HUNGARIAN UNIVERSITY OF
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GONADAL EXPERIMENTS IN CHICKEN: TESTING OF THE EFFECT OF HEAT TREATMENT AND THE CAPACITY OF PGCS INTEGRATION

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2022

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1 INTRODUCTION AND AIMS

1.1 Introduction

In the XXI. century, the effects of human activity on Earth were already being felt. Our climate has deteriorated significantly and is expected to deteriorate further if we do not reduce the use of fossil fuels ("The Intergovernmental Panel on Climate Change: 30 Years Informing Global Climate Action," 2018). As temperature rises, so do the production of crops and livestock. As the ambient temperature is unfavourable for them, their productivity decreases (Babinszky et al., 2011; Zaboli et al., 2019). In my dissertation I analysed the changes in the reproductive system of domestic chickens as a result of the preliminary heat treatment. The biggest breakthrough would be if the pre-heat treatment process could achieve a modification in the primordial germ cells that can be passed on to the offspring.

The progenitor cells of gametes, the primordial germ cells (PGCs), are able to preserve the entire genetic material, so the role of these cells is definitive. Already at the beginning of 1990, the importance of stem cells in epigenetic inheritance was recognized. The process of epigenetic regulation in stem cells has been an area of intense research ever since. (Tajima et al., 1993; Naito et al., 1994). It was not until 2006 that a research team was able to develop the maintenance of these cells in a special medium (van de Lavoir et al., 2006)

When primordial germ cells are injected back into a developing embryo of the recipient species, they are able to integrate into its gonads thus creating a germline chimera. Finding an ideal recipient breed which can be applied widely among breeds is an important task. In my dissertation, I summarized the research work in which I examined the changes in the expression of heat shock factors and heat shock proteins in the gonads of domestic chickens and

the ability of the progenitor cells to integrate into the gonads of three old Hungarian breeds.

1.2 Aims

- ❖ Molecular biological study of the effect of heat treatment in the reproductive organs of domestic chickens:
  - Investigation of the expression of heat shock proteins and heat shock factors in control and heat-treated male and female chicks.
  - Investigation of the expression of heat shock proteins and heat shock factors in control and heat-treated adult male and female chickens.

- ❖ Findings the ideal recipient breed:
  - Establishment of GFP-expressing PGC cultures.
  - *In vitro* characterization of GFP-expressing PGCs.
  - Re-injection of GFP-expressing PGCs into recipient breeds and examination of the integration efficiency.

2 METHODS

The experiments were done in the National Centre for Biodiversity and Gene Conservation – Institute for Gene Conservation Science and Small Animal Research (NBGK-HGI) at Gödöllő, and in the Hungarian University of Agriculture and Life Science – Genetics and Biotechnology (MATE-GBI) Institute. Both institutes had the permission for experiments conducted on animals. NBGK-HGI (License number: PE/EA197-4/2016), MATE-GBI (License number: 106685/4/2005).

2.1 Methods of the heat-treatment experiments

Preliminary results for the heat treatment experiments were already available and performed at HGI and made available to me. In the preliminary results, the Transylvanian Naked-neck chickens were also exposed to a longer-term (2 weeks) heat stress after heat-treatment, and then the productivity parameters were determined. In the case of roosters, the spermatological parameters and in case of hens the egg production parameters were determined.

The second heat treatment was performed under the same parameters as the preliminary heat treatment experiment (60% humidity at 37.8 oC) involving 30-30 individuals. After direct treatment, 15 heat-treated and 15 control animals were sampled. A total of 5 samples were collected (brain, liver, left-right genitals and muscle). The sex of each individual was determined by molecular biological methods (sex PCR). After RNA isolation from the samples, real-time qPCR gene expression studies were performed for two heat shock proteins (*HSP70, HSP90*) and 4 heat shock factors (*HSF1*, *HSF2*, *HSF3* and *HSF4*). Relative expression values were determined using Genex MultiD

software, where GAPDH was the reference gene and chicken embryonic fibroblast (CEF) was used as a reference.

2.2 Methods of the fluorescently labelled PGC culture characterization

The primordial germ cell (PGC) lines were established from the eggs of chickens expressing the green fluorescent (GFP) protein designed and used by the Roslin Institute in Scotland. Approximately 1-1.5 µl of blood containing PGCs were aspirated from the embryo using a glass microcapillary attached to a mouth pipette. The isolated blood was placed in a special culture medium that only aids in the development of PGCs. Cultivation was performed in a CO_2 thermostat at 38 ^oC with a CO_2 level of 5%. In the case of a sufficient amount of PGCs, the primordial germ cells were cryopreserved with a special freezing medium and stored in liquid nitrogen or in a freezer at minus 150 ^oC. The sex of PGCs were determined (sex PCR) using the P2-P8 primer pair.

The primordial germ cells have been characterized by several methods. Firstly, the RNA was isolated from the cells using the RNAqueous™ Micro Total RNA Isolation Kit (Thermo Fisher Scientific, MA, USA). After that the complementary DNA-written and was used for real-time qPCR. The relative expression of two germ cell-specific (CVH, DAZL) and three stem cell-specific (OCT4, POUV, miR302a) markers were determined.

