Examination the effect of post-hatch heat-treatment and heat-stress in Transylvanian Naked Neck chicken

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Research

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Abstract

Background

One of the most critical global problem nowadays is the increased environmental temperature. Agriculture is very susceptible to this adverse effect because the productivity of animals and poultry decreased. Although several studies reported the effects of heat-stress in chicken, the expression profile of heat-shock proteins and heat shock factors was not investigated in the gonads and germ cells of Transylvanian Naked Neck chickens.

Methods

In the first experiment, 24 hours after hatching 80 chicks were heat treated on 38.5°C ambient temperature with 60% humidity for 12 hours. After maturation, their primary productivity parameters, such as egg production, abnormalities in embryo development, sperm quantity, concentration, and motility were studied following two weeks of heat-stress on 30 °C room temperature. In the second experiment, the thermal manipulation of 60 chicks was the same but 15 treated and 15 control chicks were sacrificed immediately after the treatment. The other 15–15 chickens were raised to maturity. Expression levels for two heat-shock proteins and four heat shock factors were determined by real-time PCR in the gonads of heat-treated and heat-stressed chickens.

Results

We found that the heat-treated layers had significantly higher egg production than the control group in heat-stressed conditions. In cockerels, the sperm quality did not differ significantly between the heat-treated and heat-stressed group and the heat-stressed but not heat-treated group. We examined the expression pattern of HSPs and HSFs in the gonads. We found that the expression of HSP90 and HSF4 increased significantly (p < 0.05) in heat-treated female chick gonads but in adult females the expression of HSF2 and HSF3 were significantly lower compared to the control. In case of adult heat-treated males, the HSP70, HSF1 and HSF3 expression levels showed a significant increase in both gonads, compared to the control expression levels (P < 0.05).

Conclusion

Heat shock proteins and heat-shock factors protect cells against different stressors, including heat stress. Our findings show a significant effect on egg production but not on the sperm quality after post-hatch heat treatment in heat stress condition. The presented significant differences might be related to the increased expression level of HSP90 and HSF4 in heat-treated chickens.

Background

The most important environmental stress factor is the increased average temperature caused by climate change. The agriculture is very sensitive to the climate variability and extreme weather [1]. Increasing number of articles were published in previous years about the effects of climate change in agriculture [2–4] and in animal husbandry [1, 5–7]. Animal exposure to hot environments deleteriously affects their reproductive functions [8]. The chickens are homeothermic animals and their body temperature is maintained in the range of 41 to 42 °C [9]. Higher temperature negatively impacts the feed intake, the reproductive function, the hatchability, and the meat and egg production [10–15]. In chickens, the high ambient temperature affects their endocrine system, reproductive and egg-laying performance, too [16].

Many researchers examined the effect of thermal manipulation in chicken. Two types of thermal manipulation are known: during the incubation period or after hatching. According to Al-Rubika et al. [17], the thermal manipulation during the late embryogenesis has not affected the hatchability, but the body weight of thermally manipulated embryos was higher than the control group. Walstra et al. [18] investigated the effect of temperature manipulation on the behaviour of layer chickens. The
thermally manipulated embryos were incubated at 37.8 °C, but between the 14th and 18th embryonic day the embryos were exposed to 40 °C for 4 hours. The manipulated chicks preferred the lower ambient temperature, but no effect of thermal manipulation on behaviour and performance were observed [18]. The effect of thermal manipulation was not detected in case of body weight in broiler, but the manipulation induced the up-regulation of muscle grow factors and muscle marker genes [19]. Vinoth et al. [20] investigated the effect of thermal manipulation and thermal stress on HSP gene expression, DNA methylation in brain tissue of Naked Neck and Punjab Broiler-2 chicken breeds. Naked Neck gene is very common in the hot regions [21]. They found that the DNA methylation level was lower, and the gene expression was higher in case of heat stressed but not heat-treated chickens, compared with other experimental groups (non-heat-treated but heat stressed; heat-treated and not heat stressed; heat-treated and stressed). Rajkumar and his collages [22] demonstrated, that the Naked Neck breeds have better growth performance in higher temperature than the normal siblings, and the Heterophil/Lymphocyte ratio was significantly lower, what is indicating that Naked Neck chickens were less stressed in higher temperature.

