# The Thesis of doctoral (PhD) dissertation

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# DEVELOPMENT OF *IN VITRO* GENE CONSERVATION METHODS IN GOOSE SPECIES *(ANSER ANSER DOMESTICA)* USING EARLY EMBRYONIC CELLS.

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# 1. INTRODUCTION

## 1.1. Antecedents of the work

The population of our planet has grown significantly in recent decades. In 2000 it was only 6.1 billion, whereas in 2021 it was almost 7.8 billion. This large-scale population growth is also affecting humanity's growing need for territory and food. Intensive selection to increase animal production and cross-breeding programmes to maximize meat yields have also led to gene erosion in poultry breeding (*Bessei, 1989*). One of the unfortunate aspects of decline in genetic diversity is that with the spreading of intensive species, indigenous species –including poultry – are effaced.

We have several possible solutions to prevent the loss of valuable, rare alleles and genetic diversity. *In situ, in vivo* gene conservation means, that the species/variety is kept and bred in its natural habitat, while in *ex situ, in vivo* gene conservation nucleus populations are formed and kept in farms, zoos, not in their original habitat. The Institute for Farm Animal Conservation of the National Centre for Biodiversity and Gene Conservation (NBGK-HGI) has been home to native Hungarian poultry and waterfowl species (*Biszkup and Beke, 1951; Báldy, 1954*) as a living gene bank since the 1950s. However, maintaining these live stocks can have several threats so this method alone cannot be suitable for the safe long-term conservation of valuable populations. Therefore, it is also necessary to establish and maintain *ex situ, in vitro* gene banks, where we are able to the long-term preservation of frozen male and female cells, embryos, embryonic cells / tissues, early reproductive tissues and DNA samples from these stocks.

In Hungary, the goose breeding and keeping is traditionally an important agricultural sector, so it was necessary to develop an easy-to-apply, efficient gene conservation procedure for both native and commercial lines with valuable genetic material. An important consideration was that in avian species, the female is heterogametic, therefore the genetic material of the W chromosome as well as mitochondrial DNA can only be preserved by involving the female in the gene conservation procedure. In the case of birds, the solution is the storage of different types of stem cells and then creating germline chimeric individuals by injecting them into recipient individuals. The donor genotype can be regained by cross-breeding these individuals with each other or with the donor breed. As the goose species is a problematic experimental animal in several aspects, there is very few previous research of it.

## 1.2. Objectives

Isolation and long-term maintenance of primordial germ cells in cell culture in the domestic hen species have been developed (*Van de Lavoir et al. 2006; Whyte et al. 2015; Tonus et al. 2016*). This open the way for various manipulations of PG cells in some bird species; when injected back into the circulatory system of recipient embryos, they are able to migrate to the gonads (*Yasuda et al. 1992*) and colonize them (*Ono et al. 1998; Tajima et al. 1993*). However, the culture mediums required to maintain PG cell lines are species-specific, and the extraction, purification, and multiplication of PG cells from donor embryos require high level of infrastructure and qualified human resources which are not available in all laboratories. It became necessary to develop a bridging solution for most poultry and waterfowl species. Unfortunately, the injection of blastodermal cells into the embryo in a very early stage (stage X) is less effective for producing germline chimeras; therefore my primary goal was to develop a new technique combining the two methods. I injected blastodermal cell suspension into the bloodstream of a 3-day-old goose embryo (HH14-17) during the migration period of PG cells into the embryonic gonads. The starting point was that there are already primordial germ cells among the early embryonic stem cells, which can migrate to their final destination after being injected into the recipient embryo at the appropriate time, thus increasing the proportion of germline chimeras (*Patakiné et al. 2017*).

During my work I set the following goals:

- Precise localisation of the period of goose embryo development when the primordial germ cells migrate in the circulation system of the embryo (HH14-17).
- Immunostaining visualization of the blastodermal cell suspension contains PG cells and that cells can migrate in the bloodstream of an appropriate developing goose embryo.
- Examination of harmful effects of the injection method on embryonic development.
- Determination of the presence and location of donor cells using microsatellite marker analysis on dead recipient embryos and hatched day-old geese.
- Finally, investigation of the optimal timing for most effective chimera production.

# 2. MATERIALS AND METHODS

## 2.1. Establishment of goose breeding pair groups based on microsatellite marker studies

Microsatellite markers are available to detect the presence of donor cells in recipient individuals (*Zhou and Lamont, 1999*). I selected individuals into each family, which have different alleles in homozygous form in case of a goose-specific microsatellite marker of my choice, the Bcaμ3 (*Buchholz et al. 1998).*

I took 500-700 µl of blood from the brachial vein of 144 animals into a 1.5 µl eppendorf tube containing sodium citrate. DNA was isolated from the blood samples using a conventional salting out method (*Miller et al. 1988*). The dried DNA was redissolved in 50 µl distilled water at 37ºC overnight. After measuring the DNA concentrations of the samples, I equilibrated them to the required 5 ng/µl and stored them at -20°C until genotyping. PCR products were prepared using CY5 fluorescently labelled primers and visualised on polyacrylamide gel using an Alf Express II automatic DNA analyser.

Based on the results obtained, I formed the donor and recipient experimental groups. The 28 selected individuals of the donor stock contained the 155bp allele in homozygous form, while the 35 selected individuals of the recipient stock carried another allele of 159bp, also in homozygous form.

## 2.2. Determining the embryonic developmental stages of the Hungarian goose

To inject goose embryonic cells, first I had to determine the embryonic developmental stages when primordial germ cell migration occurs. In domestic hens, the embryo reaches these stages of development after 52-58 hours (HH13-16) of incubation. However, since the embryonic development of the domestic goose lasts 29-31 days compared to 21 days in the hen, I had to investigate when the goose embryo reaches the developmental stages HH14-16 for injection. There were no data available about this in the literature.

Freshly laid Hungarian goose eggs were washed with 0.25% hypochlorite solution and incubated at 37.7ºC, 70% humidity, and rotated 90° per hour in a PL Machine MIDI F500S incubator according to the requirements of the species.

From hour 60 to hour 89 of incubation, I removed an egg from the incubator every 10 minutes, opened a window in the lateral side with egg-breaking forceps in the middle, and examined the developing embryo after removing the shell membrane using a Leica Axioscope stereomicroscope. For

the correct assessment of the number of somites, Fast Green dye was injected under the embryonic body. Based on this examination, I determined the developmental stages of goose embryos, using the domestic hen developmental stages elaborated by *Hamburger & Hamilton* (1951). I noted the length of incubation and the developmental stage of the embryo. I summarized the results in a database and created photo documentation. A total of 745 goose embryos were examined.