PG cells were also characterized by immunohistochemistry. After reaching the optimal cell number, staining was performed with primary antibodies CVH, P63 and SSEA1. TO-PRO™-3 nuclear stain was used to label the nucleus. The rate of PG cell proliferation and the ratio of apoptotic cells were determined using an ImageXpress Pico EC (Molecular Devices, LLC, San Jose, CA, USA) automated fluorescence imaging and image analysis

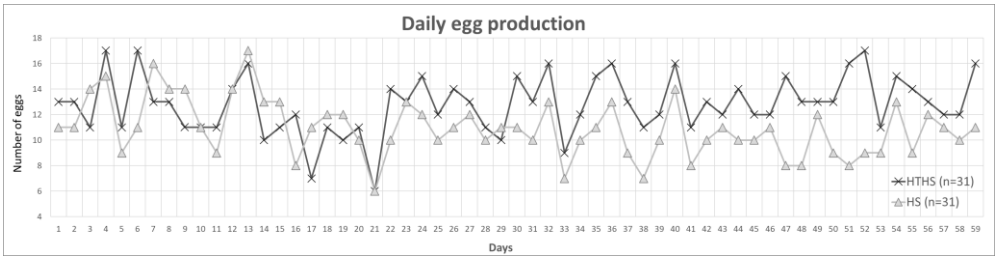
microscope. Fluorescently labelled (GFP) PGCs were re-injected into three native Hungarian domestic chicken breeds (white Hungarian, yellow Hungarian, and partridge-coloured Hungarian). After determining the appropriate cell number, 1 µl of the cell suspension was injected into the embryonic bloodstream using a glass microcapillary connected to a mouth pipette. The ratio of integration was determined on day 14 of embryonic development. The gonads were examined first with a Leica confocal microscope (Leica TCS SP8, Leica Ltd., Germany) and then with the fluorescent Leica stereomicroscope of our laboratory (Leica M205 FCA, Leica Ltd., Germany). The gonads were fixed in 4% PFA for later examination. The gonads were embedded in 7.5% gelatine according to a protocol developed by Dr. Nándor Nagy, head of the Laboratory of the Institute of Anatomy, Histology and Development at Semmelweis University of Medicine. Organs embedded in gelatine were frozen in minus 45 ^{o}C in isopentane. The frozen gelatine cube was mounted to a cryostat sample plate (MicroM GmbH D-6900 Heidelberg, Germany) where sections of 10–15µm were made. The sections were placed on a Polyzine slide, which guaranteed that the sections would not slip off the plate during the first step of immunostaining during rehydration. Primary antibodies P63 and CVH were used in the immunohistochemistry to detect PGCs. The obtained data were represented and analysed using RStudio (1.0.136), R (R-3.2.2), Genex 7.0 (MultiD Analysis AB, Gothenburg, Sweden) and Excel (MS Office) software.

3 RESULTS

3.1 Results of the heat-treatment experiments

3.1.1 Preliminary-results (NBGK-HGI)

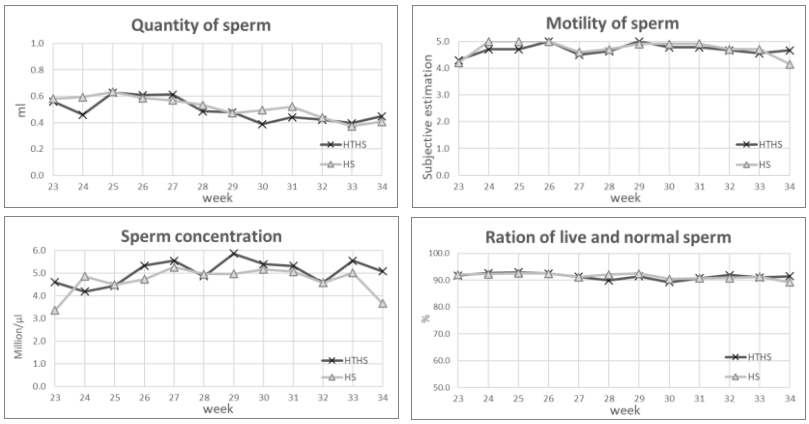
Data from preliminary experiments were already available before the start of my experiments. In the case of layers, two parameters were examined by NBGK-HGI staff in 2015–2016. Daily egg production was compared between pre-heat-treated animals and untreated laying hens. They found that pre-heat-treated hens were produced significantly mores eggs than the control group. A total of 1654 eggs were examined. Heat-treated laying hens produced 890 (54%) eggs at higher ambient temperatures, while control animals produced 764 (46%) eggs **(Fig. 1)**. After egg candling on day 7 of the incubation, 6.18% (55 eggs) of the pre-treated hens' eggs and 5.46% of the control hens' eggs (42 eggs) had to be removed because embryonic development did not begin. Further examination of the eggs showed that 10.91% of the eggs of the heat-treated hens and 42.86% of the eggs of the control hens were infertile. (Anand et al., 2016; Tóth et al., 2021).