In case of heat stress, a lot of heat shock proteins (HSPs) and heat shock factors (HSFs) start to be expressed to protect the cells from the effect of heat stress. According to their molecular weights six HSP families are known (small HSPs, HSP40, HSP60, HSP70, HSP90 and HSP100) [23]. HSP70 and HSP90 are highly conserved ATP-dependent molecular chaperons which are essential for the eucaryote systems in unstressed conditions [24]. Of the many HSPs, the HSP70 correlates the best with thermotolerance [25]. The knockdown HSP70 mutant resulted an altered lens phenotype in zebrash embryo [26]. The HSP90 chaperone is present in bacteria and all of eucaryotes. HSP90 participates in collaboration with the HSP70 chaperon system in protein folding and activation [27–29]. Increased HSP70 expression was detected in Japanese quail's myocardial tissue in case of isolation in darkness, loud noise and cold temperature [30]. These results denoted that the heat shock proteins are expressing in different types of stress. Under stress condition in poultries all heat shock factors are activated. In animals, four HSFs was known till 2018, when Saju and his collages found [31] the fifth HSF, HSF5 in Danio rerio. This HSF5 is essential for the spermatogenesis in zebrafish. Two isoforms of HSF1 was discovered in gonads and liver of Danio [32]. Mezger and his colleagues [33] found that the HSF2 is essential in development of mouse, because they observed HSF2-like DNA binding activity at blastocyst stage. However, the HSF2 knock-out mutant mice was viable [34]. In chicken, the HSF1, HSF2 and HSF3 genes were isolated by cross-hybridization with mouse hsf1 cDNA probe [35]. HSF1 was mapped to the 2nd chromosome, the HSF2 to the 3rd, the HSF3 to the 4th, while the HSF4 gene to the 11th chromosome in chicken [36]. Zhang and his colleagues [37] examined the effects of acute heat stress in two different Chinese chicken breeds. They found that with increasing heat treatment time, both HSF3 and HSP70 expression first decreased then showed a significant increase in both breeds. They found that the expression of HSF3 and HSP70 is species-specific and tissue-specific during heat treatment. HSF4 is an important heat shock factor for the formation of mammalian lens development [26]. Although both mouse HSF4a and HSF4b form trimers in the absence of stress, HSF4a acts as an inhibitor of the constitutive expression of heat shock genes, and HSF4b acts as a transcriptional activator. Furthermore, HSF4b complements the viability defect, but not HSF4a [37].

The transition during heat-stress has different effects on various poultry breeds. The broiler breeds are more sensitive, than the local breeds. The results of heat tolerance indexes suggested that with age the local breeds easily overcome heat stress, while the other chickens become increasingly vulnerable. These results have been confirmed by the high mortality rate observed in the commercial stock under heat stress, while there was no mortality among the local chickens [38].

In our study we investigated the effect of heat treatment and heat stress on the egg production, sperm quality, heat-stress protein and heat-shock factor expression profile in Transylvanian Naked Neck Hungarian chicken breed. We found that the post-hatch (24 hours after hatching) heat manipulation had an influence mainly on the female reproductive parameters, while in adult animals, we found significant difference in heat-stress protein and heat-shock factor expressions in both genders.

Methods

Eggs of Speckled Transylvanian Naked Neck (STN) chickens were from the National Centre for Biodiversity and Gene Conservation - Institute for Farm Animal Gene Conservation (NBGK-HGI), Gödöllő, Hungary. Hatching and heat treatment took place in the experimental hatchery, the comparative study under heat stress was done in the animal house of this institution.
All applied methods in NBGK-HGI were approved by the Directorate of Food Safety and Animal Health of the Government Office of Pest County (License number: PE/EA197-4/2016) and by the Institutional Ethical Review Board.

### 3.1. Heat treatment

The eggs were incubated in a PL Machine 500 incubator in regular way. For the first 24 hours after hatching, the chicks were placed under an infrared lamp at 32 °C on absorbent paper litter with *ad libitum* starter feed and water. Then the chicks were placed back to the hatcher for heat treatment. The temperature was set to 38.5 °C and the humidity was 60% for 12 hours. Drinking water and feed were *ad libitum* inside. Then in Experiment I. the animals were kept and raised up together with the control group, in Experiment II. chicks were immediately sacrificed for DNA analysis.

### 3.2. Heat stress

In Experiment I. 80–80 animals were in the treated and control groups. We randomly selected 31–31 layers with 3–3 males in the 24th life week for the further reproductive biology examinations. Both of them were kept on a wood chips / zeolite mixture litter with *ad libitum* layer feed and water under 16-hour lighting, with egg nests (10) and perches. 10–10 roosters were placed in the same air space, in individual cages, for semen examinations, with *ad libitum* rooster feed and water. Ventilation was provided 10 minutes per hour. The average temperature in the pen at the height of the birds' habitat was constantly around 30 °C.

#### 3.2.1. Examination of embryonic abnormalities

Eggs were collected daily and marked with group number and date. Every week 20–20 eggs were candled on the 7th day of incubation and if any kind of developmental abnormality was observed they were opened and checked. The ratio of infertile eggs as well as the phenotype of dead or abnormal embryos was determined [39]. The other ones were incubated the regular way.

#### 3.2.2. Semen collection and classification

Sperm-donor animals were selected on the basis of responsiveness to semen collection, followed by individual semen evaluation data. Semen collection was performed using Burrows and Quinn's [40] dorso-abdominal massage technique twice a week for 2 months - from 23 weeks of age to 34 weeks of age - following a two-week training period. Semen was classified weekly for the following spermatological parameters:

- **Volume (ml):** determined with a pipette.
- **Motility:** determined by subjective estimation using a light microscope (Leica) on a scoring scale from 0 to 5 at 40x magnification. The test was always performed by the same experienced person.
- **Concentration:** determined with a spectrophotometer (Accucell IMV, France). At the beginning of the experiment, the instrument was calibrated. A concentration curve was established by comparing the spectrophotometer data of the samples in a dilution series with the concentrations determined using the Makler chamber.
- **Type of morphological abnormalities and live / dead cell ratio:** the study was performed using eosin-aniline blue vital staining [41].

### 3.3 Collection of gonadal tissues

The chickens were euthanized by cervical dislocation after the experiment. The tissue samples were collected in sterile plastic dishes. Small pieces from each gonad were placed into RNAlater™ Solution (Invitrogen, Thermo Fisher Scientific). We collected thigh muscle samples from each chicken for DNA analysis. The samples were transferred into TRizol™ reagent after two days. The samples were incubated in TRizol™ for 10 minutes on room temperature than stored on -80 °C.

