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INVESTIGATION OF THE EXPRESSION OF HEAT STRESS INDUCED MICRORNAS RESULTING FROM THE EFFECT OF HEAT TREATMENT OF CHICKEN AT A YOUNG AGE

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1. Background and objectives of the research

The importance of poultry farming is growing year by year all over the world. According to the United Nations Food and Agriculture Organization (FAO - The Food and Agriculture Organization), products produced from domestic chickens dominated the market in 2018, with annual consumption reaching 114 million tons (*FAOSTAT*, 2023). Poultry products are the most consumed animal products in the world because they are not subject to religious or cultural restrictions (*OECD-FAO Agricultural Outlook 2020-2029*, 2020). The production of the most efficient animal protein is made possible by the poultry meat and egg production sector, which plays a key economic role in agricultural production. The ever-increasing quantitative demand resulted in qualitative changes in breeding and husbandry technology.

At the same time, the number of threatened and extinct species is increasing worldwide. This fact is increasingly transforming the outlook of humanity, the effort to maintain biodiversity, both in the flora and fauna, has become accepted. One important element of this is manifested in the rescue and preservation of rare genes (FAO, 2007).

Large-scale production is trying to satisfy the growing demand, which is greatly hampered by the increased ambient temperature due to climate change (Thornton, 2010; Rojas-Downing *et al.*, 2017). Changes in environmental factors, temperature and humidity affect animal metabolism and thermoregulation mechanisms. Maintaining homeostasis is a set of energy-demanding processes, which are further increased by unfavorable changes in environmental factors, and beyond a limit can lead to the death of animals (Leinonen, Williams and Kyriazakis, 2014). Climate change is therefore one of the problems that the livestock industry will have to deal with in the coming years. Among the negative effects caused by climate change, heat stress is considered to be the most serious environmental stressor for poultry farming worldwide.

Researchers have long been concerned with how to solve the acclimatization of hens. Today, even in many temperate regions, summers are very long and hot, so the damage caused by heat stress is now also significant in the case of large domestic plants. The increased adaptability of animals will play a major role in the development of efficient agriculture of the future (Nawab *et al.*, 2018). A molecular genetic comparative study of hen breeds that are more resistant and sensitive to heat stress is

needed. In the light of the data, by developing an optimal juvenile heat treatment method, a herd capable of tolerating higher ambient temperatures could be created (Dunnington and Siegel, 1984; Loyau *et al.*, 2016).

In our research, we focused on the domestic fowl as a poultry model animal, where after heat conditioning we performed the molecular biological analysis of the tissues from the parent flock and the offspring generation. The data obtained from the parental generation can help to learn about the molecular pathways activated as a result of the treatments, while the genetic analysis of the primordial gametes of the offspring generation and the primordial germ cell lines created from them can provide information about the inheritance mechanisms passed down through the generations.

During my research, my goal was to map the molecular processes underlying the heat adaptation ability of domestic hens. I would like to learn about the molecular mechanisms that take place as a result of a special heat treatment process, which help mitigate the consequences of stress caused by high environmental temperatures, thereby improving the heat tolerance of the treated poultry stock.

In the course of my research, I examined the expression pattern of messenger RNAs (mRNAs) and microRNAs (miRNAs) involved in the defense mechanisms activated by heat treatment in the brain tissue samples of the Speckled Transylvanian Naked Neck hen with hemp seeds.

Another important element of my research was the study and characterization of primordial germ cells (PGC), and the long-term maintenance of the established cultures, as well as the optimization of the necessary culture conditions. My goal was to support the selection of suitable breeding parameters with the results of biotechnological and molecular biological tests, and to help create and maintain the domestic fowl gene bank more effectively with the breeding methods developed in this way.

My long-term goal is to help domestic gene preservation, to create a germ cell-based gene bank and to store it optimally for a long time in the case of domestic fowl.

I aimed to analyze the gene expression of the changes caused by the heat conditioning applied in the parental generation, as well as to detect the epigenetic changes caused by these treatments and to verify their heritability.

2. Materials and methods

2.1. Keeping and heat treatment of experimental stock of domestic fowl

The animal experiments were carried out at the National Center for Biodiversity and Gene Preservation, Farm Animal Gene Preservation Institute (NBGK-HGI) in accordance with Act XXVIII of 1998 on the protection and welfare of animals. in accordance with the law, for which the Animal Health and Animal Protection Directorate of the National Food Chain Safety Office granted a license (license number: PE/EA197-4/2016).

For the heat conditioning experiment, the chicks (n=60) were kept on absorbent paper litter under an infrared lamp (at 32°C) during the first 24 hours after hatching (Tóth et al., 2021). On the second day after hatching, half of the chicks (n=30) were returned to the incubator for heat conditioning. The temperature was set at 38.5°C and the humidity at 60% for 12 hours. After the treatment, cervical dislocation was performed on the chickens, and then we started to jointly collect tissue samples from half of the control (Ctrl-C, n=15) and heat-conditioned/heat-treated chicks (HT-C, n=15). In the control and treated groups, the other half of the animals (Ctrl-A, n=15 and HK-F, n=15) were raised until sexual maturity (up to 23 weeks of age) per group in an air space, on a mixture of wood shavings and zeolite, under 16-hour lighting. Drinking water and feed were available ad libitum to the animals throughout the experiment. For sampling, cervical dislocation was applied to adults (Ctrl-A, n=9 and HT-A, n=12), the brainstem and mesencephalon were cut out and placed in sterile freezer tubes containing RNAlater™ solution (Thermo Fisher Scientific, 145 Waltham, MA, USA) we placed (Tokodyné Szabadi *et al.*, 2024). After two days, the samples were transferred to TRIzol® (#15596026, Thermo Fisher Scientific, Waltham, MA) and then stored at -70°C until RNA isolation.

I also carried out animal experiments for the study of molecular pathways activated as a result of heat stress together with the colleagues of NBGK-HGI. In the first 24 hours after hatching, the chicks were placed under an infrared lamp at 32°C on absorbent paper litter. Then, a third of the 2-day-old chicks were returned to the incubator for heat treatment. The temperature was set at 38.5°C and the humidity at 60% for 12 hours. The other chicks were kept in deep litter, with ad libitum feeding, at 32°C. Then, heat-conditioned and untreated animals were reared under the same

conditions. After sexual maturity (at 23 weeks), the heat-conditioned group (heat treatment and heat stressed - HTHS) and half of the untreated animals (heat stressed - HS) were heat-stressed, by keeping them at 30°C for 12 weeks, in an air space, on wood chip and zeolite mixture litter, with 16 hours of lighting, feed and water were available ad libitum. The animals that received neither heat conditioning nor heat stress were considered as control (Ctrl) group. To examine the offspring generation, fertilized eggs were collected from domestic hens classified into three groups.

2.2. Collection and cultivation of PG cells

My colleague Bence Lázár founded cell cultures from the embryos of the next generations of Speckled Transylvanian Naked Neck and Black Transylvanian Naked Neck hens, and then placed the successfully propagated lines in a gene bank. The starting point were the eggs of Speckled Transylvanian Naked Neck (47 pcs) that participated in the heat treatment experiment, from which 26 primordial germ cell lines were created.

I had the opportunity to thaw these cryopreserved cell cultures, then culture them and examine them.

2.3. Characterization of PG cells

Proliferation test with ImageXpress Micro XLS

The cell count of the domestic chicken PGC lines was completed before the cell proliferation test was performed using an Arthur™ Novel Fluorescence Cell Counter (NanoEnTek, Pleasanton, CA, USA). During the measurement, I worked with four lines of Black Transylvanian Naked Neck hens (two males M1: #508-ZZ; M2: #512-ZZ and two females F1: #509-ZW; F2: #513-ZW), where three types of treatment (Ctrl - control; 5P - gga-miR-302b-5P inhibition; 3P - gga-miR-302b-3P inhibition; 5P/3P - gga-miR-302b-5P and gga-miR-302b-3P inhibition) and a control I applied and examined each case on 6 parallel holes (Lázár *et al.*, 2021).