1. figure: Representation of the number of eggs per day changed by heat treatment in the heat-stressed animals. HS: Heat-stressed animals, HTHS: Pre-heat-treated and heat-stressed animals (Tóth et al., 2021).

The results of spermatological examinations of roosters show a high degree of similarity between the performance of previously heat-treated and control animals. Fertility results of ten heat-treated roosters and ten control roosters were analysed based on four parameters (quality, concentration,

motility, and live-dead ratio) **(Fig. 2)**. There was no significant difference in ejaculate volume between the two groups (p = 0.5075). There was no difference in sperm concentration between the two groups (p = 0.1077), nor was there a difference in motility (p = 0.6972). Finally, there was no significant change in the proportion of live-dead sperm ratio in the two groups (p = 0.8816). In summary, spermatological results show that pre-treatment did not affect rooster productivity and spermatological parameters.



2. Figure: Effect of pre-heat treatment on spermatological parameters of rooster in case of heat stress. HS: Heat-stressed animals, HTHS: Pre-heat-treated and heat-stressed animals (Tóth et al., 2021).

3.1.2 Effect of heat-treatment on the molecular biology level

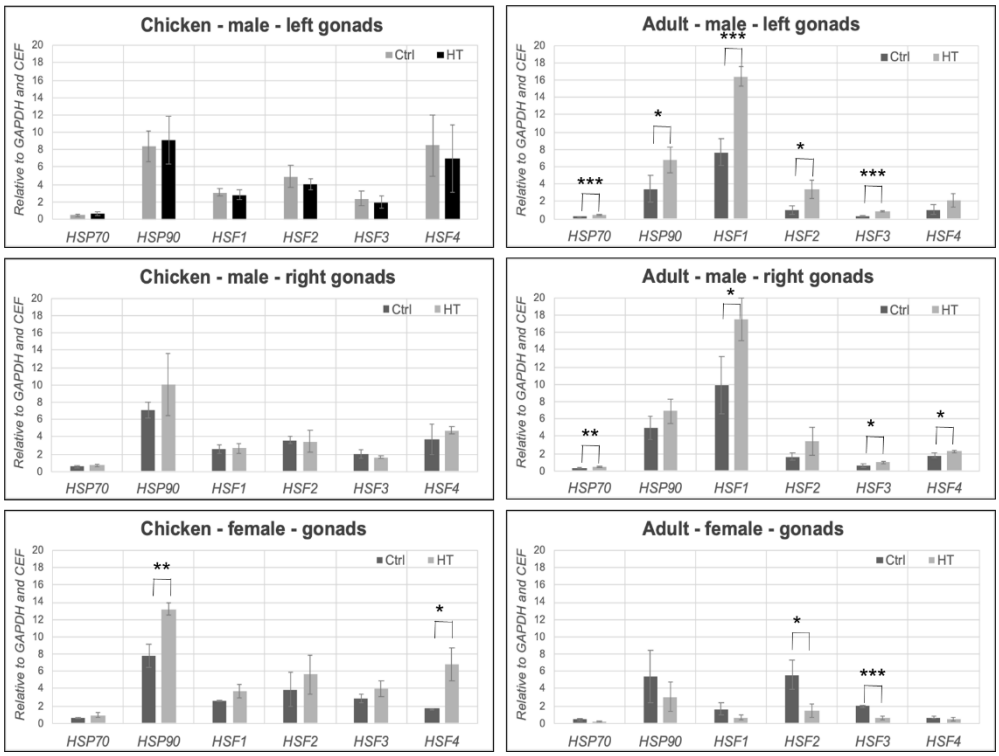
30 - 30 chickens participated in the second heat-treatment experiment. The first 30 animals were pre-heat treated while the other 30 were kept at average ambient temperature. Immediately after the heat treatment, 15 to 15 animals were randomly selected from the samples, and the other animals were kept up until sexual maturity. Samples were grouped by sex (rooster, hen), age group (chick, mature), and treatment (heat-treated, control). Each group contained at least 4 individuals. RNA samples from the individuals in the groups were examined both individually and pooled. The mean of the individually measured values as well as the results of the pooled samples showed great

similarity. Delta Ct values were compared based on individual data. There was a significant difference in the delta Ct values of *HSP70* ($p = 0.0289$) and *HSF3* ($p = 0.0482$) between the heat-treated and control groups in the male left gonads at adult age. Between the chick and the sexually mature groups in the male left gonads, all six genes showed significant differences. In the male right gonads, there was also a significant difference in the delta Ct values of *HSP70* ($p = 0.0136$) and *HSF3* ($p = 0.0349$) in mature individuals, between the treated and untreated groups. The expression profiles of the right genital of control roosters *HSP70* ($p = 0.023$), *HSF1* ($p = 0.0007$), and *HSF3* ($p = 0.0013$) differed significantly between chicks and mature individuals.