#### 3.3.1. DNA isolation and Sex determination
The thigh muscle samples were digested using 0.1% Proteinase-K Lysis Buffer solution and incubated at 55 °C for 3 hours. After the incubation, the Proteinase-K was inactivated at 99 °C for 10 minutes. Total DNA was extracted using the Phenol-chloroform DNA isolation protocol. The isolated DNA was quantified by measuring the absorbance at 260 nm using a NanoDrop One Spectrophotometer (Thermo-Scientific, USA). The purity was assessed by determining the ratio of the absorbance at 260 and 280 nm.

The sex of treated and control chickens were determined using CHD1 (Chromosome Helicase DNA binding protein 1) primer set (Table 1). The isolated DNA was diluted to 25 ng/µl for the PCR and gel electrophoresis. PCR was performed using MyTaq Red Mix. 13 µL of the reaction solution was used: 6.75 µL MyTaq Mix; 0.5 µL reverse CHD1 primer (10 µmol); 0.5 µL forward CHD1 primer (10 µmol); 4.25 µL sterile water and finally 1 µL DNA sample. The cycling parameters were 95 °C for 1 min. 28 cycles of 95 °C for 15 s followed 30 s at 48 °C and 72 °C for 10 sec. It was finally melting at 72 °C for 5 min. The PCR products were separated by electrophoresis, using 1.5% agarose gel stained with ethidium bromide, at 100 V for 30 minutes. The DNA bands were visualized under UV illumination and photographed.

### 3.3.2. RNA isolation, cDNA writing, and Real-time qPCR

Total RNA extraction and purification from cells collected in the TRizol Reagent was following the manufacturers’ protocol. The RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop One Spectrophotometer (Thermo-Scientific, USA), and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm. Total RNA (15 µL) was reverse transcribed using a cDNA synthesis kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). SYBR Green PCR master mix was applied for the qPCR as a double-stranded fluorescent DNA-specific dye according to the manufacturer’s instructions (Applied Biosystems, Life Technologies, Carlsbad, US). The primers used for real-time PCR are displayed in Supplementary Table 1. Amplification was carried out in a total volume of 15 µL containing Power SYBR Green PCR Master Mix (Applied Biosystem, Thermo Fisher Scientific), forward and reverse primers (0.1 µg/µL), sterile water (Ambion by Life Technologies) and 0.75 µL of cDNA. After an initial 10 min denaturation step at 95 °C, the reactions were cycled 40 times under the following parameters: 95 °C for 15 s, 60 °C for 40 s, and 68 °C for 20 s. Optical detection was carried out at 68 °C.

We tested the expression of two housekeeping genes (GAPDH and ß-Actin) [12]. We compared the average Ct values of GAPDH and ß-Actin in control and heat-treated samples. We decided to use GAPDH as we found lower Ct values in the case of GAPDH, and the standard deviation were higher using ß-Actin (Supplementary Fig. 1A). There was not significant difference between the control and heat-treated samples (p = 0.523) comparing the average GAPDH Ct values. We used chicken embryonic fibroblast as reference sample [12, 42, 43]. All reactions were performed in triplicate. The number of used samples is indicated in Supplementary Table 1.B.

### 3.3. Statistical analysis

To evaluate and analyse the collected data RStudio (1.0.136), R (R-3.2.2), GeneEx (6.0) and Excel (Microsoft Office) software were used. For the data obtained from the qPCR runs, expression changes of the target genes were calculated compared to the expression of the housekeeping gene with the standard \(2^{\Delta \Delta Ct}\) method, where Ct = cycle threshold; \(\Delta Ct = Ct (target\ gene) - Ct (housekeeping\ gene)\) and \(\Delta \Delta Ct = \Delta Ct (test\ sample) - \Delta Ct (control\ sample)\).

The mean values of the different sample groups were compared with t-tests, furthermore the categorical data was tested with Chi-squared tests. Significance levels were set as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

### Results

1. Analysis of the effect of heat stress on reproductive parameters of heat-treated chickens

1.1 Spermatological analysis in roosters
The reproductivity parameters of heat-treated and heat-stressed (HTHS) Transylvanian Naked Neck roosters were examined compared to the heat-stressed but not heat-treated (HS) ones. In the spermatological analysis, 10 HTHS and 10 HS rooster were used. Four parameters (quantity, concentration, motility and live-dead ratio of sperm was determined (Fig. 1.). The volume of the semen was measured by pipetting. We could not find significant difference between the two experimental groups (p = 0.5075). In the collected ejaculate, the sperm concentration was defined by a calibrated spectrophotometer. According the sperm concentration, no significant difference was found between the HTHS and HS groups (p = 0.1077). No significant difference was found in the motility rate between the two groups (p = 0.6972). Finally, in the live-dead sperm ratio no significant difference was found (p = 0.8816) between the two experimental groups. In summary, we can conclude that the pre heat-treatment has no effect on sperm quantity and quality.