## 2.3 Fluorescent immunodetection of primordial germ cells in whole goose embryos

Goose embryos at HH16 stage of development were removed from the surface of the yolk using a filter paper ring. I washed them in phosphate buffered saline (PBS) and fixed in PBS solution containing 4% paraformaldehyde (PFA) overnight at 4°C. The embryos were then washed 3 times on a shaking table in PBS containing 0.01% bovine serum albumin /BSA/ for 30 min each time. Embryos were permeabilized with PBS solution containing 1% BSA and 0.1% Triton-X-100 overnight at 4°C. Then, also at 4°C, the embryos were incubated with anti-CVH primary antibody at a dilution of 1:100 in PBS containing 0.1% BSA overnight. The next day, the samples were washed 3 times consecutively for 30 min in a shaking thermostat in PBS containing 0.01% BSA at room temperature, then incubated with fluorescein isothiocyanate secondary antibody in PBS containing 0.1% BSA overnight at 4°C. Thereafter, two washes of 30 min each were performed at room temperature in PBS containing 0.01% BSA. Nucleus staining was performed in a darkroom at room temperature for 15 min using 1 µM TO-PRO™-3 in PBS solution. This was followed by three washes in PBS for 30 min. Samples were stored in 0.01% BSA-PBS at 4°C until microscopic examination.

For taking the photographs, the samples were placed on a slide, covered with a cover slip and Vectashield cover solution, and then examined with a confocal microscope .

## 2.4 Immunostaining of goose blastodermal cell suspensions with primordial germ cell-specific markers

Blastodermal cells isolated as described in subsection 2.6 were suspended in PBS solution and fixed in 4% PFA solution for 10 min at 4°C. After three washes in PBS solution containing 0.01% BSA for 5-5 min each time, cells were blocked with PBS containing 0.1% Triton-X-100 and 2.5% donkey serum for 45 min at room temperature. Cells were then incubated overnight in a vapour chamber at 4°C with a 1:100 dilution of anti-CVH primary antibody. After incubation, I washed the samples three times for 5-5 min with PBS solution containing 0.01% BSA. Then I incubated the cells with IgG FITC

secondary antibody in a dark vapour chamber at 37°C for 1 hour and after washing with 0.01% BSA PBS solution, I stained the nucleus with TO-PRO™-3 dye. Slides were covered using a coverslip and 10 µl of Vectashield coverslip solution, and then samples were examined under a confocal microscope.

## 2.5. Management of recipient eggs before injection

Once a week, 30 recipient eggs were placed in the hatching machine. I incubated the goose eggs under conditions suitable for the species. Injection of recipient eggs was started after 72 hours of incubation.

## 2.6. Isolation of donor blastodermal cells

The donor-derived blastodermal cells used for injection in the experiment were obtained from freshly laid, unincubated, fertile eggs from the donor family.

Eggs until use, but for a maximum of 4 days are stored in an egg storage room at 16-18°C. For the experiment, cells were isolated from the germinal discs of 14-16 eggs. To avoid the risk of infection, the eggs were rinsed with 70% ethanol and then carefully cracked. A sterile filter paper ring was placed on the germinal disc, which, after adhering sufficiently to the perivitelline membrane, was cut around with scissors and the blastodermal cells were washed from the removed membrane piece into a sterile centrifuge tube with DMEM "high glucose" medium using a sterile syringe and needle. The collected cells were carefully suspended in the culture medium using a pipette and centrifuged at 2300 rpm for 7 min at 4°C. Then the cells were carefully aspirated from the upper layer of the precipitate and resuspended in 2.5 ml of sterile DMEM medium. To remove debris and yolk I repeated this step once again. After the second centrifugation, most of the supernatant was removed, leaving only 0.5 ml of medium containing the cells. Cell viability was determined by live-dead staining with Chicago Sky Blue dye (*Kútvölgyi et al, 2006*), and by counting with a Makler chamber. The prepared cell suspension was stained with 10µl Fast Green dye and stored in a thermostat at 37°C until use.

## 2.7 Injection of donor early embryonic cells into 3-day-old recipient goose embryos

After 72 hours of incubation, I started removing the recipient eggs one by one from the hatcher. The lateral side and the blunt end of the egg were disinfected with Betadine solution to prevent infection. A 1 mm diameter hole was made in the side of the air chamber with a sharp tweezer, through which 0.5 ml of albumen was sucked.

This was necessary for the easier availability of the developing embryo during the injection. Then, on the lateral side of the egg, a hole was made no larger than 15x15 mm and then the shell membrane was removed. The egg was placed into a petri dish lined with sterile paper wadding, which served as a support. Approximately 0.5 ml of sterile PBS solution containing 10% penicillin - streptomycin at 37°C was dropped into the open part of the egg to prevent infection and to avoid drying out of the embryo. Then I placed the prepared egg under the stereomicroscope. I attached a 10 µl Hamilton syringe to the micromanipulator, which I had set up earlier, with a drawn glass capillary at the end. Using the micromanipulator, after puncturing the extraembryonic membranes, I injected 1-1.5 µl of donor cell suspension (containing approximately 600-800 cells) into the heart tube of the recipient embryo. After successful injection, a few drops of the antibiotic-containing PBS solution were again dropped into the egg, and the window was sealed with double layers of parafilm. I sealed the small hole opened at the blunt end with paper glue. I individually marked the eggs and immediately placed them back into the incubator.

## 2.8. Verifying chimerism by microsatellite marker analysis

The DNA analyses were performed on samples from embryos and different embryonic organs. Brain, heart, liver and gonad samples for microsatellite marker analysis were collected from hatchlings and dead embryos at later stages. Only dead embryos that had reached or exceeded the day 10 of embryonic development were analysed. DNA was isolated from all tissues using the previously described method. The tissues were incubated in 300 µl of nucleus lysis buffer with 10 µl of proteinase K enzyme and 10% SDS (*Miller et al. 1988*), following a protocol optimized for poultry tissues. Then I used the DNA isolation protocol applied to the blood samples. A total of 191 samples were analysed and genotyped, of which 171 were from dead embryos and the rest were from hatched and slaughtered day-old geese. I searched for heterozygote animals, carrying both donor (155 bp) and recipient (159 bp) alleles together, to verify the chimerism.

## 2.9. Control experiments

In order to study the harmful effects of the injection method to the embryo development, two control experiments were performed on 100 fertile goose eggs per experiment. I named them control groups K1 and K2.

The K1 control group was the so-called "window" control group. Since no similar studies have yet been carried out in goose eggs, implementation of K1 control experiment was necessary.

In this experiment, the lateral sides of the eggs were disinfected with Betadine solution, then a little hole was opened at the area of the air chamber to suck out 0.5 mL albumen with a syringe and then the eggshell and the shell membrane were removed from a 15 mm x 15 mm area. The "windows" were sealed with double laboratory parafilm, and it was perforated two days before hatching. The eggs were marked individually. The aim of this control group: To determine the effect that the making of a window itself (without the injection) has on the survival of the embryos.

