reproduction and significantly influences breeding progress and preserving genetic diversity. Moreover, *ex situ* conservation is the only method which allows to collect unique genetic material and use it after the death of its donor.

At the United Nations Conference on Environment and Development in Rio de Janeiro, which was held in 1992, 167 countries being a member of the United Nations signed the Convention on Biological Diversity and international actions for the conservation of farm animal genetic resources were taken (www.cbd.int). Poland ratified the convention and by virtue of the ordinance of the Ministry of Agriculture and Rural Development as of 6 June 2008 (Journal of Laws No. 108, item 691), the National Research Institute of Animal Production became an entity authorized to coordinate the activities related to genetic resources conservation in Poland as well as storing and collecting biological material derived from individual species of farm animals.

The origins of *ex situ* conservation of genetic resources in the National Research Institute of Animal Production date back to the 1960s. In 1968, the Ministry of Agriculture established the Central Sperm Bank (present name: the Bank of Biological Material) under the Institute. The main task of the Central Sperm Bank was to store semen coming from young bulls, which was initially delivered to the Bank from all insemination centres in Poland. Moreover, because of the growing import of semen, the Ministry of Agriculture commissioned the Central Sperm Bank to conduct evaluation and qualification of the semen for insemination within the country. In 1968, the first biological material was stored at the Central Sperm Bank in the form of semen coming from a bull of Polish Red breed. During the 60 years of functioning of the bank, its resources were steadily expanded by the semen of bulls of such breeds as: Polish Red, Polish Red-and-White, Polish Black-and-White and White-backed.

Thanks to this, a unique and the only in Poland collection of biological material was created, which was original in terms of both genotype and the amount of the collected material. At present, the collection of the Bank of Biological Material consists of 63 240 semen portions coming from 165 native bull breeds.

The majority of the material is composed of the semen obtained before breeding activities were implemented, that is the material derived from the animals which were not subject to absorptive crossing with high-yielding breeds of dairy cattle. The stored collection of biological material of native breeds is used as an element of the activities in breeding programmes regarding *in situ* conservation of cattle genetic resources in Poland (Table 1).

The National Bank of Biological Material was established within the National Research Institute of Animal Production in 2014 and aimed at collecting biological material in the form of semen, embryos and oocytes originating from several species of farm animals: pigs, horses, sheep, goats and cattle. Bull semen and embryos of Puławska and Złotnicka Spotted pigs have been collected at the Bank to the present day. It must be noted that technologies concerning semen cryopreservation, among others semen from rams (Kareta and Wierzbowski, 1993; Gogol et al., 2019) and boars (Trzcińska and Bryła, 2015) as well as freezing of pig embryos have been developed in the Department of Reproductive Biotechnology and Cryoconservation for many years. Studies conducted in recent years on cryopreservation of boar semen led to obtaining Patent No PL 228192 B1 for an invention: "Diluent for boar semen freezing and the method of freezing semen" in 2018. Thanks to the patented method it is possible to obtain high quality frozen semen and use it in genetic resources conservation programmes as well as in specialist breeding programmes, which aim at preserving chosen characteristics in breeding, also those that were lost as a result of intensive farming (Trzcińska et al., 2015). Moreover, securing biological material of pigs is a current issue due to an increased risk of the destruction of the whole herds in case of the appearance of African Swine Fever. The problem concerns especially few and closely related herds of native pig breeds, i.e. Puławska, Złotnicka White and Złotnicka Spotted. Collecting biological material in the form of boar semen of these breeds is the only possibility of preserving gene pool of the population in case of its depletion, decreased variability or extinction.

Males and ejaculates earmarked for cryopreservation undergo selection in order to obtain high quality post-thawing semen. The selection of proper males for freezing semen is relatively easy in case of a large animal population. However, in case of endangered or breeding animal species, included in biodiversity conservation programmes, we are dealing with a low number of males in the population within which the choice of proper semen donor with high cryoresistance may be difficult. That is why, the Institute pursues research on the development of effective methods of cryopreservation of male sperm irrespective of the quality of biological material. For this purpose, new technological solutions are applied with the use of high hydrostatic pressure (Bryła and Trzcińska, 2018) to improve the quality of male and female gametes as well as embryos (Pribenszky et al., 2010) and stem cells (Dinnyes et al., 2010). Previous studies have shown that using high hydrostatic pressure in assisted reproduction technologies improves the cryotolerance of the oocytes, blastocysts (Pribenszky et al., 2008) and also spermatozoa (Pribenszky et al., 2008; Huang et al., 2009).