1.2 Examination of the egg production and fertilization rate in hens

In the females, two parameters, the egg production and the percent of unfertilized eggs were measured (Fig. 2.). The daily egg production of heat-treated chickens (HTHS) was significantly higher than the non-heat-treated (HS) hens on high environmental temperature (30°C) (p = 0.00002) (Fig. 2.A). Altogether, 1654 eggs were collected. The HTHS group produced 890 (54%) eggs, while in case of the HS group, we could collect 764 (46%) eggs (Fig. 2.B). We also determined the ratio of unfertilized eggs. In the HTHS group, 108 eggs, while in the HS group, 172 eggs were analysed. The eggs were incubated till 3rd day of embryonic development, then we opened the eggs, and analysed the embryonic development. In the case of HTHS group from 108 eggs, 4 (3.7%) was unfertilized, while in the HS group from 172 eggs 19 (11.05%) did not contain embryo. We found all together 257 fertile eggs. 52% of fertile eggs were derived from heat-treated group, the remaining 48% originated from control group (Fig. 2.C).

2. Comparison of the expression profile of Heat Shock Proteins and Heat Shock Factors in heat-treated and control chicken gonads

2.1 Comparison of the Delta Ct values

In this experiment we collected the gonads of heat-treated (HT), and control (Ctrl), male (M) and female (F) chickens, immediately after the heat-treatment (38.5 °C for 12 hours, in 2-days-old chickens) and in adulthood. We isolated RNA from the collected gonads. We performed qPCR to analyse the expression profile of two heat shock protein (HSP70, HSP90) genes and four heat stress factor (HSF1, HSF2, HSF3, HSF4) genes in male left and right gonads, and female genital ridges. To determine whether there is any difference in the expression profile of heat treated and control groups, we pooled the RNAs of the individual samples group by group. As we found differences in the expression pattern compared to the heat treated and control groups, we performed qPCR runs from the individual samples to prove, whether these differences are statistically different or not. We compared the Delta Ct values of pooled samples with the average values of individual RNA samples in different groups. The comparison showed high similarity in expression profile of the pooled samples to the average of individual samples values (Sup. Figure 1). Comparing the Delta Ct values calculated in the individual samples, we found significant difference between the chicks and adults (Fig. 3.1 1A-6A). Significant difference was determined between the Delta Ct values of HSP70 (p = 0.0289), and HSF3 (p = 0.0482) expressions in the heat-treated and control samples in case of adult male left gonads (Fig. 3.1 1A and Fig. 3.2 5A). In case of the right gonads, significant differences were found in HSP70 (p = 0.023); HSF1 (p = 0.0007) and HSF3 (p = 0.0013) (Fig. 3.1 1B and 3B; Fig. 3. 5B) between the control chicks and control adults. Only the HSP70 showed a significant difference comparing the values of control and heat-treated right gonads in adults (p = 0.0136) (Fig. 3.1 2A).

In case of the female gonads, only HSF4 Delta Ct values defined significant difference (p = 0.0016) between the control chicks and control adults (Fig. 3.2 6C). Significant differences were found between the control and heat-treated chickens in case of HSP90 (p = 0.0355) (Fig. 3.1 2C) and HSF4 (p = 0.0342) values (Fig. 3.2 6C). In other cases, we could not find significant differences between the groups. The Delta Ct values were analysed with Chi-squared tests.

2.2 Comparison of the relative expression profile

To get more detailed information whether there is any significant difference in relative expression of HSPs and HSFs in control and treated samples, we calculated the relative expression values using the GenEx (6.0) software. The relative expression was
determined using $2^{-\Delta\Delta Ct}$ method. The mean values of the different sample groups were compared using t-tests. The HSP90 (p = 0.0094) and HSF4 (p = 0.0387) expression was significantly higher in the heat-treated female chicken gonads than in the control (Fig. 4, E). However, in the adult female gonads all of HSPs and HSFs decreased compared to the control groups, the HSF2 (p = 0.0181) and the HSF3 (p = 0.0011) were significantly lower in treated samples. (Fig. 4, F). In the case of chicks, there was no significant difference between the male left and right gonads compared to the control group (Fig. 4, A; Fig. 4, C). However, in the left gonads of adults, the HSP70 (p = 0.0002); HSF1 (p = 0.0013); HSF2 (p = 0.0217) and HSF3 (0.0014) relative expression levels showed significant increase compared to the controls (Fig. 4, D). Analysing the male right gonads in adult samples we found that the expression of all HSPs and HSFs increased. The expression of HSP70 (p = 0.0052); HSF1 (p = 0.0333); HSF3 (p = 0.0332) and HSF4 (0.0498) showed significant increase compared to the control (Fig. 4, B).

**Discussion**

El-Tarabany [44] reported the impact of high temperature humidity index. They found that the control groups had significantly greater fertility and hatchability, than the heat stressed group. It was published that in the broiler chickens the genetically lean breed is more resistant to the higher ambient temperature, than the fat counterpart [21]. Laine and her collages [45] studied the effect of higher temperature in the hypothalamic-pituitary-gonadal-liver axis of Great Tit (Parus major). They found that the zona pellucida glycoprotein 4 (ZP4) is differently expressed before and after the onset of egg-laying [45]. We could not detect difference in spermatological parameters between the heat-treated and control groups in heat-stress condition analysing four spermatological parameters; quantity, concentration, motility and live-dead ratio of sperm (Fig. 1). On the other hand, analysing the female reproductive performance, we found significantly higher egg production and fertility rate (Fig. 2). The reason why we did not find any difference among the spermatological parameters might be the effect of the Transylvanian Naked Neck breed. However, Végi and her colleagues found that the spermatological parameters decline after heat-treatment compared with the control group in Transylvanian Naked Neck chicken breed [46]. It was published that the Naked Neck chickens show better body temperature regulation and higher radiation rates from the naked neck than the covered neck breeds if they are kept in 35 °C [47].