I placed the plate in an ImageXpress Micro XLS device with a built-in incubator and its High-Content Screening imaging system performed the measurement per hole (A01-H12), in the 16 regions (S1-16) of each hole, I evaluated the data with recordings made every 4 hours for 3 days. For example, on the third day, the proliferation rate

was calculated by dividing the average cell number counted on the third day (h76) by the value measured on the second day (h52). I compared the proliferation rate of control and treated lines on the first day (28 hours/4 hours), the second day (52 hours/28 hours) and the third day (76 hours/52 hours).

Immunostaining

I performed immunostaining with primary and secondary antibodies on cell lines derived from hemp-seeded and Black Transylvanian Naked Neck hens. After fixation, the primary antibodies (SSEA-1, CVH, DAZL, P63) were used in different combinations. The following day, I washed off the primary antibodies with 0.01% BSA-PBS, then measured the secondary antibodies (anti-mouse(m)-IgM-D549(r), anti-rabbit(rab)-IgG-A488) (I left a negative control in each case). One hour after the first wash (0.01% BSA-PBS, RT, 5 min), I applied nuclear dye To-Pro-3 to each droplet of both lines. The slides were covered and stored in the dark until photography with a Leica confocal (TCS SP8) microscope.

2.4. RNA isolation and cDNA writing

In the heat conditioning experiment, RNA was isolated from the brain tissue of control and heat-treated chicks, and control and heat-treated sexually mature roosters and hens. Samples collected in TRIzol® (#15596026, Thermo Fisher Scientific, Waltham, MA) were thawed and then homogenized. Isolation was performed according to the TRIzol® manufacturer's protocol. The concentration of RNAs obtained as a result of the process was checked with a NanoDrop (ND-1000, Thermo Fisher Scientific, Waltham, MA, UV-Vis) spectrophotometer.

In order to characterize the PGC lines and to study the effect of heat stress, RNA was isolated from the cells of PGC cultures created from HH14-16 embryos developing in 2.5-day-old eggs of Speckled Transylvanian Naked Neck hens from the three treatment groups HTHS, HS and C. I thawed the freezer tubes containing the PGC samples collected in the lysis buffer by placing them in a 37^{o}C water bath for 90 seconds, and then performed RNA isolation from the PG cells using the RNAqueous™ Total RNA Isolation Kit, based on the protocol. The concentration of the RNA obtained during the isolation was also measured with a NanoDrop (ND-1000, Thermo Scientific, UV-Vis) spectrophotometer.

For the qPCR tests, cDNA was synthesized from the RNA by reverse transcription. For the reaction, the amount of RNA was diluted to a concentration of 25 ng/µl based on the values measured with the spectrophotometer. For cDNA writing, I used the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA).

2.5. RNA sequencing

RNA sequencing was performed by Bertrand Pain (Stem-Cell and Brain Research Institute, USC1361 INRA, U1208 INSERM, 69675 Bron, France) and his research team from our RNA pool samples compiled per treatment group (Ctrl, HS, HTHS).

2.6. Real-time PCR

I monitored the changes in the expression pattern of markers induced by heat treatment using the real-time PCR (qPCR) technique. For qPCR, I used a 96-well plate, the reaction was performed in a Mastercycler Realplex^4 epGradient S (Eppendorf AG, 22331 Hamburg, Germany) (Tóth *et al.*, 2021). To test the mRNA expressions, I measured 0.75 µl of the previously prepared cDNA solutions and 14.25 µl of the qPCR mix containing the SYBR Green dye and primers per well. I performed 3 parallel measurements for each sample/primer. While in the case of miRNAs, I used special TaqMan probes, where I also used the cDNA solutions, of which I measured 1µl, and 14µl of the qPCR mix containing the TaqMan master mix and the primers. I performed 3 parallel measurements for each sample/primer. I used GAPDH as an internal control (housekeeping gene), and for the markers to be tested, I used the primer pairs described in the literature (HSP70, HSP90, HSF1, HSF2, HSF3, HSF4, miR-92-3P, miR-138-5P) (Kisliouk, Yosefi and Meiri, 2011; Xie *et al.*, 2014).

2.7. DNA isolation

DNA was isolated from the cells of PGC cultures created from HH14-16 embryos developing in 2.5-day-old eggs of hens from the three treatment groups HTHS, HS and Ctrl. I performed the DNA isolation from the cell pellet using the DNA Isolation Kit for Cells and Tissues (Roche® Life Science Products) based on the associated protocol. The concentration of the DNA samples obtained during the

isolation was measured with a NanoDrop (ND-1000, Thermo Scientific, UV-Vis) spectrophotometer.

2.8. Whole genome bisulfite sequencing and methylation pattern analysis

Whole genome bisulfite sequencing performed to map the methylation pattern of DNA samples obtained from cell lines was provided by UD-GenoMed Medical Genomic Technologies Kft. (Debrecen). Whole genome bisulfite sequencing is based on the conversion of unmethylated cytosine bases to thymine. The analysis of the obtained DNA methylation data was carried out by Dr. István Likó (UD-GenoMed Medical Genomic Technologies Kft.) from the gender (ZW – female, ZZ – male) pool DNA samples compiled per treatment group (HS, HTHS) (HS-ZW, HTHS- ZW, HS-ZZ, HTHS-ZZ).

2.9. Statistical analysis

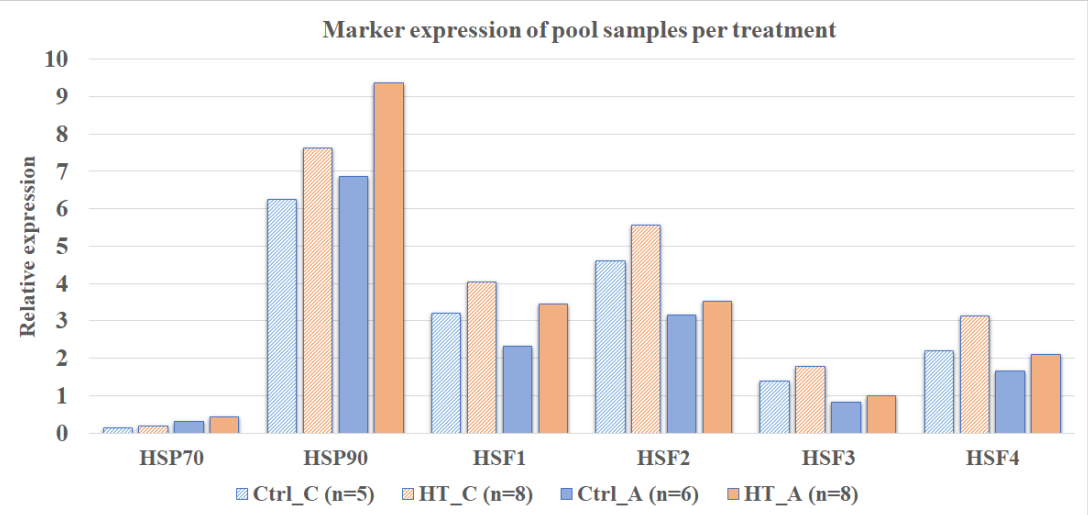
The expression or repression of the target gene compared to the internal control gene was calculated in each sample using the GenEx 7.0 program (MultiD Company) using the $2^{-\Delta\Delta Ct}$ method (Rao et al., 2013). As an internal control, I measured the expression of the constitutively expressed GAPDH (NM204305.1) gene using the primer pair 5'-AGCAATGCTTCCTGCACTAC-3' / 5'-CTGTCTTCTGTGTGGCTGTG-3'. I determined the Ct threshold value (threshold cycle) manually. The ΔCt values of each cDNA reaction were normalized based on the formula ($Ct_{target\ gene} - Ct_{internal\ control}$), and then the averages of the ΔCt values (average ΔCt_{gene}) were determined. During the determination of the ΔΔCt values, the values of the treated samples (average $\Delta Ct_{gen\text{-}treated}$) were normalized to the untreated control (average $\Delta Ct_{gen\text{-}untreated}$) (average $\Delta Ct_{gen\text{-}treated}$ - average $\Delta Ct_{gen\text{-}untreated}$).

The data was evaluated using GenEx 7.0 software. After ANOVA analysis, I used Tukey's post hoc procedure to identify the difference between the averages of which groups was significant, where $p < 0.05$ values were considered significant ($*p < 0.05$).