The K2 control group was the injected control. In the K2 control group, holes were opened (the same way as described above) at the lateral side of the eggs after disinfection and removing 0.5 mL albumen. The hearts of the recipient embryos were injected with 1-1.5 µL DMEM tissue culture medium painted with 5% Fast green dye®. The procedure was performed with a micromanipulator under a stereomicroscope. Finally, the window was covered with parafilm, the eggs were marked individually, and they were put back into the incubator. The aim of this control group was to determine the effect of the injection procedure on the survival of embryos.

## 2.10. Statistical analysis

Statistical methods were used to analyse the distribution of developmental stages of goose embryos as dependent on the length of incubation time. I used interquartile range (IQR) to characterize the temporal occurrence of each embryonic developmental stage. The IQR value represents the interval where the middle 50% of all values are. To compare the experimental groups, I used a logistic regression model, where data from both control groups (K1, K2) and the experimental group injected with blastodermal cell suspension (T) were subjected to pairwise comparisons. In the case of this method, comparisons were made using Tukey's test (*Reiczigel et al. 2007).* To determine the optimal time of injection, I used one-way analysis of variance (ANOVA). In all cases where there was a significant difference, I used Fisher's Least Significant Difference (LSD) test to compare the results of the three groups. All data obtained were evaluated using the programs R studio version 0.99.489 and Statistica 7.0 at a significance level of $P < 0.05$.

# 3. RESULTS AND DISCUSSION

## 3.1 Investigation of embryonic developmental stages and determination of the optimal period for injection in Hungarian goose

As a first step, comparative embryo development studies were performed to identify the appropriate embryonic developmental stage when migration of the PGCs takes place in the goose embryo. Statistical parameters (minimum, maximum, median, lower and upper quartiles) were determined (*Figure 1*.) from the incubation time data to characterize each developmental stage appropriately. An IQR (interquartile range) was chosen to describe when each of the Hamburger and Hamilton developmental stages are typically observed in the goose species during the embryonic development. The results are shown in *Figure 1*.

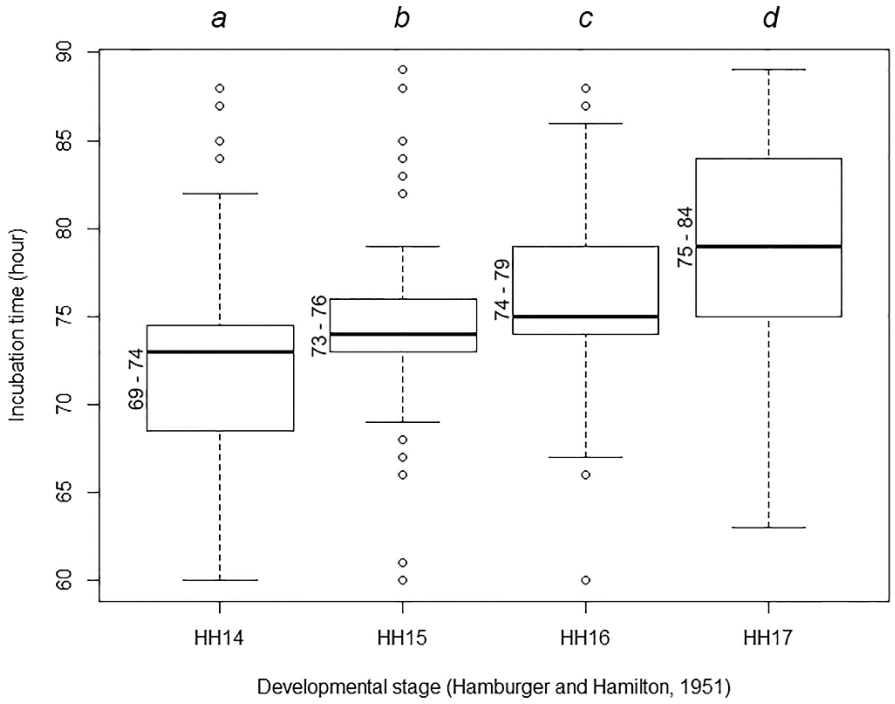


**Figure 1: Temporal distribution of the goose embryos examined at different developmental stages (Goose embryos corresponding to the Hamburger-Hamilton developmental stages shown in Figure 2).**

The square plots represent descriptive statistical parameters, the boxes show the interquartile range (IQR), which I used to characterize the typical developmental timeframes of each stage, the median is indicated by the horizontal line inside the box, the whiskers above and below the boxes show the range (lower and upper quartiles), and the outstanding datas are indicated by the circles.

a: HH14: Min.: 60.0, Median: 73.0, Max.: 88.0, IQR: 68.8-74.3;
b: HH15: Min.: 60.0, Median: 74.0, Max.: 89.0, IQR: 63.0-76.0;
c: HH16: Min.: 60.0, Median: 75.0, Max.: 88.0, IQR: 74.0-79.0;
d: HH17: Min.: 63.0, Median: 78.5, Max.: 89.0, IQR: 75.0-84.0)

To summarize goose embryos reach the HH 14-17 equivalent developmental stages between 69 to 84 hours of incubation (*Figures 1 and 2*). Among the individuals the variance in stage HH14-17 was very high (*Figure 2*.).

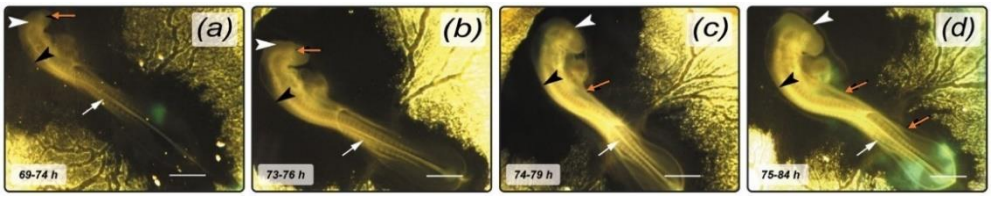


**Figure 2: Optimal developmental stages for injecting Hungarian goose embryos between HH14 and HH17** (Hamburger and Hamilton, 1951) **and illustration of the main morphological features of each stage** (scale: 1 mm)**.**

**a: HH14 stage** (69-74 hours), embryo has 22 somites (white arrow), cranial inclination angle is about 90° (white arrowhead), cervical curvature is wide (black arrowhead), primary optic vesicle begins to invaginate (orange arrow);

**b: HH15 stage** (73-76 hours), 26 somites (white arrow), skull includes sharp angle to the body (white arrowhead), cervical curvature is broad (black arrowhead), ocular ridge is fully developed, distinct double contour in the iris region is visible (orange arrow);

**c: stage HH16** (74-79 hours), when the embryo has 28 somites (white arrow), all curvatures are more pronounced (white and black arrowheads), the wing bud appears as a thickened ridge for the first time (orange arrow);

**d: stage HH17** (75-84 hours), 32 somites are observed (white arrow), the cervical curvature is more sharply curved but still includes an angle greater than 90° (black arrowhead), the epiphysis appears for the first time (white arrowhead), wing formation and foot buds are visible (orange arrows), the cranial curvature is unchanged.