Mezquita et al. [48] investigated the HSP70 expression in adult chicken testes. They found that the HSP70 was highly expressed in the left and right testes at higher temperatures (44 or 46 °C). However, at normal internal temperature the HSP70 is not expressed in the left gonad but it is present in the regressed (right) gonad [48]. We could detect low HSP70 expression level, but we found significantly higher HSP70 expression at adult age in roosters both in left and right gonads when they were heat-treated. Interestingly, we found that HSF3 expression level increased parallel with HSP70 expression. Zhang and his colleagues [11] found that the level of HSP70 declined in the heart 6 hours after the heat stress, but the HSF3 expression remained high. Tarkhan and his colleagues [49] examined the HSP70 and HSF3 expression levels in cold stress. They found decreased expression levels in the liver in case of both genes.

In AA Broiler breed (from China) the HSP90 mRNA level increased in the liver, heart and kidney after 2 hours of high temperature. The HSP90 expressed in the endothelium cells and the blood vessel walls, which influences the regulation of the blood flow [50]. Hao and Gu examined the expression of HSP90 on pectoralis major in broiler breed after acute heat stress. They found that the HSP90 expression is positively correlate with corticosterone and superoxide dismutase, but negatively correlate with the pH in pectoralis major [51].

We found high HSP90 expression in chickens compared to the HSP70 expression level. We could observe significant differences only in case of female chickens between the control and heat-treated HSP90 expression level.

It was reported that numerous transcripts in the testes expressed differentially between the heat-stressed broiler-type and layer-type chickens [52]. We found in the left gonads of the adult heat-treated males that the HSP70 HSF1, HSF2 and HSF3 relative expression levels showed significant increase compared to the controls. Whether these expression patterns associate with the heat-tolerance require a further investigation. It was found that after 2 hours of heat treatment the expression of HSP27, HSP90 and HSP70 increased in a Taiwanese country chicken rooster, but the mRNA of CDH5, CIRBP, SLA and NTF3 were downregulated in the testes [52]. Wang et al. [53] published that in the heat-stressed chicken testes the proteins that involve in autophagy and
the major HSPs (HSP90α, HSPA5, HSPA8) were upregulated but the proteins that negatively regulate apoptosis were downregulated. In the future, we plan to check the expression level of these factors in our heat-treated samples, too.

Furukawa et al. [54] shows that the HSF1 is a very important regulator in the ovarian differentiation of Medaka. They made a HSF1 knock-out animal and found that HSF1 protects the female germ cells under heat stress. We could detect significantly higher HSF1 expression in heat-treated roosters, in both left and right gonads, compared to the control, but there was no significant difference in the level of HSF1 in treated and control females.

HSF2 is very important in the development of brain and reproductive organs, but the fundamental rule is not identified yet [55]. In HSF2 knock-out B Lymphocyte cells they found that the KO line was more sensitive to the heat stress than the wild type [56]. We found higher HSF2 expression in heat-treated gonads in adult roosters, but significantly lower expression in adult females.

The mutation of HSF4 gene may cause a congenital or senile cataract in human. We found significantly higher HSF4 expression in heat-treated female chickens parallel with high HSP90 expression. In case of males, we could not detect difference in the expression profile between the heat-treated and the control ones. According to these findings, we propose that the increased HSP90 and HSF4 levels could eliminate somehow the effect of heat stress, but further analysis is needed to find the molecular pathways responsible for this effect.

**Conclusion**

The average global temperature has increased over the century. Heat shock proteins and heat-shock factors play an essential role in normal cellular physiology and protection against different stressors, including heat stress. In chicken, HSP and HSF levels are increased in almost all the tissues in response to heat stress. This increased HSP level protects cellular proteins from heat-stress induced damage. Our findings show a significant effect on egg production but not on the sperm quality after post-hatch heat treatment. The egg production is more complex, longer and energy intense process than the spermiogenesis and that could be one of the reasons why we could not find any difference in the sperm quality between the control and heat-stressed group. The found significant differences might be related to the increased expression level of HSP90 and HSF4 in heat-treated female chickens.

**Declarations**

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**Authors’ contributions**

The experiments designed by E.G and K.L. The experiments were performed by R.T., N.T.SZ, B.L, K.B., K.L., B.V., J.B. Statistical analysis was done by B.L. and E.G. The paper was written by R.T, E.G and was revised E.P.V., K.L, B.V and P.B. All read and accepted the final manuscript.

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**Availability of data and materials**

All data supporting our findings are included in the manuscript.

**Ethics approval and consent to participate**
Not applicable

**Consent for publication**

Not applicable

**Competing interest**

The authors declare that they have no competing interests.

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**References**

1. Babinszky L, Halas V, W.A. M. Impacts of climate change on animal production and quality of animal food products. Clim Chang Socioecon Eff. InTech; 2011.

2. EPA U. Climate impacts on agriculture and food supply. Clim Impacts Agric Food Supply [Internet]. 2013 [cited 2020 Feb 25];1–10. Available from: https://19january2017snapshot.epa.gov/climate-impacts/climate-impacts-agriculture-and-food-supply_.html

3. Araya A, Prasad PVV, Zambreski Z, Gowda PH, Ciampitti I, Assefa Y, et al. Spatial analysis of the impact of climate change factors and adaptation strategies on productivity of wheat in Ethiopia. Sci Total Environ [Internet]. 2020 [cited 2020 May 19];731:139094. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0048969720326115

4. Grünig M, Mazzi D, Calanca P, Karger DN, Pellissier L. Crop and forest pest metaweb shift towards increased linkage and suitability overlap under climate change. Commun Biol. Nature Research; 2020;3.