3. Results and their discussion

Molecular biological study of parental generation

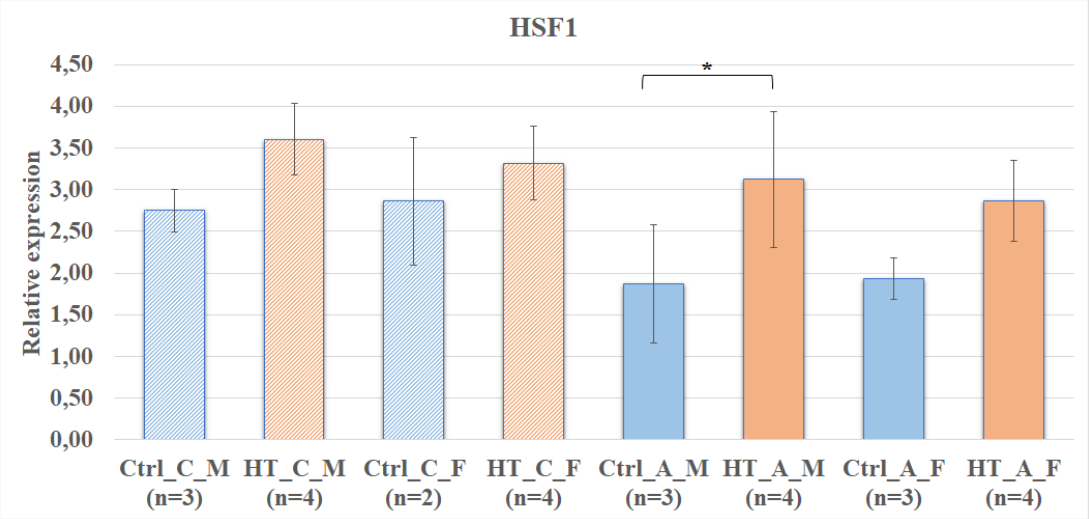
The qPCR tests were performed with pool samples per treatment (Ctrl-control; HT-heat-treated) (Figure 1) and with individual domestic hen brain tissue samples, which were represented per treatment (Ctrl; HT) and according to gender (M-male; F-female) chicks (C), or at sexually mature/adult (A) age. The expression of all markers (HSP70, HSP90, HSF1, HSF2, HSF3 and HSF4) increased in the treated groups (HT-C; HT-A) compared to the control. The increase in the expression of HSF1 in the brain tissue of sexually mature males was significant (p= 0.0478) (Figure 2) as a result of heat conditioning (Tokodyné Szabadi *et al.*, 2024).



1. figure: Gene expression levels of heat shock proteins and heat shock factors measured in brain tissue samples of chicks (C) and sexually mature/adult (A) pooled per treatment (Ctrl-control; HT-heat-treated).

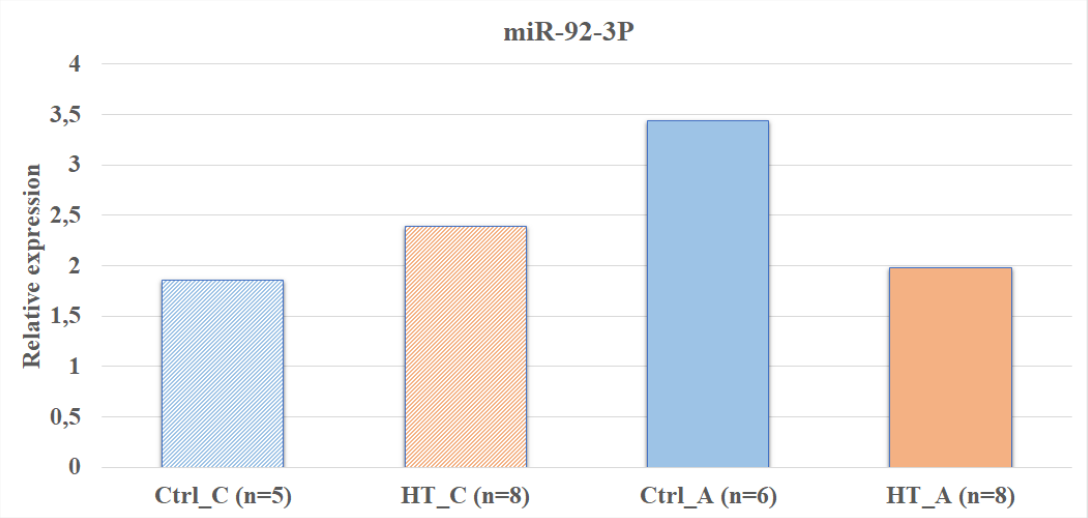
I found that the expression of heat shock proteins (HSP70, HSP90) and heat shock factors (HSF1, HSF2, HSF3 and HSF4) can be detected in the brain tissue samples of Speckled Transylvanian Naked Neck hens and roosters with hemp seeds. The heat conditioning we used caused an increase in the gene expression level of all heat shock proteins and heat shock factors at the same time in young and adult age. The temperature difference contributes to the increase in HSF1 expression, which requires a certain thermal threshold value, but its exact value has not yet been clarified (Tanabe *et al.*, 1997). Since a rapid increase in the expression of HSFs is observed upon acute heat treatment, it is thought that HSFs may be essential for the rapid transcription of HSPs (Xie *et al.*, 2014). My results confirm, in accordance with

literature data, that preliminary heat conditioning affects the expression of markers linked to heat stress.

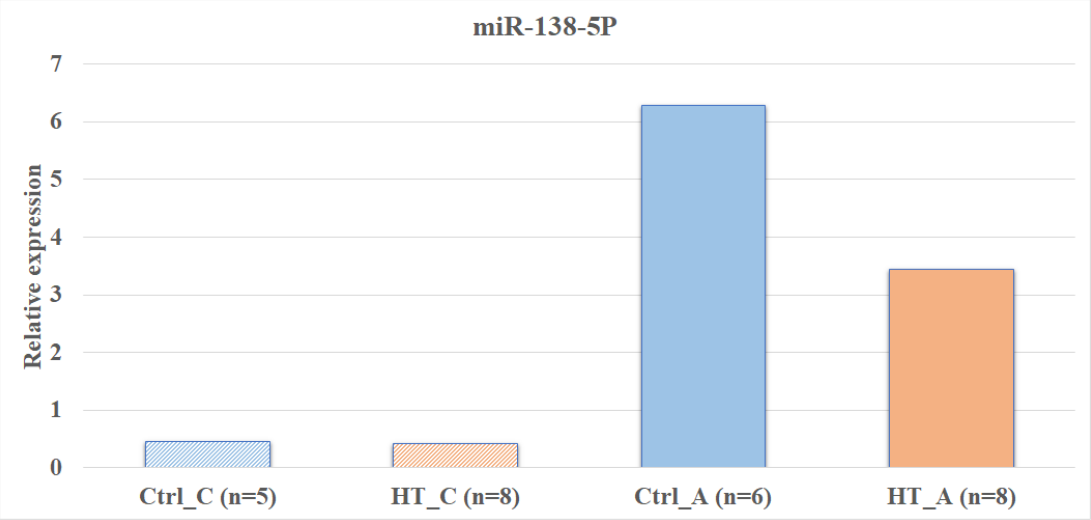


2. figure: The expression of the HSF1 marker in individual samples, which were plotted according to treatment (Ctrl; HT) and sex (M-male; F-female) in the case of chicks and sexually mature animals, where $p < 0.05$ values were considered significant ($*p < 0.05$).