Statistical analysis was carried out to define whether there is any relationship between the embryonic developmental stages and the elapsed incubation time (*Figure 1*). Moderate positive correlation was obtained reflecting the heterogeneity of the embryo quality as well as the stock itself, as the Hungarian goose species is an indigenous breed.

The above results indicate that HH 14-17 equivalent stage which likely contains PGC cells in bloodstream of goose embryo similarly to chicken occur between 69-84 hours of incubation. These results suggest that injection of blastodermal cells in this window period may be suitable for germ line chimera generation in goose.

## 3.2 Fluorescent immunodetection study of goose primordial germ cell migration in the embryo

The cornerstone of the planning experiment was the hypothesis, that on one hand primordial germ cells are present among the early blastodermal cells and on the other hand these cells can migrate to the gonads in the goose embryo at the same developmental stages as in domestic hens. Germ cell-specific immunostaining, as shown in *Figure 3*, demonstrated that the goose blastodermal cell suspension contains primordial germ cells and the migration of these PG cells in the goose embryo occurs at the same developmental stages as in domestic chickens. No such studies have been performed in goose species before.

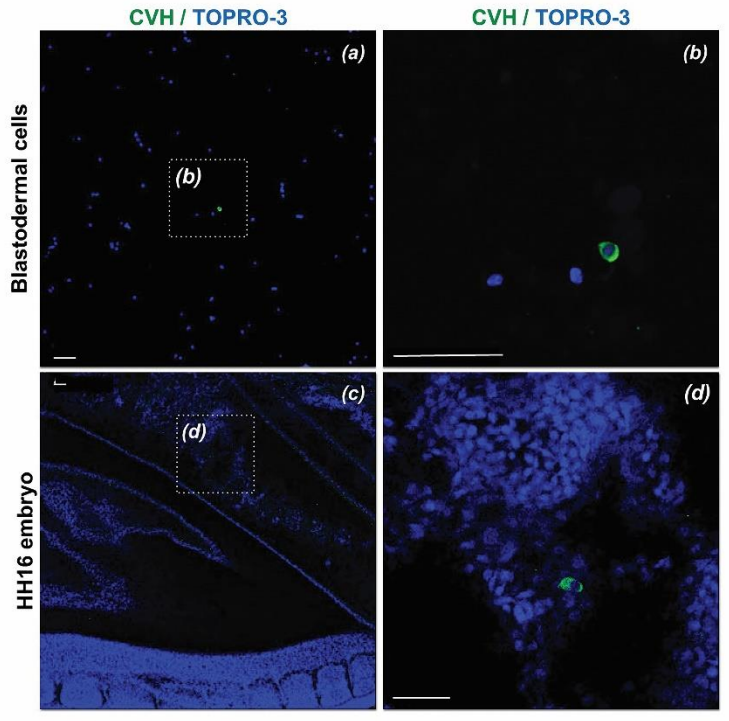


**Figure 3: Immunostaining of primordial germ cells (PGC) among blastodermal cells (A, B) and in HH16 stage goose embryo during migration (C, D).**

Photographs show chicken vasa homolog (CVH) positive PGC cells in the blastodermal cell suspension (**A, B**) and in the extraembryonic region of a HH16 stage goose embryo (**C, D**). Photographs labelled **B** and **D** are higher magnification images of the areas circled in squares in photographs **A** and **C**. Nuclei were stained with TO-PRO™-3. Scale bars: 50 µm

## 3.3. Intracardiac injection into 3-day-old goose embryos and the efficiency of chimera production

The aim of my work was to determine whether blastodermal or early PG cells collected from freshly laid unincubated eggs injected into the bloodstream of a recipient embryo with appropriate HH developmental stage can reach and colonize the embryo's gonads. During the injection experiments, 1-1.5 µl of the donor blastodermal cell suspension (600-800 cells) were injected into the heart tube of 249 healthy, appropriately developed recipient goose embryos using micromanipulator.

The trial was deemed successful, when the developing embryo reached or exceeded the $10^{th}$ day of development (*Table 1*) by which time most embryonic mortality caused by the method occurred. On this basis, 83 embryos were retained in the treated group, which led to 19 hatched gooslings (*Table 1*).

Microsatellite marker analysis was carried out with all the 171 samples collected from the 83 initial gooslings or dead embryos, which revealed the presence of donor cells in gonadal tissues of 3 embryos (one 12-day old dead embryo, two hatched gooslings; 3.6%). One additional goosling showed chimerism in its brain and heart tissue samples (13-day old, 1.2%), adding altogether to a rate of chimerism of **4.8%** of the analyzed embryos and gooslings. Chimeras were also found among the younger dead embryos ($\leq 10^{th}$ day of development), but were excluded from the calculation of rate of chimerism.

Injections were performed after 72 - 78 hours of incubation. It is worth noting that one chimera was taken from the 73 h (73 h 44 min) injected group, another from the group injected after 75 h incubation and two from the group injected after 76 h incubation.

When determining the optimal time of the injection, the procedure at hour **75** in both control groups proved to be significantly better in terms of the proportion of embryos classified as good for the experiment. This correlation was not significant in the experimental group.

| | K1 | | | K2 | | | EXPERIMENTAL GROUP (T) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **INCUBATION TIME (h)** | **number of treated eggs** | **embryonic development ≥ 10 days** | **hatched** | **number of treated eggs** | **embryonic development ≥ 10 days** | **hatched** | **number of treated eggs** | **embryonic development ≥ 10 days** | **hatched** | **chimeras developed ≥ 10 days** |
| **72** | 0 | 0 | 0 | 7 | 0 | 0 | 4 | 3 | 0 | 0 |
| **73** | 9 | 3 | 2 | 38 | 10 | 3 | 35 | 9 | 2 | **1** |
| **74** | 27 | 17 | 9 | 26 | 13 | 6 | 78 | 27 | 7 | 0 |
| **75** | 41 | 29 | 22 | 14 | 10 | 6 | 64 | 21 | 6 | **1** |
| **76** | 17 | 6 | 5 | 9 | 5 | 3 | 43 | 13 | 2 | **2** |
| **77** | 6 | 2 | 1 | 6 | 2 | 2 | 23 | 8 | 2 | 0 |
| **78** | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 |
| **Total (nr) (%)** | **100** | **57 (57%)** | **39 (39%)** | **100** | **40 (40%)** | **20 (20%)** | **249** | **83 (33,33%)** | **19 (7,63%)** | **4 (1,6%)** |
| **percentages as a function of the number of embryos in the experimental group alive ("considered good") beyond incubation day 10** | | | | | | | | **100%** | **22,9%** | **4,8%** |

**Table 1: Summary of the experimental data**

**K1:** The "windowed" control group, in which a 15x15 mm "window" was opened in the egg and then sealed with double layers of Parafilm; **K2**: Injected control group, in which I injected 1-1.5 µl of DMEM culture medium into the heart tubes of recipient embryos after the window was created. Embryos in the experimental group (**T**) were injected with blastodermal cell suspension.