In case of hens, there was a significant difference in the delta Ct of *HSP90* ($p = 0.0355$) and *HSF4* ($p = 0.0342$) immediately after heat treatment in chickens between control and heat-treated individuals. The expression value of *HSF4* alone ($p = 0.0016$) was significantly increased between control chickens and control sexually mature chickens.

Relative expression values were also determined for the expression changes of the heat shock protein (HSP) and heat shock factor (HSF) genes, where the GAPDH was the reference gene **(Fig. 3)**. The calculation was performed with Genex (7.0) software (MultiD Analyzes AB, Gothenburg, Sveden). Immediately after heat treatment, significantly higher relative expression levels of the *HSP90* ($p = 0.0094$) and *HSF4* ($p = 0.0387$) genes were observed in chicks alone. Compared to mature animals, the opposite relationship is seen. In pre-heat-treated animals, the expression levels of all heat shock-associated genes were lower, than in control animals. The difference was significant for *HSF2* ($p = 0.0181$) and *HSF3* ($p = 0.0094$) genes. There was no significant difference in testes in roosters immediately after heat treatment. At matured age, the relative expression of all genes in the left testes except *HSF4* was significantly increased. In the case of the right

gonads, the relative expression of *HSP70* (p = 0.0052), *HSF1* (p = 0.0333), *HSF3* (p = 0.0332) and *HSF4* (p = 0.0498) was significantly higher.

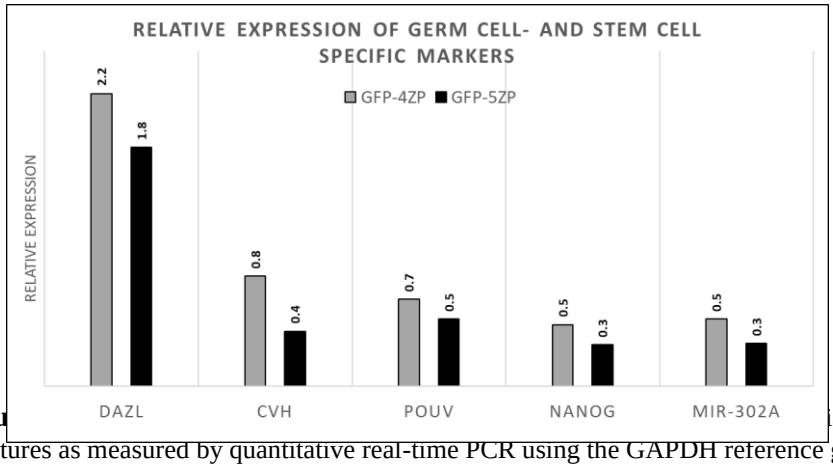


3. Figure: Relative expression values in male and female chickens. The comparison was based on the expression of all heat shock protein (*HSP70*; *HSP90*) and heat shock factor (*HSF1*; *HSF2*; *HSF3*; *HSF4*) genes in chicken embryonic fibroblast (CEF) cells. Changes in the left and right gonads, immediately after heat treatment (chick age) and sexual maturity. * < 0.05; ** < 0.01; *** < 0.001.

3.2 Results of the fluorescent labelled PGCs

GFP-expressing PGCs were established from embryos of domestic chickens containing the GFP construct prepared by the Roslin Institute. Out of a total of 19 embryos, 10 stable stem cell cultures were established. The culture was accepted as a stable line if at least 1 x 10^6 PGCs were present in the culture dish one and a half months after isolation, and then these cells were cryopreserved. At each isolation, I collected tissue samples from the embryos

from which I determined the sexes. Of the 19 isolated tissue samples, 6 (31.6%) were female and 13 (68.4%) were male. According to the sex distribution of stable cultures, 8 (80%) male and 2 (20%) female lines were established. 2 female and 2 male cultures were selected, in which the expression of stem cell (cPOUV, NANOG, miR302a) and germ cell (CVH, DAZL) specific markers was determined using real-time qPCR **(Fig. 4)**. For all four cell lines, the relative expression levels of all markers were found to be high. A chicken embryonic fibroblast (CEF) sample was also included in each qPCR measurement to provide comparability of plate results.



4. Figu ial germ cell cultures as measured by quantitative real-time PCR using the GAPDH reference gene.

Immunohistochemistry was performed on the two selected PG cell lines (4ZP = male; 5ZP = female). Both PGC lines also showed germ cell (CVH, P63) and stem cell (SSEA1) specific staining. ToPro®-3, which is blue on the images, was used as the nucleus staining. The proliferation rate of four stable GFP-expressing PG cells (4 ZP; 5 ZP; 6 ZP and 8ZP) was determined. The cell division rate was represented well the values characteristic of germ cells. It is important to note that the 4 ZP male culture had an outstandingly good division rate. The integration efficiency of GFP-expressing PGCs into three recipient Hungarian chicken breeds (yellow Hungarian, white Hungarian,

partridge coloured Hungarian) was studied. A total of 30 injection attempts were made. For each variety, there were 5-5 injection experiments with GFP-expressing PGCs, 4 ZP (male) and 5 ZP (female). A total of 365 embryos were used for injection experiments, of which 241 (66.03%) survived. Female (5ZP) GFP-PGC was injected into 198 (54.25%) embryos, of which 132 (66.67%) embryos survived. A total of 58 (43.94%) gonadal integrations were observed, of which 26 (44.83%) were male and 32 (55.17%) female recipient embryos. The efficiency of integration into the gonads of embryos occurred on day 14 of embryonic development.