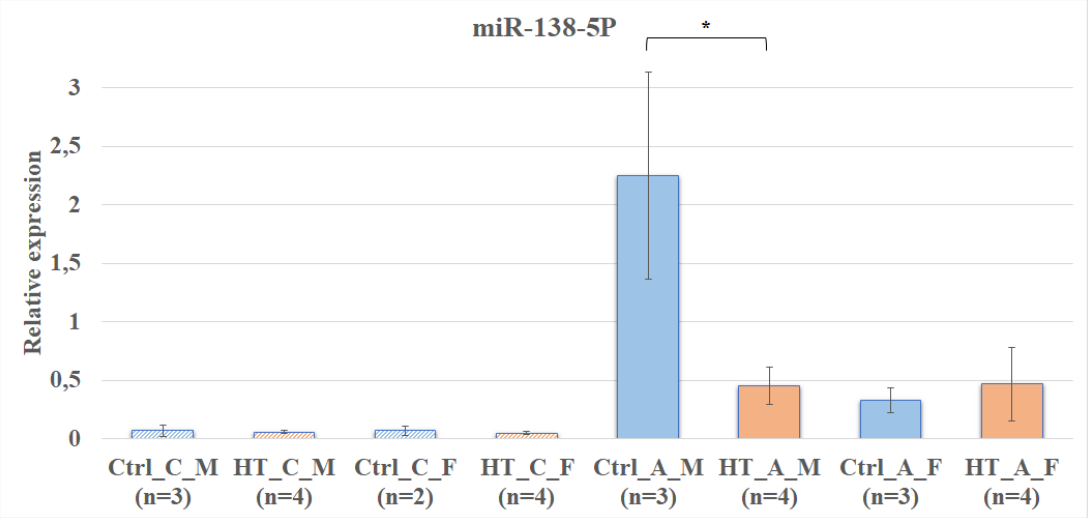
I also examined the expression pattern of two heat stress-related microRNAs (miRNAs) (miR-92-3P, miR-138-5P) in the brain tissue of treated (HT) and untreated (Ctrl) chicks (C) and sexually mature/adult (A) domestic hens. The reduced expression of miR-92-3P and miR-138-5P can be observed in the heat-conditioned groups of sexually mature age compared to the controls (Figures 3, 4). To find out if these differences are significant, I also performed the analysis from individual RNA samples pooled by sex. After that, I found that miR-138-5P expression only showed a significantly lower ($p= 0.0043$) level in the heat-conditioned individuals (HT-A-M) compared to the control (Ctrl-A-M) in the case of sexually mature roosters (Figure 5). The measurement results obtained from individual RNA samples overlap well with the results obtained with pool RNA samples (Tokodyné Szabadi *et al.*, 2024).



3. figure. Expression of the miR-92-3P marker measured in the brain tissue samples of chicks (C) and sexually mature/adult (A) pooled per treatment (Ctrl-control; HT-heat-treated).



4. figure. Expression of the miR-138-5P marker measured in the brain tissue samples of chicks (C) and sexually mature/adult (A) pooled per treatment (Ctrl-control; HT-heat-treated).



5. figure: The values of the expression of the miR-138-5P marker in the individual samples, which were plotted according to treatment (Ctrl; HT) and gender (M–male; F–female) for chicks and sexually mature animals, where $p < 0.05$ values were considered significant ($*p < 0\ 05$).

In domestic chickens, the main function of miR-138-5P is to regulate the signaling pathway responsible for regulating body temperature. Based on literature data, the role of miR-138-5P in the regulation of hypothalamic neurogenesis (Kisliouk, Cramer and Meiri, 2014) is fundamental, because miR-138-5P binds to the 3'UTR region of Reelin (RELN), and consequently inhibits the expression of RELN. which promotes neuronal cell migration. This finding also provides an explanation for the timing of the heat treatment, since neuroplasticity is still active in birds at a young age. (Kisliouk, Cramer and Meiri, 2014). In addition to the heat conditioning applied by our research group, I was able to detect miR-138-5P expression in the brain tissue of the control animals, which is significantly higher at the sexually mature age (in males $p= 0.00000016$, in females $p= 0.0186$) compared to chicks. The expression level of the miRNAs I examined decreases as a result of heat conditioning at the sexually mature age, which confirms the effectiveness of heat conditioning at chick age.

Molecular biological study of the next generation

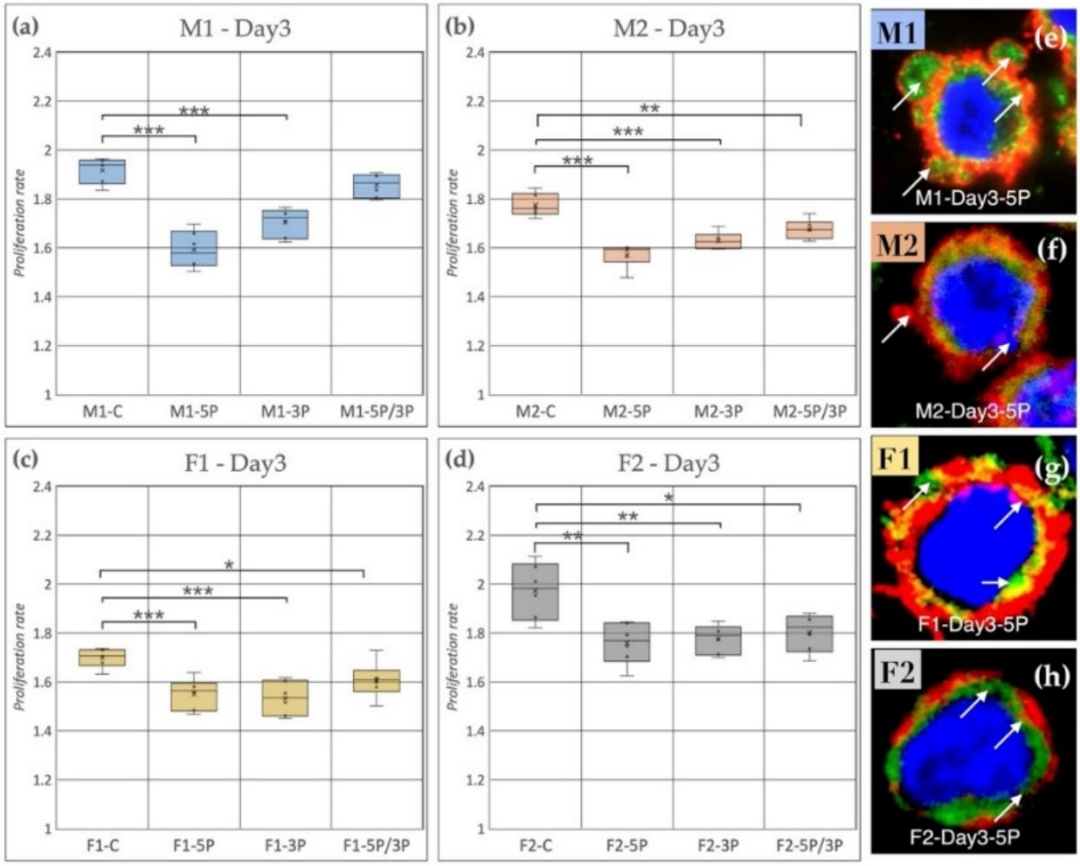
Effect of microRNA inhibition in PGCs

In this study, two male (M1: #508-ZZ; M2: #512-ZZ) and two female (F1: #509-ZW; F2: #513-ZW) PG cell lines of Black Transylvanian Naked Neck hens were used for cell line characterization. The cell number of these cell lines was measured every 4 hours for three days by an XLS device with a built-in incubator and an Imaging system. I wanted to investigate the proliferation rate of PGCs on days 1, 2 and 3 after

inhibition of gga-miR-302b-5P (5P) or gga-miR-302b-3P (3P) and anti-gga- after the combined use of miR-302b-5P and anti-gga-miR-302b-3P inhibitors (5P/3P inhibition).

Based on my results, it can be concluded that on the third day of the experiment, the inhibition with 5P and 3P drastically reduced the proliferation rate for all cell lines compared to the control, however, the time scale of the inhibition was different in these cell lines. So Figure 6 shows the comparison of proliferation rates on day 3. It can be seen that the control samples showed significantly higher proliferation in the case of M2, F1, F2 (Figure 6, b, c, d) compared to the treated samples. M1 PGCs did not differ from control after 5P/3P inhibition (Figure 6, a) (Lázár *et al.*, 2021).