5. Wolfenson D, Lew BJ, Thatcher WW, Graber Y, Meidan R. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. Anim Reprod Sci [Internet]. Anim Reprod Sci; 1997 [cited 2020 Mar 3];47:9–19. Available from: https://pubmed.ncbi.nlm.nih.gov/9233502-seasonal-and-acute-heat-stress-effects-on-steroid-production-by-dominant-follicles-in-cows/

6. Rath P, Behura N, Sahoo S, Panda P, Mandal K, Panigrahi P. Amelioration of Heat Stress for poultry welfare: A strategic approach. Int J Livest Res. ScopeMed International Medical Journal Management and Indexing System; 2015;5:1.

7. Magdi M, GL H, MA K, Ahmed G, AO A, PH P. Effect of heat stress on production parameters and immune responses of commercial laying hens. Poult Sci [Internet]. Poult Sci; 2004 [cited 2020 Mar 4];83:889–94. Available from: https://pubmed.ncbi.nlm.nih.gov/15206614-effect-of-heat-stress-on-production-parameters-and-immune-responses-of-commercial-laying-hens/

8. Cheng CY, Tu WL, Wang SH, Tang PC, Chen CF, Chen HH, et al. Annotation of differential gene expression in small yellow follicles of a broiler-type strain of Taiwan country chickens in response to acute heat stress. PLoS One [Internet]. Public Library of Science; 2015 [cited 2020 Feb 28];10. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0143418

9. Murugesan S. Heat shock protein and thermal stress in chicken. Heat Shock Proteins Vet Med Sci [Internet]. 2018. p. 179–93. Available from: https://www.researchgate.net/publication/323898457_Heat_Shock_Protein_and_Thermal_Stress_in_Chicken
10. Xie J, Tang L, Lu L, Zhang L, Lin X, Liu H, et al. Effects of acute and chronic heat stress on plasma metabolites, hormones and oxidant status in restrictedly fed broiler breeders. Poult Sci [Internet]. 2015;94:1635–44. Available from: https://www.ncbi.nlm.nih.gov/pubmed/25910904

11. Zhang WW, Kong LN, Zhang XQ, Luo QB. Alteration of HSF3 and HSP70 mRNA expression in the tissues of two chicken breeds during acute heat stress. Genet Mol Res. 2014;13:9787–94.

12. Xie J, Tang L, Lu L, Zhang L, Xi L, Liu H, et al. Differential expression of heat shock transcription factors and heat shock proteins after acute and chronic heat stress in laying chickens (Gallus gallus). PLoS One [Internet]. 2014;9:16–8. Available from: https://pubmed.ncbi.nlm.nih.gov/25072282-differential-expression-of-heat-shock-transcription-factors-and-heat-shock-proteins-after-acute-and-chronic-heat-stress-in-laying-chickens-gallus-gallus/

13. Cedraz H, Gracielle JGG, Garcia AAPJ, Filho RVF, Souza TM, Ribeiro E de O, et al. Heat stress induces expression of HSP genes in genetically divergent chickens. PLoS One [Internet]. 2017;12. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29020081

14. Y W, Saelao P, Chanthavixay K, R G, D B, Susan J L, et al. Physiological responses to heat stress in two genetically distinct chicken inbred lines. Poult Sci [Internet]. Poult Sci; 2018 [cited 2020 Feb 18];97:770–80. Available from: https://pubmed.ncbi.nlm.nih.gov/29267901-physiological-responses-to-heat-stress-in-two-genetically-distinct-chicken-inbred-lines/?from_term=Chicken+heat+treatment&from_pos=3

15. Akbarian A, Michiels J, Degroote J, Majeddin M, Golan A, De Smet S. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. J Anim Sci Biotechnol [Internet]. Journal of Animal Science and Biotechnology; 2016;7. Available from: http://dx.doi.org/10.1186/s40104-016-0097-5

16. Rozenboim I, Tako E, O G-G, J. A P, Uni Z. The effect of heat stress on ovarian function of laying hens. Poult Sci [Internet]. Poult Sci; 2007 [cited 2020 Mar 3];86:1760–5. Available from: https://pubmed.ncbi.nlm.nih.gov/17626822-the-effect-of-heat-stress-on-ovarian-function-of-laying-hens/

17. Al-Rubika RK, Al-Zghoul MB, Hananeh W, Al-Natour MQ, Abu-Basha EA. Thermal manipulation during late embryogenesis: Effect on body weight and temperature, thyroid hormones, and differential white blood cell counts in Broiler chickens. Poult Sci [Internet]. 2017 [cited 2020 Mar 4];96:234–40. Available from: https://pubmed.ncbi.nlm.nih.gov/27587725-thermal-manipulation-during-late-embryogenesis-effect-on-body-weight-and-temperature-thyroid-hormones-and-differential-white-blood-cell-counts-in-broiler-chickens/

18. Walstra I, Napel J Ten, Kemp B, van den Brand H. Temperature manipulation during layer chick embryogenesis. Poult Sci. Poult Sci; 2010;89:1502–8.