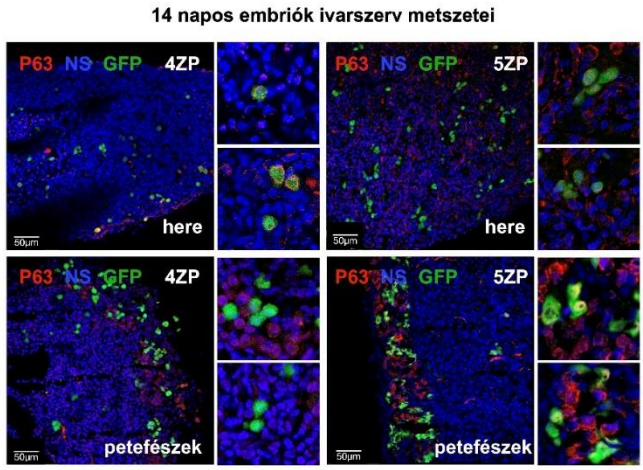
For male PG cells (4ZP), a total of 167 (45.75%) embryos were injected, of which 109 (65.27%) survived the procedure. The success of the integration was determined in 58 embryos, of which 28 (48.28%) were male embryos and 30 (51.72%) were female recipient embryos. The data by detailed varieties are shown in **Table 1.** A significantly higher integration value can be observed in the case of the partridge colour Hungarian variety compared to the other two breeds when the 5 ZP female cell line was injected. Among the injected embryos, the survival rate of the yellow Hungarian breed was significantly lower than that of the white Hungarian. It is worth mentioning that the GFP-4ZP cell line has been shown to integrate into the gonads of guinea fowl. (Molnár et al., 2019).

1. Table: Investigation of the ability of integration efficiency of female (5ZP) and male (4ZP) GFP-expressing PGC lines. I used three Hungarian breed as recipients.

5 ZP PGC injection	Injected embryo	Lived embryo	Chimrea gonads	Male recipient	Female recipient
White Hungarian	**72**	**55** (76,39%)	**19** (34,54%)	**7**	**12**
Partridge colour Hungarian	**74**	**48** (64.86%)	**30** (62,5%)	**14**	**16**
Yellow Hungaryan	**52**	**29** (55,77%)	**9** (31,03%)	**5**	**4**
Summary	**198**	**132** (66,67%)	**58** (43,93%)	**26**	**32**

4 ZP PGC injection	Injected embryo	Lived embryo	Chimrea gonads	Male recipient	Female recipient
White Hungarian	**54**	**39** (72,22%)	**21** (53,84%)	**11**	**10**
Partridge colour Hungarian	**64**	**44** (68,75%)	**22** (50,0%)	**8**	**14**
Yellow Hungaryan	**49**	**26** (53,06%)	**15** (57,69%)	**9**	**6**
Summary	**167**	**109** (65,27%)	**58** (53,21%)	**28**	**30**

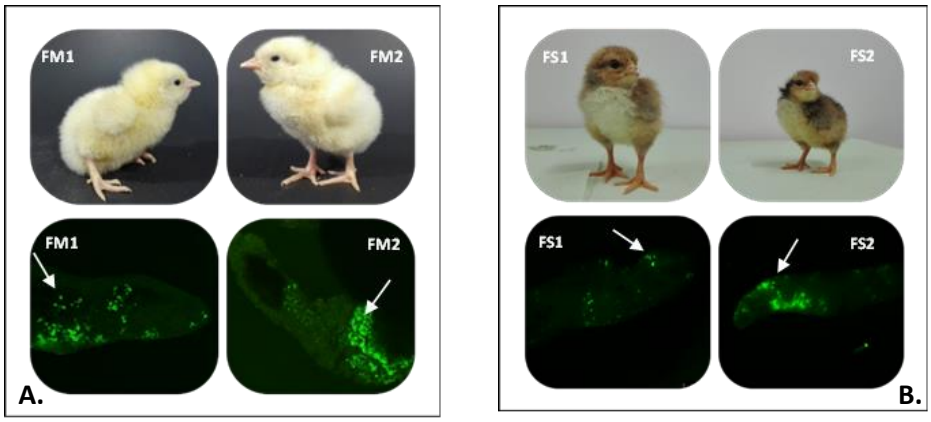
In both sexes, immunohistochemistry confirmed the presence of integrated GFP-PGC, and germ cell ratios could be determined by staining PGCs in the gonads of the recipient breeds. Two germ cell-specific antibodies were used (CVH, P63). It can be clearly seen in **Figure 5.** that in the ovary, PGCs are found mainly in the *cortical* part, while in the testis the integration is localized in the *medullary* region, inner parts of the testes.