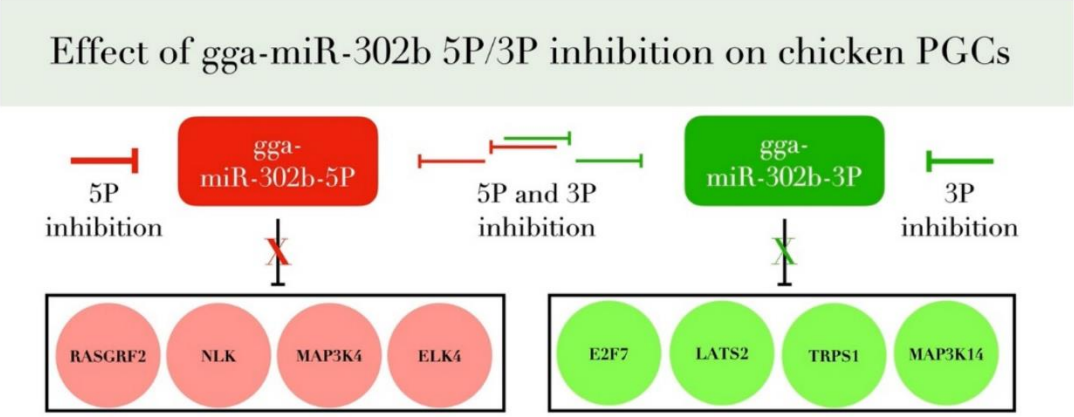
After three days of cultivation, I fixed the lines and performed immunostaining, which also shows that membrane differentiation occurred in the lines inhibited in the different combinations, which, based on the staining, only contain cytoplasm, so these are cytoplasmic vesicles, nuclear degradation was not observed. Most vesicular cells appeared in the 5P inhibition treatment (Fig. 6, e, f, g, h) (Lázár *et al.*, 2021).



6. figure: Comparison of the proliferation rates of M1 (a), M2 (b) (ZZ genotype) and F1 (c), F2 (d) (ZW genotype) PGC lines at day 3. I performed immunostaining with SSEA1 (red), CVH (green) and TO-PRO™-3 nuclear staining (blue). Arrows indicate vesicles on the cell surface.

In previous studies, our research group reported that gga-miR-302b-5P showed a higher expression compared to gga-miR-302b-3P in cells with a higher proliferation rate (Lázár *et al.*, 2018). Based on this information, I continued my work, in which I found that inhibition of the miR-302b-5P arm significantly reduced proliferation, suggesting a role for miR-302b-5P in cell proliferation. In the case of cells inhibited with gga-miR-302b-5P, the proportion of apoptotic cells was lower than in the case of cells receiving double inhibition (Lázár *et al.*, 2021).

This result is consistent with previous literature data (Wu *et al.*, 2019), where miR-302b-5P acted as a proliferation promoter and oncomiR. Inhibition of miR-302b-5P reduced the proliferation rate and moderated the proportion of apoptotic cells. This confirms the role of miR-302b-5P miRNA in regulating the proliferation of PGCs *in vitro* and *in vivo*. Overall, my results confirmed the decrease in the proliferation rate after inhibition of gga-miR-302b-5P and gga-miR-302b-3P (Figure 7).

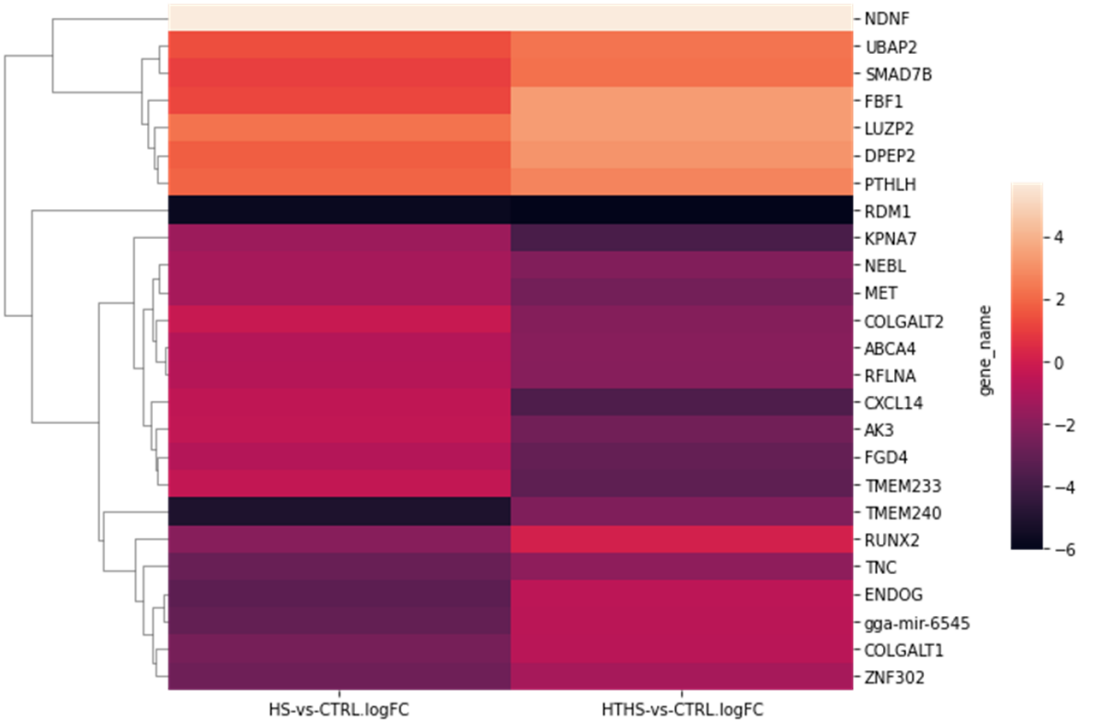


	control	5P inhibition	3P inhibition	5P/3P inhibition
cell proliferation rate	++++	+	++	+++
apoptosis rate	++	+	++	++++
cell membrane abormalities	+	++++	++	+++

7. figure: The most relevant target genes of gga-miR-302b-5P and gga-miR-302b-3P, based on previously published data (Cataldo és mtsai., 2016; Lázár és mtsai., 2018; Sun és mtsai., 2015).

Molecular diagnostic use of PGCs established from embryos of heat-treated domestic chickens to explore signaling pathways

In my research, I used the Speckled Transylvanian Naked Neck hen PGC lines established by our research group for molecular diagnostic tests. RNA samples from PGCs were pooled by treatment group and then sequenced. The heat map summarizing the results contains the genes detected by RNA sequencing and whose expression changed in response to stress. Where the left-hand column indicates the expression values measured in PGC samples from the heat-stressed group (HS) with color intensity compared to the control (Ctrl) values, while the right-hand column shows the data of the PGC samples created from the heat-conditioned and heat-stressed group (HTHS) also compared to the control (Ctrl) compared to. A light color indicates an increase in the expression level detected for the given gene, a dark color indicates a decrease in the expression level (Figure 8). Using RNA sequencing, we obtained changes in the expression levels of several genes that, based on literature data, play an important role in the molecular processes activated during heat stress. (Tavares *et al.*, 2018). The miR-6545, RUNX2, and ENDOG, which are differentially expressed in PG cell cultures derived from embryos of heat-conditioned and then heat-stressed domestic chickens and only heat-stressed animals. It can be said that the heat effect and heat effects applied by our research group changed the gene expression pattern.