## 3.4. Effect of injection on mortality of recipient embryos

Comparing the embryonic development and hatching rates in the **K1** (57/100 and 39/100) and **K2** (40/100 and 20/100) control groups with the results of the injected (**T**) group (83/249 and 19/249), it can be concluded that the number of surviving and hatched gooslings beyond 10 days of incubation decreased with the invasiveness of the procedure (*Table 1.*). The Tukey test showed significant differences between groups T and K1 ($P < 0.001$) and between control groups K1 and K2 ($P = 0.0288$), but no significant difference in the number of embryos that died after 10 days nor in the percentage of hatched gooslings between groups T and K2. These suggest that the injection of blastodermal cells did not significantly increase embryo mortality compared to the injection of dye only (*Figure 4.*).

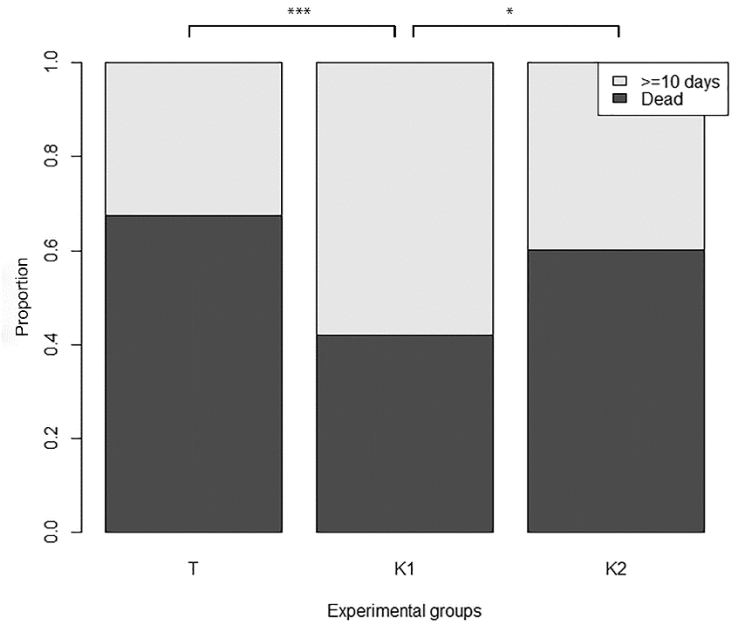


**Figure 4: Embryonic mortality rates in the different experimental groups**

**T** is the treated group, in which donor blastodermal cell suspensions were injected into recipient embryos through a 15x15 mm window opened in the eggshell, and the window was closed with double layers of parafilm. In control group **K1**- the "windowed" control group- I opened only the 15x15 mm "window", then closed it back with double layers of parafilm. In control group **K2**- injected control group- I injected 1-1.5 µl of DMEM medium into the heart tube of embryos after opening the "window", and then closed the window with double layers of parafilm. Mortality data from the treated (**T**) and the two control groups (**K1, K2**) were compared using logistic regression with Tukey's test. There was a significant difference between the values of the treated (**T**) and control group **K1** (***P <0.001), and between the data of the two control groups (*P=0.0288).

Based on statistical analysis, the optimal time for manipulation is around **75 hours** of incubation. In the two control groups obtained the highest number of embryos with a "good" classification (embryos surviving 10 days of incubation, *Figure 5*.) in this time interval, but this correlation was not significant for the experimental group (T). Since the hatched chimeras were obtained from embryos injected after 74-76 hours of incubation, this time interval is expected to have the highest probability of successful intracardiac injection.

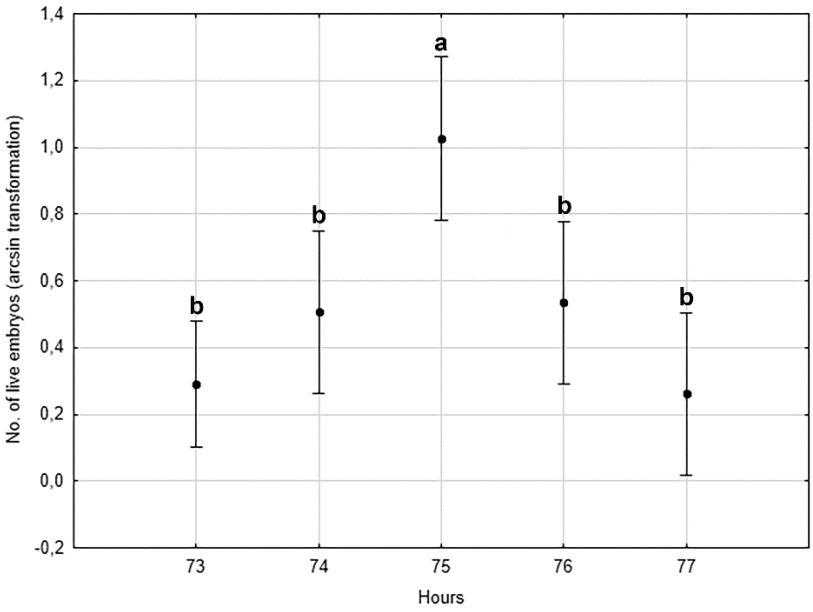


**Figure 5: The rate of live embryos (≥ 10 days) in the different time groups (a; b: $p < 0.001$)**

The initial data was statistically analysed by Arcsin transformation, One-Way Anova and Fisher LSD methods. According to the analysis there were significantly more embryos, which reached day 10 (after the injection) in the 75-hour group than in any other group. (Numbers on the X axis indicate the length of the incubation.)

# 4. CONCLUSIONS AND SUGGESTIONS

## 4.1 Embryonic development studies in Hungarian goose

Embryo developmental studies from oviposition onwards, similar to the HH embryo developmental stages of the domestic hen, are high importance for researches with domestic goose stem cells. These studies are important not only at species level but also at breed level, since on the one hand the hatching period of the domestic goose differs significantly from that of the domestic hen and on the other hand, experience shows that there is a high variability - especially in native breeds-even at individual level. In domestic fowl, the presence of primordial germ cells in the circulatory system reaches its maximum at the HH14 stage (50-53 h after oviposition) (*Tajima et al. 1999*). Migrating PG cells exit the circulation in the intermediate mesoderm and begin to concentrate from the HH15 - HH17 stage (52-64 hours after egg laying in chicken) (*Nakamura et al. 2007*). The determination of this so-called "window period", when PG cells migrate in the bloodstream is especially important regarding my research. I assumed that this period occurs at the same embryonic developmental stages as in the domestic hen species, but is shifted due to the longer incubation period. At the time of starting my experiments, I could not find any results in the relevant literature on the detailed study of this period in domestic geese.