19. Al-Zghoul MB, El-Bahr SM. Thermal manipulation of the broilers embryos: Expression of muscle markers genes and weights of body and internal organs during embryonic and post-hatch days. BMC Vet Res. BioMed Central Ltd.; 2019;15.

20. Vinoth A, Thirunalasundari T, Shanmugam M, Uthrakumar A, Suji S, Rajkumar U. Evaluation of DNA methylation and mRNA expression of heat shock proteins in thermal manipulated chicken. Cell Stress Chaperones. Cell Stress and Chaperones; 2018;23:235–52.

21. Geraert PA, Guillaumin S, Leclercq B. Are genetically lean broilers more resistant to hot climate? Br Poult Sci. Br Poult Sci; 1993;34:643–53.

22. Rajkumar U, Reddy MR, Rama Rao S V., Radhika K, Shanmugam M. Evaluation of growth, carcass, immune response and stress parameters in naked neck chicken and their normal siblings under tropical winter and summer temperatures. Asian-Australasian J Anim Sci. Asian-Australasian Association of Animal Production Societies; 2011;24:509–16.

23. Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and Ecological Physiology. Annu Rev Physiol [Internet]. Annual Reviews; 1999 [cited 2020 May 19];61:243–82. Available from: http://www.jstor.org/stable/23496643?seq=1#page_scan_tab_contents

24. Genest O, Wickner S, Doyle SM. Hsp90 and Hsp70 chaperons: Collaborators in protein remodelling. J Biol Chem [Internet]. 2019;294. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30401745

25. Li GC, Mak JY. Re-induction of hsp70 synthesis: An assay for thermotolerance. Int J Hyperth. 2009;25:249–57.
26. Swan CL, Evans TG, Sylvain N, Krone PH. Zebrash HSF4: A novel protein that shares features of both HSF1 and HSF4 of mammals. Cell Stress Chaperones. Cell Stress Chaperones; 2012;17:623–37.

27. Johnson JL. Evolution and function of diverse Hsp90 homologs and cochaperone proteins. Biochim. Biophys. Acta - Mol. Cell Res. 2012. p. 607–13.

28. Stankiewicz M, Mayer MP. The universe of Hsp90. Biomol. Concepts. De Gruyter Mouton; 2012. p. 79–97.

29. Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: Emerging mechanistic insights. Nat. Rev. Mol. Cell Biol. 2010. p. 515–28.

30. Hoekstra KA, Iwama GK, Nichols CR, Godin D V, Cheng KM. Increased heat shock protein expression after stress in Japanese quail. Stress. Harwood Academic Publishers GmbH; 1998;2:265–72.

31. Saju JM, Hossain MS, Liew WC, Pradhan A, Thevasagayam NM, Tan LSE, et al. Heat Shock Factor 5 Is Essential for Spermatogenesis in Zebrash. Cell Rep. 2018;

32. Christina M R, Susanna A, Arto S, HV B, T J, Mikko N, et al. Tissue-specific expression of zebrash (Danio rerio) heat shock factor 1 mRNAs in response to heat stress. J Exp Biol. 2000;203:1817–24.

33. Mezger V, Rallu M, Morimoto RI, Morange M, Renard JP. Heat shock factor 2-like activity in mouse blastocysts. Dev Biol. Academic Press; 1994;166:819–22.

34. McMillan DR, Christians E, Forster M, Xiao X, Connell P, Plumier J-C, et al. Heat Shock Transcription Factor 2 Is Not Essential for Embryonic Development, Fertility, or Adult Cognitive and Psychomotor Function in Mice. Mol Cell Biol. American Society for Microbiology; 2002;22:8005–14.

35. Nakai A, Morimoto RI. Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. Mol Cell Biol. American Society for Microbiology; 1993;13:1983–97.

36. Fujimoto M, Nakai A. The heat shock factor family and adaptation to proteotoxic stress. FEBS J [Internet]. Blackwell Publishing Ltd; 2010 [cited 2020 Feb 20];277:4112–25. Available from: http://doi.wiley.com/10.1111/j.1742-4658.2010.07827.x

37. Huang M, Li D, Huang Y, Cui X, Liao S, Wang J, et al. HSF4 promotes G1/S arrest in human lens epithelial cells by stabilizing p53. Biochim Biophys Acta - Mol Cell Res. Elsevier; 2015;1853:1808–17.

38. Melesse A, Maak S, Schmidt R, von Lengerken G. Effect of long-term heat stress on some performance traits and plasma enzyme activities in Naked-neck chickens and their F1 crosses with commercial layer breeds. Livest Sci. 2011;141:227–31.

39. Liptoi K, Hidas A. Investigation of possible genetic background of early embryonic mortality in poultry. Worlds Poult Sci J. Informa UK Limited; 2006;62:326–37.

40. Burrows WH, Quinn JP. The collection of spermatozoa from the Domestic Fowl and Turkey. Poult Sci. Elsevier BV; 1937;16:19–24.

41. Váradi É, Drobnyák Á, Végi B, Liptói K, Kiss C, Barna J. Cryopreservation of gander semen in cryovials-comparative study. Acta Vet Hung. 2019;67:246–55.