5. Figure: Immunohistochemical labelling of GFP-PGC integration in the reproductive organs (testes-ovaries) of embryos of domestic chickens examined on day 14 of embryonic development. Green shows GFP-PGC, red shows P63 germ cell specific antibody, and blue shows nuclear staining (ToPro®-3).

The integration efficiency of the re-injected PG cells was also tested in hatched chicks. It is expected that healthy, phenotypically non-defective animals of the white Hungarian and partridge colour Hungarian breed will hatch from the eggs. As the Institute does not have permits for transgenic organisms, gonad autopsy was performed on the animals 6 hours after hatching with euthanise. We allowed 9 chicks from the white Hungarian breed

and 3 chicks from the partridge colour Hungarian breed to hatch. In the case of the white Hungarian breed, 4 chicks hatched when injected with male PGCs, and 2 were injected with female PGCs in both cases. In the case of the partridge colour Hungarian breed, 3 chicks were allowed to hatch, two of them hatched but the third died immediately before the hatch, however all three gonads contained GFP-expressing PG cells. No phenotypic defects were observed in the hatched chicks. A total of 5 gonadal chimeric chicks hatched from the eggs (**Fig. 6)**. 2 female and 3 male recipients. Female GFP-PGC was integrated into 1 female (5ZP) and 3 male recipients, while the male (4ZP) GFP-PGC was integrated into only one male recipient.



6. Figure: Hatched white Hungarian (A.) and partridge colour Hungarian (B.) chicken breeds. The bottom row shows the integrated GFP-PGCs marked with arrows in the gonads of the recipient, and the top row shows the 1-day-old chick of the recipient breed.

3.3 New scientific achievements

1. I found that *HSP90* and *HSF4* were significantly more strongly expressed in the gonads of pre-heat-treated female chicks after treatment than in control animals, whereas the expression levels of *HSF2* and *HSF3* were significantly lower in adult animals than in control chickens.
2. I demonstrated that the expression levels of *HSP70*, *HSF1* and *HSF3* in both sides of the gonads of sexually mature roosters were significantly increased in heat-treated animals compared to control roosters.
3. First time in Hungary, I successfully established GFP expressing primordial germ cell lines from embryos, and proved that they express both germ cell and stem cell specific markers and are suitable for the generation of germline chimeras.
4. I found that the integration rate of injected GFP-4ZP PGCs did not differ in the case of the examined native breeds, but the proportion of embryos containing female gonadal chimera was higher in the case of GFP-5ZP PGCs. In the case of the white Hungarian and yellow Hungarian breed, the integration efficiency of 5ZP was significantly lower than that of the partridge colour Hungarian breed.
5. After injection, the yellow Hungarian variety had a significantly lower survival rate than the white Hungarian breed.
6. Embryos containing male and female chimeric gonads were obtained in almost equal proportions after 4ZP PGC injection, while the proportion of female chimeric gonads was higher after 5ZP PGC injection. In the case of a white Hungarian recipient breed, this difference was notable.
7. I demonstrated that both GFP-4ZP male and GFP-5ZP female PG cells are able to integrate into both the female and male reproductive organs. The majority of female GFP-5ZP PG cells showed positive P63 expression in the gonad of the female recipient.

4 DISCUSSION AND RECOMMENDATION

One of the most serious problems today is the global warming. Unfortunately, it has a major impact on humanity and nature alike and we can only change it over a long period of time. Until humanity can stop the rise in average annual temperature and then turn it back to the optimal range, we must try to adapt to extreme weather conditions. Extreme temperatures make life difficult not only for humans but also for crops and livestock. Beyond the comfort zone, productivity as well as the fitness of the animals begin to decline (Babinszky et al., 2011; Rath et al., 2015; Vandana et al., 2021). A better understanding of these negative processes provides an opportunity to indirectly protect our animals and plants from the negative impact. Numerous studies have shown that heat stress has a negative effect on egg production, egg weight and egg quality (Emery et al., 1984; Magdi et al., 2004; Star et al., 2008).

In my dissertation, I performed gonadal examinations on domestic chickens. Individuals of the Transylvanian naked-neck breed were heat-treated and the changes in the reproductive organs were examined. I measured the expression levels of heat shock proteins and heat shock factors. The progeny of germ cells in the reproductive organs are the mature gametes that are able to pass on to the next generation the complete genetic information of the animal. I also examined this cell type more thoroughly. Heat treatment research was carried out at the current Farm Animal Gene Conservation Institute as early as the 1990s (Molnár, 1990) led by Dr. Andrea Molnár and Dr. Krisztina Liptói, for both geese and ducks. In my dissertation, I examined one of the most effective of these protocols. Changes in gene expression as a result of the method have not been studied. The transferability of the effect of this treatment to the offspring has not been studied so far, so we would like to prove this as well.

For the heat treatment experiment, I used an article published in 2014 by Xie et al (Xie et al., 2014). Acute and chronic heat treatment was performed on broiler chickens. After heat effects, the expression levels of two heat shock proteins and four heat shock factors in myocardium, skeletal muscle, and liver were measured. After acute heat stress, the expression levels of all four heat shock factors were significantly increased in the heart. *HSF1* expression was unchanged in the liver, in contrast to other heat shock factors that had remarkably high expression.