*Liptói* (2005) described the embryonic development of the domestic goose from oviposition to hatching; however, this study does not describe the development of the goose embryo during hatching in comparison to the HH embryonic development stages, but only on a daily time scale.

*Łukaszewicz et al*. (2017) investigated the embryo development in white koluda geese with unincubated eggs and eggs after 4, 8, 12, and 16 h incubation, in relation to HH and EG&K stages of domestic fowls from EG&K stage X. onwards. In their work, they concluded that goose embryos from unincubated eggs were at stage X and XI of EG&K. According to their observations the individuals already showed a wide variation in development at this stage. After 16 hours of incubation, the embryos were between HH2 and HH4 developmental stages. Their results show that while the developmental stages of the embryo in the unincubated goose egg are similar to that of the hen, the goose embryo is slightly larger in diameter. Following incubation, the goose embryo develops more slowly than the chicken embryo until the end of the 16-hour incubation period they studied. This study analyses the early embryo development of the domestic goose in much more details, but the period during which the peak of PG cell migration is observed in the bloodstream (equivalent to HH14-17) was not investigated. However, this research group also found that there is a high variation in embryonic

development between goose embryos within species, even at very early developmental stages.

*Sellier et al*. (2006) compared embryonic development of several bird species from oviposition to 72 hours of incubation, based on EG&K and HH developmental stages. Embryo development was assessed in every 6 hours, based on 145 eggs from domestic hens. They concluded that while the chicken embryo was at EG&K stage X at the time of egg laying, the Landaise goose they studied was at EG&K stage XI. Due to its longer incubation period (28-30 days), it reaches the respective HH stages in a longer incubation period compared to the domestic fowl. The HH14-17 developmental stage, which is particularly important for my studies, was observed to be reached by chicken embryos between 48 and 60 h of incubation, while the 251 goose embryos they studied reached HH14-16 stages between 60 and 72 h of incubation. Their data are the same as mine in order of magnitude, which shows that the majority of native Hungarian goose embryos reach the HH14-17 stage of development after 68.8-84 hours of incubation. However, an important difference between the two studies is that while *Sellier's* research team examined development every six hours, I monitored embryo development every 10 minutes from the 60th hour of incubation until the 89th hour, examining a total of 745 embryos. A further important difference between the two studies is that the rate of embryo development in native species shows considerable individual variation, so it was even more justified to carry out this study.

*Li* and colleagues (*2019*) studied the complete embryo development and compared it to the HH embryo developmental stages of chicken in ducks and geese, but they studied embryos of *Anser cygnoides* (swan goose) rather than *Anser anser domestica* (domestic goose), the species I studied. A total of 600 goose eggs were examined, and embryo development was examined every hour for the first 72 hours of incubation, and then every six hours from day three to day ten. For the stages relevant to my research, they found that HH13 stage was reached between 55 and 62 hours, HH14 between 61 and 65 hours, HH15 between 63 and 69 hours, HH16 between 67 and 73 hours, and HH17 stage between 71 and 86 hours, which are similar to my results. Although this study is about a different species of goose, the more thorough and comprehensive embryonic development studies carried out by *Li's* research team so far provide a good basis for comparison with my own studies.

## 4.2 Developing a new method for gene conservation using blastodermal cells by producing germline chimeras in Hungarian goose

The International Union for Conservation of Nature’s Red List shows a total of 11,147 bird species, of which 13.3%, or 1,486 species, are classified as critically endangered, endangered or vulnerable (IUCN Red List).

According to the United Nations Food and Agriculture Organization (FAO) surveys of domestic animals, 7% of the nearly 8,800 listed breeds of 40 species are extinct and a further 24% are threatened with extinction (*FAO 2019, http://www.fao.org/resources/infographics/ infographics-details/en/c/174199*). There are 3,689 poultry breeds registered worldwide, of which 2,222 are indigenous. Of these species, 28% are endangered, vulnerable or extinct (FAO, DAD-IS). These data show that a high percentage of domesticated poultry and wild bird species fall into some risk category. Therefore, active action and appropriate conservation strategies are needed as soon as possible in order to slow down the current trend and to stop it in the future. Conserving the valuable and rare genetic information of native poultry species is of paramount importance for maintaining agricultural biodiversity (*Wang et al. 2017*).

In Hungary, goose breeding is a traditional and economically important sector of animal husbandry, and that is why it is important to preserve the genetic stock of the indigenous Hungarian goose. During my research I developed a method that allows the long-term conservation of the genetic material (W chromosome and mitochondrial DNA) of both sexes of the species. At the beginning of my studies and research work (2012), very few research had been carried out on avian primordial germ cells, only partial results had been obtained, and only in domestic fowl. Our institute has been involved in gene conservation research with blastodermal cells in several bird species for a long time, so I aimed to develop this method for the goose species. In my studies, I have sought to combine the simplicity of blastodermal cell isolation with the ability of PG cells to migrate to the gonads. Thus, I developed a new method to efficiently inject stem cells into domestic geese.

As there is only few literature on the use of embryonic cells of the domestic geese in gene conservation research (both blastodermal cells and PG cells), I will compare my results with similar studies on embryos of other bird species.

Chimera production experiments by injecting donor blastodermal cells into recipient EG&K stage X recipients have been previously performed by several research groups in several species. In goose species, only *Bednarczyk* and colleagues (*Bednarczyk et al. 2003*) reported the injection of donor blastodermal cells into the subgerminal cavity of recipient EG&K stage X-XII embryos, where 6.7% of treated eggs hatched, but none of the hatched gooslings proved to be germline chimeras. In chickens, *Carsience* and his research group (1993) conducted a study in which approximately 100 or 200-400 blastodermal cells from Barred Plymouth Rock fertile donor eggs were injected into untreated and treated with different doses of γ-irradiation. White Leghorn EG&K stage X recipients. Somatic chimerism was determined by feather colour, while germline chimerism was determined by backcrossing hatched individuals with Barred Plymouth Rock. The frequency of germline