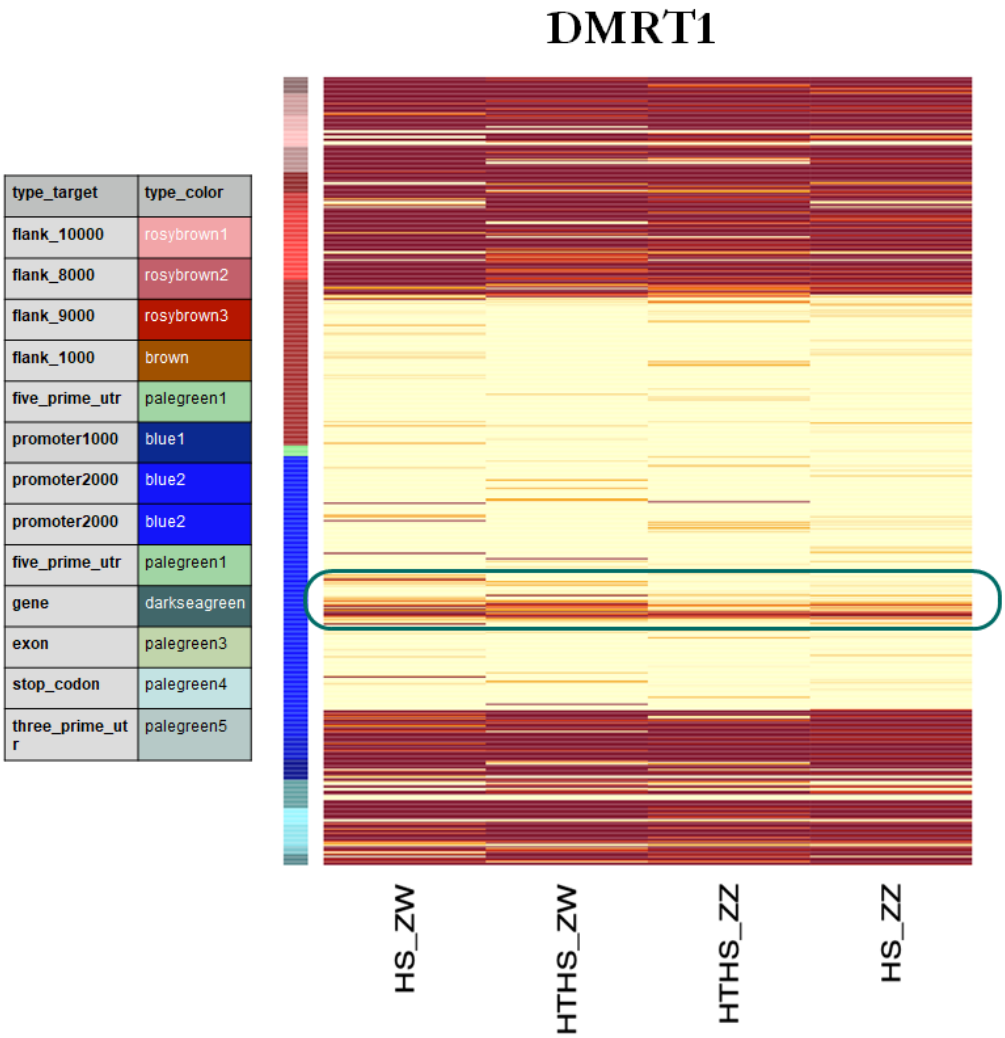


8. figure: Heat map diagram of RNA sequencing data.

DNA samples from PGCs were pooled by treatment group and sex, and then sent for whole genome bisulfite methylation sequencing (WGBS) analysis.

Based on RNA sequencing, the expression of miR-6545 is affected by heat conditioning. miR-6545 has a target site on the *DMRT1* gene (Doublesex and Mab-3 Related Transcription Factor 1), so its presence or absence affects the expression of this gene (Prastowo and Ratriyanto, 2021). Based on these, I examined the methylation pattern of the genomic region of the *DMRT1* gene. In the heat map, the methylation pattern of *DMRT1* is shown by treatment and in the case of pools according to gender, where the red parts are highly methylated, while the gene transcription is active in the yellow regions. It is clear that there is a difference in the degree of methylation in the promoter region of the gene, the gene expression in males is twice as high as in females, which is also confirmed by the localization of the gene, since the *DMRT1* gene is located on the Z chromosome. Also, methylation is lower in the HTHS group compared to the HS groups, so the expression of the *DMRT1* gene is higher in the HTHS group (Figure 9).

The fact that the genetic changes appearing in the offspring are actually the result of epigenetic inheritance necessitated further confirmation with molecular biotechnological methods. Therefore, I examined the methylation pattern of several development-specific (miR-92-3P, miR-6545, *DMRT1*) and heat stress-related (miR-138-5P) markers in the data obtained by whole-genome bisulfite methylation sequencing of the DNA samples of PGC cultures established from the progeny generation of domestic chickens that participated in the experiment. I found differences in the CpG methylation pattern for several genes (miR-92-3P, miR-138-5P, miR-6545, *DMRT1*), which are already documented to play a role in the processes regulating embryonic development and the molecular mechanisms activated by heat stress. in signaling pathways (Tavares *et al.*, 2018). Others have described the relationship between miRNAs and their mRNA targets after database analysis and found that many miRNAs have target sites on the domestic fowl *DMRT1* gene (Dunislawska *et al.*, 2021; Prastowo and Ratriyanto, 2021). From this list, we found changes in the methylation pattern of miR-92-3P, miR-138-5P and miR-6545 in our experimental samples.

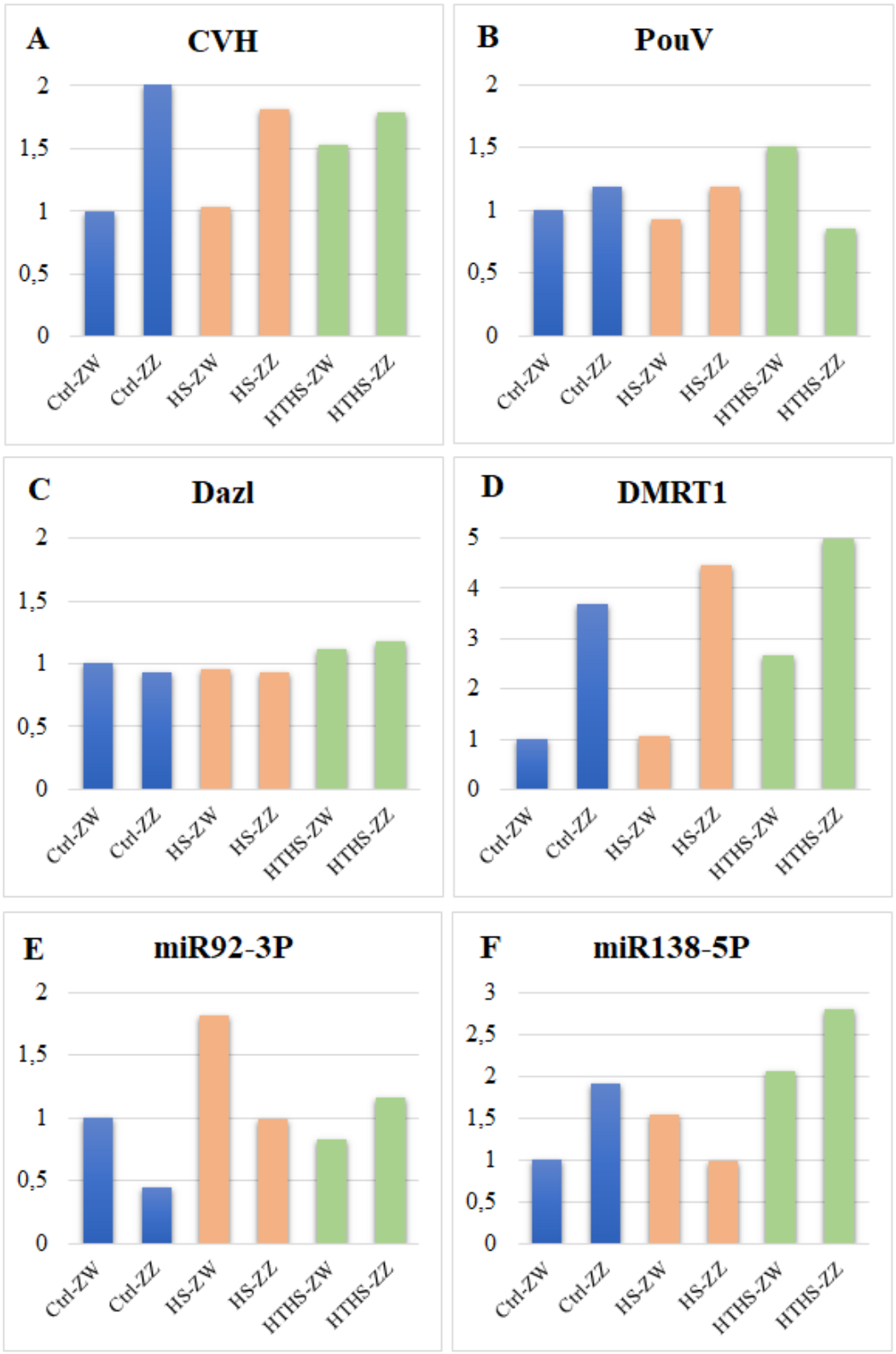


9. figure: The methylation pattern obtained during the WGBS analysis on the genomic regions of *DMRT1*, in the treatment groups (HS, HTHS) according to gender (ZW, ZZ).