Wang and colleagues found elevated heat shock protein and heat shock factor expression in the testes of mature roosters of Taiwanese domestic chicken (Wang et al., 2013). The function of more than 300 genes has been studied using a microarray. Based on their results, 169 genes were overexpressed, while the expression of 140 genes was decreased.

Kang et al. studied the results of pre-heat treatment of broiler chickens at sexual maturity under heat stress. Expression of heat shock proteins was determined from blood taken from the wing vein as well as the liver. The results of the two heat-stressed groups were normalized to the *GAPDH* reference gene. Significantly higher expression was found for *HSP70* and *HSP27* in the pretreated and heat stressed group compared to the control, and HSP70 expression was significantly higher than in animals treated with heat stress alone. Expression levels of *HSP60* and *HSF3* were significantly lower in heat-stressed animals than in controls (Kang et al., 2019).

Anand and colleagues heat-treated a Transylvanian naked-neck breed. The results of the heat treatment were examined in three groups. The control group was maintained at an average ambient temperature, the heat-treated group was exposed to heat stress at 38.5 ^{o}C for 12 hours at two days of age and then at 30 oC for 12 weeks at matured age. The third group did not receive any pre-heat conditioning, only heat stress at 30 ^{o}C for 12 weeks. PGC cultures were

established by taking blood from F2 generation embryos, and on day 10 of embryonic development, the gonads were dissected and the HSP70 expression pattern was examined. Genital organs from previously heat-treated and heat-stressed animals, as well as from heat-exposed animals only, showed higher *HSP70* expression than in the control group, but this was not considered to be a significant result (Anand et al., 2016).

The heat treatment procedure described in the dissertation caused a positive effect in the case of the Transylvanian naked-neck breed, which was confirmed by molecular biological methods. I was unable to compare HSP and HSF expression values because no literature data are available. The molecular changes in the reproductive organs of domestic chickens exposed to heat stress without prior heat treatment have been studied by several research groups. (Xie et al., 2014; Zhang et al., 2014; Kang et al., 2019). Their results are well comparable with the results of the tests I obtained after the direct heat treatment.

More in-depth study of germ cells has become a major research topic following the creation of a culture medium developed by Van de Lavoir et al. (van de Lavoir et al., 2012). Several publications have been published in the last decade on the establishment, culture and characterization of primordial germ cells. (Rikimaru et al., 2011; Tonus et al., 2016; Wang et al., 2017; Yu et al., 2019). The best culture medium currently in use, which also supports the maintenance of female PGC cultures without feeder cells, is developed by Whyte et al. (Whyte et al., 2015).

Yu et al. isolated primordial germ cells from Chinese meiling laying hens and characterized them by immunohistochemical staining. PG cells were isolated from stage HH14-16 embryos and purified from the blood after Nycodenz gradient centrifugation. Germ cell-specific marker expression levels (DAZL, CVH) were examined on primordial germ cell lines using real-time PCR.

Characterized PG cells were stained with PKH26 and then injected back into the dorsal aorta of the recipient broiler breed. Four mature roosters were created, which they crossed with the original meiling breed. A total of 12.6% received progeny of the original donor breed (Yu et al., 2019).

Fluorescently labelled stem cell lines were generated for better traceability of integration into the recipient breed. With these illuminated PG cells, the integration of PGCs into the gonads could be better investigated. Park et al. transfected primordial germ cells from chicken and quail species using the piggyBac transposon construct. After characterization of the produced cell lines, the GFP-expressing PGCs were injected back into the recipient breed. A total of 228 (52.2%) of the 459 hatched chicks in the F2 generation expressed GFP (Park and Han, 2012). Macdonald et al. isolated stem cells from embryos expressing GFP and then re-injected them into the bloodstream of the recipient variety. A total of 26 embryos were injected with male GFP-expressing primordial germ cells, of which 12 (46.15%) embryos survived the procedure. Transgenic surviving roosters were growing up and then examined for their spermatological properties. Backcrossing of male transgenic roosters with non-transgenic hens resulted in 2–16% transgenic offspring. Laying transgenic hens were cross-bred with non-transgenic roosters and 0% received transgenic offspring. No GFP-positive cells were found in the ovaries after genital examination. From this, it was concluded that male PGCs remain functionally active in the male recipient, whereas they are eliminated from the ovaries during oogenesis in laying hens. (Macdonald et al., 2010).

Reinjection of female PGCs was performed by Tagami et al. PGCs were isolated from the White Leghorn variety and returned to the Barred Plymouth Rock breed, and vice versa. Chimeras were prepared with efficiencies between 4.9 and 77.6%. Mixed-sex PGCs were injected back into the recipient breed, and the roosters were selected from the hatched chicks and examined for

spermatology. A region specific for the W chromosome sequence was found by Southern hybridization between sperm of chimeric roosters. This suggests that female PGCs are able to engage in spermatogenesis and produce mature sperm in the gonads of the male recipient species, but most female PGCs are unable to differentiate into spermatozoa. (Tagami et al., 1997).