Tabela 1. Izolowany materiał biologiczny zgromadzony w Instytucie Zootechniki Państwowym Instytucie Badawczym
Table 1. Biological material stored at the National Research Institute of Animal Production

Gatunek Species	Nazwa rasy Name of breed	Rodzaj materiału Type of material	Liczba słomek lub kulek Number of straws/pellets	Liczba dawców Number of male donors	Liczba dawczyń Number of female donors	Data pierwszego zdeponowania First year of storage	Data ostatniego zdeponowania Last year of storage
		BANK MATERIAŁÓW BIOLOGICZNYCH BANK OF BIOLOGICAL MATERIAL					
BYDŁO CATTLE	POLSKA CZERWONA POLISH RED	nasienie semen	48211	127	–	1966	2003
	POLSKA CZARNO-BIAŁA POLISH BLACK AND WHITE		9522	16	–	1973	1985
	POLSKA CZERWONO-BIAŁA POLISH RED AND WHITE		5457	21	–	1974	2001
	BIAŁOGRZBIETE WHITEBACKS		50	1	–	2007	2007
		KRAJOWY BANK MATERIAŁÓW BIOLOGICZNYCH NATIONAL BANK OF BIOLOGICAL MATERIAL					
BYDŁO CATTLE	POLSKA CZERWONA POLISH RED	nasienie semen	10725	53	–	2006	2017
	POLSKA CZARNO-BIAŁA POLISH BLACK AND WHITE		2870	16	–	2009	2017
	POLSKA CZERWONO-BIAŁA POLISH RED AND WHITE		3989	19	–	2008	2017
	BIAŁOGRZBIETE WHITEBACKS		1933	34	–	2005	2018
	SIMENTAL SIMMENTAL		860	4	–	2009	2016
	LIMOUSINE LIMOUSINE		200	1	–	2018	2018

	POLSKA HOLSZTYŃSKO-FRYZYJSKA ODMIANA CZARNO-BIAŁEJ POLISH HOLSTEIN-FRIESIAN BLACK AND WHITE		2298	3	–	2007	2011
	POLSKA HOLSZTYŃSKO-FRYZYJSKA ODMIANA CZERWONO-BIAŁEJ POLISH HOLSTEIN-FRESIAN RED AND WHITE		14714	14	–	2009	2014
	PUŁAWSKA		143	4	15	2017	2018
ŚWINIE	PULAWSKA	zarodki					
PIGS	ZŁOTNICKA PSTRA ZLOTNICKA SPOTTED	embryos	34	2	3	2018	2018

In reference to pigs, the development of the method of vitrification of swine embryos in chosen vitrification mixtures (Gajda and Smorag, 2002) obtained *in vitro* and *in vivo* (Gajda and Smorąg, 2004) was also of paramount importance. As a result of the conducted research, Poland's first offspring following the transplantation of vitrified pig embryos was obtained in 2003 (Gajda and Smorąg, 2004). In the following years, research has been undertaken, which enabled to determine the quantity and quality of lipid compounds in particular development stages of *in vivo*-derived pig embryos and those following *in vitro* culture (Romek et al., 2009, 2010, 2011a, b) and the influence of pig embryo culture conditions on their susceptibility to cryopreservation (Gajda and Smorąg, 2002).

Apart from the research on the improvement of cryopreservation technology, work was carried out in order to develop reliable, comprehensive methods of the quality assessment of biological material. In view of long-term storage of biological material, it is essential that the stored biological material is possibly comprehensively assessed in terms of quality. The effectiveness of the practical usage of the collected biological material depends ultimately on its initial quality.

Comprehensive andrological diagnostic methods applied in the Institute are based not only on the standard methods of the evaluation of motility and morphology by means of Computer Assisted Sperm Analysis, but also on the fluorescent evaluation of apoptotic changes. Methods of the evaluation of apoptotic-like changes in the spermatozoa as an indicator of their fertilizing ability have been developed (Trzcińska et al., 2011). Author's own research indicates that the detection of apoptotic-like changes in the spermatozoa makes it possible to determine their fertilizing ability *in vivo* (Bryła and Trzcińska, 2015; Bryła et al., 2009; Trzcińska et al., 2011; Trzcińska et al., 2015; Trzcińska and Bryła, 2015). In the apoptosis process, mitochondrial megachannels are opened and mitochondrial transmembrane potential decreases. Author's own research (Trzcińska et al., 2008) on boar spermatozoa has evidenced a positive correlation between motility, a decline in mitochondrial potential and the percentage of apoptotic spermatozoa. The opening of megachannels and the outflow of ions of calcium from the mitochondria are preceded by other changes characteristic for apoptosis such as chromatin condensation and DNA fragmentation. The evaluation of sperm chromatin structure is performed by Sperm Chromatin Structure Assay (SCSA) (Bochenek et al., 2001; Gogol et al., 2000; Gogol et al., 2002). Tests carried out by Bochenek et al. (2001) showed the presence of high correlation between susceptibility of bull sperm chromatin to denaturation and fertility obtained following the insemination with this semen. Sperm DNA fragmentation is evaluated using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method. Author's own research on boar semen indicated that the procedure of freezing and thawing sperm does not induce DNA fragmentation (Trzcińska and Bryła, 2015).

The Institute has also pursued research on the influence of environmental and technological factors on sperm plasma membrane stability and photon emission as a biophysical phenomenon related to the intensity and correctness of metabolic processes within a cell. Work carried out demonstrated the usefulness of the measurement of photon emission of spermatozoa to assess semen quality (Gogol et al., 2007; Gogol et al., 2009).

Multidirectional activity of the Institute consisting in the development and use of biotechnological methods in animal reproduction and storing biological material allows for the implementation of the objectives of *ex situ* conservation programme for livestock genetic resources in Poland.

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Summary

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