I confirmed the results of RNA sequencing and WGBS with qPCR analyzes and data described in the literature, based on which it can be said that miR-6545 affects the expression of DMRT1, while the presence of miR-138-5P affects the expression of RUNX2 (Kisliouk et al. 2011; Tavares et al. 2018; Prastowo and Ratriyanto 2021).

I examined the expression pattern of gamete-specific (CVH, PouV, DAZL) markers and heat stress-related (miR-92-3P, miR-138-5P, *DMRT1*) markers in the PGC cultures established from the offspring of the domestic hens that participated in the experiment. The expression of *CVH* (Fig. 10, A) and *PouV* (Fig. 10, B) markers showed a gender difference, while *Dazl* (Fig. 10, C) was evenly expressed in all groups, in both sexes. Among the genes affected by heat, I found a change in the expression level of miR-92-3P, miR-138-5P and DMRT1 (Figure 10, D, E, F). Further

studies are needed to confirm the significant difference in these expression level changes.



10. figure: Germ cell-specific markers (A) CVH, (B) PouV, (C) DAZL, and heat stress-related (D) DMRT1, (E) miR-92-3P and (F) miR-138-5P markers expression pattern of bar charts summarizing.

4. Conclusions and recommendations

Poultry meat consumption accounts for an increasing share of global meat consumption every year. In order to satisfy the increased market demands, faster-growing and higher-yielding breeds are being introduced to the market. At the same time, as a result of global climate change, the resistance of the new varieties to increased environmental temperatures has drastically decreased. In the case of high ambient temperatures, the meat quality deteriorates, a decrease in fertility and egg quantity can occur, as well as the death of the stock, which generates increasingly serious losses in the poultry sector. Most of the literature on heat stress focuses on indirect protection strategies, analyzing the effects of optimizing housing technology and using nutritional supplements (Benton *et al.*, 1998; Daghir, 2009; Varasteh *et al.*, 2015).

Recently, new methods have come to the fore, including selection of poultry breeds based on genetic markers. The use of molecular techniques in poultry farming can lead to a sustainable economy (Rajkumar *et al.*, 2011; Felver-Gant *et al.*, 2012).

Developments in the field of biotechnology and genomics are increasingly promoting the success of breeding work in practice. Today, we already have the complete chicken genome sequence, with millions of already identified single nucleotide polymorphisms (Hillier, 2004; Wong, 2004). However, it is important to note that genome analyzes will not replace traditional selection methods, rather they will enable close cooperation between the two, increasing efficiency. Innovation should be seen as an opportunity, which could even forge the institutions of business - education - research and development into a well-functioning network (Horn, 2008).

Molecular biology tools facilitate the understanding of signaling pathways, physiological processes and immune responses, which can help better adapt species to a changing climate (Borges *et al.*, 2004; Cheng *et al.*, 2015; Wang *et al.*, 2015).

The role of HSPs in heat stress-activated responses has already been proven. The expression of HSPs increases, inducing signaling pathways to prevent protein damage (Xie *et al.*, 2014; Murugesan, Ullengala and Amirthalingam, 2017). Recent research highlights the adaptive benefits of epigenetics (Deans and Maggert, 2015; Li *et al.*, 2015) during heat conditioning (Kisliouk, Cramer and Meiri, 2017), however further experiments are needed to optimize this treatment, and a more detailed understanding of the interaction between HSPs and other molecular signaling pathways involved in

thermoregulation is also important. After that, it would be possible to develop a procedure that can be uniformly applied to all species.

Heat treatment has already been used in several different combinations in the literature. It was used in an earlier time interval, e.g.: still in eggs, by raising the hatching temperature, but this was not effective enough (Loyau *et al.*, 2016). It has also been described that if the temperature is increased stepwise, there is no significant difference compared to the effect achieved with a single heat treatment (Xie *et al.*, 2014).

Based on the results of our previous research, it can be said that the reproductive properties were less impaired in the case of heat stress compared to the control, if heat conditioning was applied beforehand. The rate of egg production was also significantly higher in the heat-conditioned group in the case of heat stress than in animals that only underwent heat stress (Anand *et al.*, 2016; Tóth *et al.*, 2021). During my research, I confirmed the claim that heat conditioning improves the proportion of viable embryos in heat-stressed conditions for domestic hens.

I think that the changes in the expression pattern of miRNAs caused by heat treatment can provide direct information about the molecular processes underlying the adaptive capacity of animals (Gebert and MacRae, 2019; Safdar and Özaslan, 2023). These results support the importance of heat conditioning at a young age. So, by optimizing the heat treatment process, we can improve the adaptability of domestic hens.

To characterize PGCs, I investigated the effect of stem cell-specific miR-302b-5P and miR-302b-3P on cell proliferation and apoptosis, using a technique based on miRNA inhibition. Inhibition of miR-302 with antagomirs (anti-miR) resulted in a decrease in the self-renewal rate of hESCs, hiPSCs, which was verified using a cell colony formation detection assay (Wu *et al.*, 2019).

Nowadays, more and more research is aimed at determining the heritability of the acclimatization trait, which can be realized through the transfer of epigenetic changes created in gametes. Adaptation is influenced by several genes, there are dominantly and recessively inherited genes. There are genes that are inherited in a sex-linked manner, such as the *DMRT1* gene that I also highlighted. The *DMRT1* gene is located on the Z sex chromosome, so after a few generations in the female line, the adaptability of the herd decreases without repeated heat conditioning (Prastowo and Ratriyanto, 2021).

Since the heritability of this ability was also confirmed in the methylation pattern studies, we believe that epigenetic inheritance can be the determinant of heredity. The epigenetic pattern is also influenced by many parameters during the course of an individual's life. Although the methylation patterns that determine the resistance to the treatment can be demonstrated over several generations, the treatment must be repeated after one or two generations to keep the actually economically useful phenotype stable.

Transgenerational epigenetic transmission from one generation to the next is unquestionable only if the effect is detected in the F3 generation or beyond (Skinner, 2011). During the study of endocrinological signaling pathways in mammals, it was confirmed that the effect of treatments applied to the parental generation can also be detected in individuals of the F3 generation (Anway *et al.*, 2005; Wolstenholme *et al.*, 2012).

During my research, I used PG cell cultures as a model system, which can be used to determine the molecular processes that take place in the next generation as a result of heat treatment. In addition to the expansion of the gene bank, I also used the cultivation and maintenance of PG cell cultures for these biotechnological tests. Its use as a model system provided the opportunity to investigate many molecular processes, such as the discovery of epigenetic modifications, which took place as a result of treatments affecting the parental generation.

All in all, heat conditioning and heat stress cause detectable gene expression level changes in the primordial germ cells, which suggests that the epigenetic changes caused by the treatment are passed on to subsequent generations. So, even with the experimental parameters used by our research group, I was able to show the expression level changes of the factors involved during heat stress in the offspring generation as well, which supports the inheritance of the animals' ability to adapt.

The results of our research can also be used economically in the case of domestic chickens. The practical applicability of the heat treatment system is available, because the heat treatment protocol we use does not require a large amount of investment, there is no need to use food supplements and vaccines, which can accumulate in meat and eggs. Industrial-scale incubators are used in farms serving economic needs, so heat treatment can be carried out in these machines, by placing the hatched chicks back at the age of two days, for 12 hours at 38^{o}C with 60% humidity, providing drinking water

and food. After the heat treatment, the normal chick rearing protocol could be continued.

If the heat treatment protocol could also be applied to other breeds of domestic fowl and other poultry species, it would be ensured that our results could contribute to increasing the efficiency of economic production on a large scale. Therefore, it can directly help the adaptability of heat-conditioned animals, regarding growth parameters and egg production.