chimerism after injection of approximately 100 donor cells using irradiated recipients was significantly ($P<0.001$) higher (3/24 cells, 12.5%) than for untreated recipients (2/106 cells, 1.9%). Increasing the number of cells injected had no effect on the frequency of chimerism in non-irradiated recipients. Injection of 200-400 cells into irradiated recipients significantly ($P<0.01$) increased the incidence of germline chimerism to 8/14 (57.1%). The rate of germline chimeras was related to the number of hatched individuals. Overall, it was found that irradiation caused embryos to be slightly retarded in development, produced a more prolonged hatching, and hatched a significantly lower percentage of total eggs, but a higher proportion of them had germline chimeras than untreated recipients, based on the number of hatchlings. The germline chimerism rate (1.9%) obtained for the non-irradiated hatchlings in relation to the total number of hatchlings is most comparable to the germline chimerism rate obtained by myself, where **10.52%** (2 animals) of the hatched individuals had germline chimeras relative to the number of hatched individuals (19 animals). Thus, my technology development of injecting the blastodermal cell suspension not into the subgerminal cavity of the blastoderm, but into the heart tube/bloodstream of the 3-day-old embryo, significantly increased the obtained germline chimera ratio. *Kino et al* (1997) used fresh and frozen-thawed blastodermal cells. Approximately 500 blastodermal cells from Barred Plymouth Rock were injected into the subgerminal cavity of irradiated EG&K stage X White Leghorn recipient embryos. Injections were also performed with fresh and Percoll-Nycoprep gradient centrifuged freeze-thawed blastodermal cells. After injection, hatching was performed in a surrogate eggshell, which was 20-25 g larger than the original recipient egg. Somatic chimerism was determined by colour and germline chimerism by test crosses. Fresh blastodermal cell suspensions were used to inject 59 recipients, of which 24 hatched and 22 of which were probably somatic chimeras. Test crosses were performed on 16 animals, resulting in 9 confirmed as germline chimeras (56% of all test crosses). A lower efficiency was obtained with frozen-thawed cells, but the comparison with my experiments is not relevant in this case, as I was working only with freshly isolated blastodermal cells. The comparability of the results is complicated by the fact that *Kino's* team used irradiated recipients, which increases the chance of donor cells integrating, and that they placed the developing embryos in a different, larger eggshell during hatching. In addition, as an experimental animal, the chicken is more resistant to manipulation than the goose and is much less sensitive to hatching conditions and various stresses. *Li et al* (2002) attempted to create interspecific chimeras using donor Maya duck and White Leghorn hen recipient breeds. Duck blastodermal cells of donor origin were injected into the subgerminal cavity of EG&K stage X recipient hen embryos. The experiment was performed on a 600 rad γ irradiated group of 112 recipient injected individuals (group 1) and

an untreated (non-irradiated) control group of 121 recipient individuals (group 2). Of the 112 animals in the treated group, 14 hatched (12.5%), and of the embryos and hatchlings that developed beyond 13 days, 18 (16%) were likely to be somatic chimeras based on feather colour, but only 4 (3.57%) hatched. Of the 121 animals in the non-irradiated control group, 21 hatched (17.4%), 12 (9.9%) were likely to have somatic chimerism based on feather colour, of which 7 hatched (5.78%) and the rest died before hatching. After backcrossing with the donor, it was concluded that only one male (0.82%) proved to be a germline chimera, which was from the untreated group. This experiment shows that it is possible to recover an endangered species by creating interspecific chimeras, and also provides an interesting result on the issue of irradiated recipients. Hatchability and developmental vigours are clearly impaired by irradiation, as described in previous studies, but interestingly the only germline chimera they obtained was not from the irradiated recipient group, although previous studies have largely found that irradiation of recipients increases the likelihood of chimera (both somatic and germline) being produced.

At present, the maintenance of efficient, long-lasting cell cultures capable of preserving their gamete-generating capacity is only possible in the domestic fowl species (*Van De Lavoir et al. 2006; Macdonald et al. 2010; Whyte et al. 2015; Oishi et al. 2016; Tonus et al. 2016; Kong et al. 2018*). For other bird species, there are very few successful attempts to culture and maintain primordial germ cells.

Goose primordial germ cell culture and maintenance (*Doddamani, 2019*) is the only previous research, but the method is still under development. They were able to maintain the PG cells in their serum-free BMP4-containing medium for more than three months, but the proliferation rate was lower than that of domestic fowl cell cultures. Also, in this study, no *in vivo* testing of donor-derived PG cells was performed, i.e. the cultures were only graded by proliferation assays, gene expression assays, and no reimplantation of cells into the recipient was performed.

Zebra finch (*Taeniopygia guttata*) PG cell lines were maintained in culture for more than 30 days by Jung et al. (*Jung et al. 2019*) using a feeder cell layer and high FBS concentration (10%). *Gessara et al*. aimed to establish a culture medium for zebra finch primordial germ cells that was free of feeder cells and contained minimal FBS to avoid early differentiation (*Gessara et al. 2020*). Under the conditions they used, cells in cell culture started to destruct after 15-20 days. About 500 of the GFP-labelled cells they cultured *in vitro* were injected under the blastodiscs of freshly laid, fertile recipient eggs. Out of 22 injected eggs, 10 individuals (45.4%) hatched in mixed sex (6 females and 4 males), all of which had the donor-derived, cultured; GFP-labelled PG cells in their gonads.

*Yakhkeshi et al. (2018)* isolated Japanese quail (*Coturnix japonica*) primordial germ cells from the blood of HH13-15 stage embryos and gonads of HH28-30 stage embryos. These cells were cultured on a quail embryonic fibroblast conditioned medium. After 40-50 days, the number of cells in the PG cell culture isolated from blood increased 100-fold, while the number of gonad-derived PG cells increased 400-fold, so the latter were used for their experiments. In addition to the proliferation and gene expression assays of the cells, 1000 GFP+ PG cells were injected into the heart tubes of HH13-15 stage recipient embryos to study their migration and incorporation efficiency. Of the 310 embryos, 59% survival was observed on day 6 of incubation and 34% survival on day 16. PG cells containing GFP were found in 16% of embryos alive on day 6 and in 24% of embryos alive on day 16. It was concluded that the cultured PG cells had normal migratory and incorporation capacity.

*Tagami* and colleagues *(2017*) found that EG&K stage X (freshly laid) domestic hen embryos already contain early PG cells, and I hypothesized by analogy that this is also the case for geese. My subsequent immunostaining studies confirmed this hypothesis. In my experiment, 83 of the 249 injected recipient embryos developed beyond the 10-day of incubation (33%). I considered this number of individuals as successful manipulations for the purpose of the experiment. Out of 83 individuals, 19 hatched (22.9%) and in 4 of the 83 embryos I was able to detect the allele confirming the presence of donor cells (**4.8%**). If I project the success rate to the number of hatched individuals, **10.52%** of the live individuals proved to be germline chimeras (2). Exactly this type of experiment has not yet been done in any species, but the PG cell injection experiment into the subgerminal cavity in zebra finch by *Gessara* and colleagues is the "reverse" of my study. Their hatching rate was very high (45.4%, 10 out of 22 animals), but the number of embryos treated was low compared to my study. Another interesting result was that the presence of donor PG cells was detected in the gonads of all hatched individuals. They did not publish data for the unhatched animals, which might have given more insight into the success of the experiment, and since no other literature reports 100% germline chimeras in any species, this somewhat calls this result into question. Comparing the results of *Yakhkeshi's* research group's experiment on quail with my own results, I conclude that although they injected cultured PG cells into the bloodstream of recipient embryos, the survival in their experiment (59% at day 6 and 34% at day 16) was similar to my survival (33%) for incubation times longer than 10 days, and the number of recipient embryos injected was much higher (310 individuals). They achieved better results than me in terms of PG cell incorporation (4.8% of individuals surviving 10 days of incubation showed germline chimeras), but no hatchlings were reported, unlike in my research.