The establishment rate of GFP-expressing primordial germ cells was 52.62%, which is in good agreement with the PGC establishment results found in the literature (Vantress heritage breed: 40-56%; White Leghorn breed: 49-82%; yellow Hungarian breed: 50%; white Hungarian variety 47.6%) (Nandi et al., 2016; Woodcock et al., 2019; Lázár et al., 2021). The established cell lines were analyzed by germ cell-specific and stem cell-specific markers (*SSEA1*, *CVH*, *DAZL*, *P63*), which show good agreement with the results found in the literature. (Tonus et al., 2016; Lázár et al., 2018, 2021; Yu et al., 2019).

Injection results of GFP-expressing PG cells in both survival and germline chimeria production reflect the data from the literature (Macdonald et al., 2012; Park and Han, 2012). The integration of the injected male and female GFP-PG cells into male and female recipient embryos gave similar results to those obtained by Macdonald et al. and Tagami et al. I found integration in both sexes, and by immunohistochemistry on the 14^{th} day of embryonic development I was able to determine the PG cells in the gonads that are likely to be able to form the germ cells of a mature chicken.

4.1 Recommendation

Heat treatment methods can generally improve the heat tolerance of animals. The heat-treatment method I described in my dissertation can be successfully applied to large-scale farms so that the daily egg production and fertility rate do not decrease during hot summers. In my research, I extensively examined the expression levels of factors in the gonads of domestic chickens using real-time qPCR. Immediately after heat treatment, the expression levels of heat shock proteins (HSP) and heat shock factors (HSF) change. I have tried to prove the existence of these changes in adult domestic chickens.

Primordial germ cells, or PGCs, are capable of transmitting this information, so I considered it important to learn more about these cell types. An epigenetic study of our PGC lines is also underway in collaboration with INSERM staff in Lyon. If it is proven that this acquired trait can be inherited through PGCs, it is likely to attract the attention of large-scale livestock keepers as well. Because of the very hot summers this simple pre-heat-treatment will be more important, because they have an effect not only on productivity but also on fitness. This allows animals to withstand extremely high ambient temperatures.

The cultivation of chicken primordial germ cells has already been solved. Finding the optimal recipient breed is an important task for the future, with the help of which mortality can be reduced during injection, and a higher integration rate can be achieved, which can even help the recovery of endangered species. Another important goal is that a given genetic modification (e.g., heat tolerance) can be successfully transferred to a recipient breed with PG cells, so that individuals whose cells contain the desired modification can be crossed through their offspring.

5 SCIENTIFIC PUBLICATIONS

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- Lázár B., Tokodyné Szabadi N., Mahek A., **Tóth R.,** Ecker A., Urbán M., Maria Terese, S. A., Ganna S., Hegyi Z., Homolya L., Várkonyi E., Bertrand P., Gócza E. (2022): Effect of miR-302b MicroRNA inhibition on chicken primordial germ cell proliferation and apoptosis rate. Genes, 2022 13 (1) 82. https://doi.org/10.3390/genes13010082: **Q2** IF: **3.886** független idéző közlemények száma: **0**

- Lázár B., Molnár M., Sztán N., Végi B., Drobnyák Á., **Tóth R.**, Tokodyné Szabadi N., McGrew MJ., Gócza E., Patakiné Várkonyi E. (2021): Successful cryopreservation and regeneration of a partridge coloured Hungarian native chicken breed using primordial germ cells. Poultry Science 2021 Aug;100(8):10120. Animals Science and Zoology 25/419: **D1**, IF: **2.752**, független idéző közlemények száma: **0**

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<u>Poster presentations related to the topic of the thesis:</u>

- TOKODYNÉ SZABADI NIKOLETT, **TÓTH ROLAND**, LÁZÁR BENCE, BUDA KITTI, MOLNÁR MARIANN, PATAKINÉ VÁRKONYI ESZTER, LIPTÓI KRISZTINA, GÓCZA ELEN (2021): A hőkezelés hatásának vizsgálata a kezelésen átesett házityúkok mRNS és miRNS expressziós profiljának tanulmányozásával. Szaporodásbiológiai Találkozó 2021. november 5-6. Marina Port Hotel, Balatonkenese.

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- **TÓTH R.**, STEFANIE ALTGILBERS, WILFRIED A. KUES, HOFFMANN O., URBÁN M., GOCZA E. (2021): Vénusz és mCherry transzfektált őscsírasejtek vizsgálata. GBI-NAPOK Gödöllő, 2021. 12. 14-15.

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- **TÓTH ROLAND**, URBÁN MARTIN, BODROGI LILLA, PINTÉR TÍMEA, ECKER ANDRÁS, PEER GABRIELLA, BABARCZI BIANKA, SZŐKE ZSUZSANNA, GÓCZA ELEN (2021): Mikotoxinok hatásának vizsgálata a házityúk és nyúl embriók fejlődésére. Szaporodásbiológiai Találkozó 2021. november 5-6. Marina Port Hotel, Balatonkenese.

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