In addition, in order to reduce the number of animals used in animal experiments, we plan to develop an *in vitro* heat treatment procedure by performing the heat treatment on PGC cultures. With the help of this, the changes in molecular signaling pathways caused by heat treatment can be studied even without animal experiments.

5. New scientific results

1. I proved that preliminary heat conditioning has an effect on the expression of several heat stress-related genes in domestic chicken brain tissue samples. HSP70 and HSP90 expression increased in all groups as a result of heat conditioning, while HSF2 and HSF3 expression decreased at puberty, which was moderated by heat conditioning. The expression of HSF1 and HSF4 increases as a result of the treatment in all groups. The expression of HSF1 showed a significant increase at the sexually mature age, in males, as a result of the heat treatment.

2. I demonstrated a significant decrease in miR-138-5P expression in the brain tissue of sexually mature males, thus confirming the long-term effect of 2-day heat conditioning.

3. I proved the role of miR-302b-3P and miR-302b-5P in the regulation of the proliferation of primordial germ cells (PGCs) by using the inhibitors of these miRNAs individually or together. In parallel with the decrease in miR-302-5P expression, the cell proliferation rate decreased in both ZW and ZZ genotype PGCs.

4. PGC cultures were used as a model system for the detection of heat treatment and heat stress linked markers. I proved that the expression of miR-92-3P increases in females as a result of heat stress, while in males it increases in the heat-stressed groups as well in the heat-conditioned and non-pretreated groups. The expression of miR-138-5P was higher in PGC cultures established from the offspring of heat-conditioned and heat-stressed animals in both sexes.

5. In the case of PGC cultures, I verified that the expression level of DMRT1, RUNX2, miR-6545 increased significantly after the heat conditioning we used.

6. I proved that in the case of PGC cultures created from the embryos of heat-treated domestic chickens, the methylation level of the promoter region of the *DMRT1* gene decreased, compared to that measured in the control cultures. Due to the decrease in the methylation level, the expression of *DMRT1* increased, which was also supported by the results of qPCR and RNA sequencing. This proved that the effect of epigenetic changes in gametes caused by heat treatment can also be detected in the next generation.

6. Publications

Publications from the topic of the dissertation

- **Tokodyné Szabadi Nikolett**, Tóth Roland, Lázár Bence, Ecker András, Urbán Martin, Várkonyi Eszter, Liptói Krisztina, Gócza Elen (2024). „Hőkondicionálás hatására létrejövő molekuláris változások vizsgálata a házityúkok agyszövetében/ Investigation of molecular changes in the brain tissue of Transylvanian naked neck chicken caused by heat conditioning" Magyar Állatorvosok Lapja, 146 (2), 67-75. 10.56385/magyallorv.2024.02.67-75.

- **Tokodyné Szabadi Nikolett**; Tóth Roland; Lázár Bence; Várkonyi Eszter; Liptói Krisztina; Tokody Dániel; Ady László; Gócza Elen (2023). „Klímaváltozás hatása a házityúk reproduktív rendszerére" Animal welfare etológia és tartástechnológia / Animal welfare ethology and housing systems 19: 1 pp. 92-101., 10 p.

- Bence Lázár*, **Nikolett Tokodyné Szabadi***, Mahek Anand, Roland Tóth, András Ecker, Martin Urbán, Maria Teresa Salinas Aponte, Ganna Stepanova, Zoltán Hegyi, László Homolya, Eszter Patakiné Várkonyi, Bertrand Pain, Elen Gócza (2022). "Effect of miR-302b MicroRNA Inhibition on Chicken Primordial Germ Cell Proliferation and Apoptosis Rate" Genes 13, no. 1: 82. https://doi.org/10.3390/genes13010082.

- Roland Tóth, **Nikolett Tokodyné Szabadi**, Bence Lázár, Kitti Buda, Barbara Végi, Judit Barna, Eszter Patakiné Várkonyi, Krisztina Liptói, Bertrand Pain, Elen Gócza (2021). "Effect of Post-Hatch Heat-Treatment in Heat-Stressed Transylvanian Naked Neck Chicken" Animals 11, no. 6: 1575. https://doi.org/10.3390/ani11061575.

- **Tokodyné Szabadi Nikolett**; Sima Krisztina; Tóth Roland; Lázár Bence; Patakiné Várkonyi Eszter; Liptói Krisztina; Gócza, Elen (2020). „Hőstressz hatására aktiválódó fiziológiai válaszok házityúkban, a hőháztartás fenntartása érdekében" Állattenyésztés és takarmányozás" 69: 1 pp. 41-52., 12 p.

- Maraghechi Pouneh, Maria Teresa Salinas Aponte, András Ecker, Bence Lázár, Roland Tóth, **Nikolett Tokodyné Szabadi**, Elen Gócza (2023). "Pluripotency-Associated microRNAs in Early Vertebrate Embryos and Stem Cells" Genes 14, no. 7: 1434. https://doi.org/10.3390/genes14071434

- **Tokodyné Szabadi Nikolett**; Tóth Roland; Lázár Bence; Ecker András; Várkonyi Eszter; Liptói Krisztina; Gócza Elen (2023). „Házityúk ősivarsejt tenyészetek, mint a biotechnológiai kutatás modellrendszerei" In: Dániel, Molnár; Dóra, Molnár (szerk.). XXVI. Tavaszi Szél Konferencia 2023: Tanulmánykötet I. Budapest: Doktoranduszok Országos Szövetsége (DOSZ) 572 p. pp 15-25., 11p.

- **Tokodyné Szabadi Nikolett**; Sima Krisztina; Tóth Roland; Lázár Bence; Molnár Mariann; Patakiné Várkonyi Eszter; Gócza Elen (2021). „Házityúk esetében alkalmazható in vitro génmegőrzés, az ősivarsejt tenyészetek jelentősége” In: Szabó, Péter; Simon, Brigitta; Soós, Adrienn; Faludi, Gergely; Fitos, Gábor (szerk.). Kutatás-fejlesztés-innováció az agrárium szolgálatában II. kötet. Budapest, Magyarország: Doktoranduszok Országos Szövetsége (DOSZ) 205 p. pp. 167-180., 14 p.

- **Tokodyné Szabadi Nikolett**; Tóth Roland; Lázár Bence; Gócza Elen. (2020) „KLÍMAVÁLTOZÁS KÁROS HATÁSAINAK KIVÉDÉSE BAROMFIBAN” In: Bihari, Erika; Molnár, Dániel; Szikszai-Németh, Ketrin (szerk.). Tavaszi Szél 2019 Konferencia = Spring Wind 2019: Konferenciakötet I. Budapest, Magyarország: Doktoranduszok Országos Szövetsége (DOSZ) 641 p. pp. 164-171., 8 p.

Publications outside the scope of the dissertation

- András Ecker, Bence Lázár, Roland Tóth, Martin Urbán, **Nikolett Tokodyné Szabadi**, Maria Teresa Salinas Aponte, Mahek Adnan, Eszter Várkonyi, Elen Gócza (2023). The Effects of Freezing Media on the Characteristics of Male and Female Chicken Primordial Germ Cell Lines. Life (Basel, Switzerland), 13(4), 867. https://doi.org/10.3390/life13040867

- Bence Lázár, Mariann Molnár, Nikolett Sztán, Barbara Végi, Árpád Drobnyák, Roland Tóth, **Nikolett Tokodyné Szabadi**, Michael J. McGrew, Elen Gócza, Eszter Patakiné Várkonyi (2021). Successful cryopreservation and regeneration of a partridge colored Hungarian native chicken breed using primordial germ cells. Poultry science, 100(8), 101207. https://doi.org/10.1016/j.psj.2021.101207

- Tóth, Roland; Lázár, Bence; **Tokodyné, Szabadi Nikolett**; Patakiné, Várkonyi Eszter; Gócza, Elen (2019). „Őshonos magyar tyúkfajták, mint lehetséges univerzális recipiensek az ősivarsejt alapú génmegőrzésben/ Indigenous Hungarian chicken breeds as universal recipients for primordial germ cell-based gene conservation” Magyar Állatorvosok Lapja, 141 (7), 439-447., 9 p.

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