The new method used in my experiment works more efficiently than the method based on the use of blastodermal cells isolated at stage X of EG&K and injected back into the subgerminal cavity of the recipient embryo at the same stage, because the cells are not integrated mosaically throughout the body, creating somatic chimeras, but colonize the recipient gonads much more efficiently, and thus produce higher proportion of germline chimeras. Developing a culture medium suitable for goose PG cells and then efficiently maintaining cell cultures over the long time period is more difficult, more expensive and requires more human and laboratory resources than my method. In conclusion, the method I have developed - injection of blastodermal cell suspension containing early PG cells isolated from freshly laid eggs into the heart tube of a recipient goose embryo of HH14-17 development - I find suitable for gene conservation with stem cells in geese.

## 4.3. Suggestions

Goose embryo development studies should be extended to more goose breeds from EG&K stage X to hatching, preferably with as many embryos as possible, with maximum hourly monitoring of development. In this way, a database could be created to provide a better overview of the differences in embryonic development between breeds. I would also consider it useful doing the embryo development study more complex by monitoring egg size, weight and laying time within the cycle, as these values are related to the rate of development. This could be useful data for further researches.

As for the development of the method, I would suggest fluorescent labelling of the donor cells used, so that their migration and colonisation of the recipient embryo can be investigated. Furthermore, the method could be more simplified by using a mouth pipette for injection. In the future, an experiment using frozen/thawed blastodermal cells could be considered. In case a goose primordial germ cell culture medium is developed to allow long-term maintenance, the ability of the maintained cell lines to produce gametes should be tested by re-injection, the results of which could be compared with those obtained by my own experiments.

As for the use of this method, I would suggest the storage of embryonic cells of indigenous and producing breeds and lines in the Genebank of our Institute. This would be of great importance, firstly because goose flocks are under constant threat due to avian influenza disease, which occurs every year to some breeders. On the other hand, there is a demand from goose breeders to maintain the genetic material of their lines that are not currently being used, not in live, but *in vitro* form. This is because keeping geese is extremely expensive, especially if no income is generated. The importance of preserving the genetic material of indigenous breeds is much discussed nowadays, but a detailed discussion of this issue is beyond the scope of this paper.

However, the sperm, blastodermal cell and primordial germ cell samples of the Hungarian native goose flock maintained at our Institute have been deposited in the Genebank.

# 5. NEW SCIENTIFIC RESULTS

1. I determined that Hungarian goose embryos reach the HH14-17 developmental stage after **69-84** hours of incubation. This is the period when the injection of blastodermal cells is the most efficient.

2. I demonstrated using germ cell-specific immunostaining that the blastodermal cell suspension isolated from EG&K stage X (freshly laid) goose eggs contains primordial germ cells (PGC).

3. I demonstrated the presence of migrating primordial germ cells in the bloodstream of goose embryos incubated for 69-84 hours by using germ cell-specific immunostaining.

4. I elaborated a new method for germline chimera production by intracardiac injection of blastodermal cells into the heart tubes of 3-day-old embryos, which is cost-effective and does not require very complex and expensive laboratory equipment. I have found that the optimal period for the injection is between **74-76** hours of incubation in goose species.

5. I successfully produced germline chimeras in Hungarian goose breeds by this developed method, and confirmed the chimerism using molecular genetic markers.

# 6. PUBLICATIONS ON THE SUBJECT OF THE PhD. THESIS

**Scientific papers with impact factor:**

**Sztán, N.**, Patakiné Várkonyi, E., Liptói, K., Barna, J. (2012): Baromfifajok embrionális sejtjeinek kezelésével szerzett tapasztalatok/Observations of embryonic cell manipulations in different poultry species **MAGYAR ÁLLATORVOSOK LAPJA**:(8) pp. 475-481. **Q3, IF: 0.108**

Patakiné Várkonyi, E., Horváth, G., **Sztán, N.**, Váradi, É., Barna, J. (2012): Vitrification of Early Avian Blastodermal Cells with a New Type of Cryocontainer **ACTA VETERINARIA HUNGARICA** 60: (4) pp. 501-509. Doi: https://doi.org/10.1556/avet.2012.044 **Q2, IF: 1.202**

Patakiné Várkonyi, E., Molnár, M., **Sztán, N.**, Váradi, É., Végi, B., Pusztai, P. (2016): Egy értékes hazai baromfifajtánk, a magyar parlagi gyöngytyúk (*Numida meleagris*) embrionális blasztodermasejtjeinek mélyhűtése génmegőrzés céljából / Cryopreservation of embryonic blastodermal cells of a valuable domestic poultry breed, the Hungarian landrace guinea fowl (*Numida meleagris*) as a biodiversity preservation method **MAGYAR ÁLLATORVOSOK LAPJA** 138/11: pp. 673-680. **Q4, IF: 0.031**

**Sztán, N.**, Lázár, B., Bodzsár, N., Végi, B., Liptói, K., Pain, B., Patakiné Várkonyi, E. (2017): Successful chimera production in the Hungarian goose (*Anser anser domestica*) by intracardiac injection of blastodermal cells in 3-day-old embryos. **REPRODUCTION, FERTILITY AND DEVELOPMENT**. Doi: 10.1071/RD16289. **Q1, IF: 2.656**

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Pálinkás-Bodzsár, N., Sztán, N., Edviné Meleg, E., Hidas, A. (2021): Molekuláris genetikai vizsgálatok őshonos magyar baromfi fajtákon a génmegőrzés tekintetében *In:* ***ÁLLATTENYÉSZTÉS ÉS TAKARMÁNYOZÁS*** (0230-1814): 70 3 175-184. p.

**Book chapter on the subject of the PhD. thesis:**

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**Papers published in conference proceedings:**

**Sztán, N**., Bodzsár, N., Liptói, K., Hidas, A. (2010): Biotechnológiai módszerek alkalmazása a tyúk fajban genetikai diverzitás megőrzése céljából. *In: oral presentation: Genetikai Műhelyek Magyarországon, IX. Minikonferencia,* Szeged, 2010.09.03. Apperance: Hungary

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