42. Borowska D, Rothwell L, Bailey R, Watson K, Kaiser P. Identification of stable reference genes for quantitative PCR in cells derived from chicken lymphoid organs. Vet Immunol Immunopathol [Internet]. Elsevier B.V.; 2016 [cited 2020 Nov 10];170:20–4. Available from: https://pubmed.ncbi.nlm.nih.gov/26872627/

43. Zhang J, Gao Y-Y, Huang Y-Q, Fan Q, Lu X-T, Wang C-K. Selection of housekeeping genes for quantitative gene expression analysis in yellow-feathered broilers. Ital J Anim Sci [Internet]. Taylor and Francis Ltd.; 2018 [cited 2020 Nov 10];17:540–6. Available from: https://www.tandfonline.com/doi/full/10.1080/1828051X.2017.1365633

44. El-Tarabany MS. Effect of thermal stress on fertility and egg quality of Japanese quail. J Therm Biol. Elsevier Ltd; 2016;61:38–43.

45. Laine VN, Verhagen I, Mateman AC, Pijl A, Williams TD, Gienapp P, et al. Exploration of tissue-specific gene expression patterns underlying timing of breeding in contrasting temperature environments in a song bird. BMC Genomics. BioMed Central Ltd.; 2019;20:1–16.
46. Barbara V, Éva V, Zsuzsanna S, Szőke Zsuzsanna F, Molnár Andrea K, Judit B. A hőkezelés hatása hímivarú baromfifélék spermatológiai mutatóira. AWETH [Internet]. Gödöllő: Különszám; 2008 [cited 2020 Nov 18];4:401–8. Available from: moz-extension://8f6d2f54-98d8-447d-874d-e0b76597efa9/enhanced-reader.html?openApp&pdf=http%3A%2F%2Fepa.oszk.hu%2F02000%2F02067%2F00011%2Fpdf%2FEPA02067_AWETH2008401408.pdf

47. Yahav S, Luger D, Cahaner A, Dotan M, Rusal M, Hurwitz S. Thermoregulation in naked neck chickens subjected to different ambient temperatures. Br Poult Sci. Taylor and Francis Ltd.; 1998;39:133–8.

48. Mezquita B, Mezquita J, Durfort M, Mezquita C. Constitutive and heat-shock induced expression of Hsp70 mRNA during chicken testicular development and regression. J Cell Biochem [Internet]. 2001;82:480–90. Available from: https://www.ncbi.nlm.nih.gov/pubmed/11500924

49. Tarkhan AH, Saleh KMM, Al-Zghoul MB. HSF3 and Hsp70 Expression during Post-Hatch Cold Stress in Broiler Chickens Subjected to Embryonic Thermal Manipulation. Vet Sci [Internet]. MDPI AG; 2020 [cited 2020 Nov 10];7:49. Available from: /pmc/articles/PMC7356021/?report=abstract

50. Lei L, Yu J, Bao E. Expression of heat shock protein 90 (Hsp90) and transcription of its corresponding mRNA in broilers exposed to high temperature. Br Poult Sci [Internet]. 2009 [cited 2020 Mar 27];50:504–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19735020

51. Hao Y, Gu XH. Effects of heat shock protein 90 expression on pectoralis major oxidation in broilers exposed to acute heat stress. Poult Sci [Internet]. 2014;93:2709–17. Available from: https://www.ncbi.nlm.nih.gov/pubmed/25239533

52. Wang SH, Cheng CY, Tang PC, Chen CF, Chen HH, Lee YP, et al. Acute heat stress induces differential gene expressions in the testes of a broiler-type strain of Taiwan country chickens. PLoS One. Public Library of Science; 2015;10.

53. Wang SH, Cheng CY, Chen CJ, Chan HL, Chen HH, Tang PC, et al. Acute Heat Stress Changes Protein Expression in the Testes of a Broiler-Type Strain of Taiwan Country Chickens. Anim Biotechnol. Taylor and Francis Inc.; 2019;30:129–45.

54. Furukawa F, Hamasaki S, Hara S, Uchimura T, Shiraishi E, Osafune N, et al. Heat shock factor 1 protects germ cell proliferation during early ovarian differentiation in medaka. Sci Rep [Internet]. 2019;9:1–10. Available from: https://www.nature.com/articles/s41598-019-43472-4

55. Shinkawa T, Tan K, Fujimoto M, Hayashida N, Yamamoto K, Takaki E, et al. Heat shock factor 2 is required for maintaining proteostasis against febrile-range thermal stress and polyglutamine aggregation. Mol Biol Cell [Internet]. Mol Biol Cell; 2011 [cited 2020 Nov 10];22:3571–83. Available from: https://pubmed.ncbi.nlm.nih.gov/21813737/

56. Joutsen J, Da Silva AJ, Luoto JC, Budzynski MA, Nylund AS, de Thonel A, et al. Heat Shock Factor 2 Protects against Proteotoxicity by Maintaining Cell-Cell Adhesion. Cell Rep. Elsevier B.V.; 2020;30:583-597.e6.

57. Lee JCI, Tsai LC, Hwa PY, Chan CL, Huang A, Chin SC, et al. A novel strategy for avian species and gender identification using the CHD gene. Mol Cell Probes [Internet]. Mol Cell Probes; 2010 [cited 2020 Nov 10];24:27–31. Available from: https://pubmed.ncbi.nlm.nih.gov/19716876/

Figures
Figure 1

In this figure the results of heat-treated and heat stressed (HTHS) and only heat stressed but not treated (HS) mature cockerel's sperm parameters are summarized. Significant difference between the HT and HTHS group was not found. A: The quantity of sperm (ml) collected with dorso-abdominalis massage from cockerels. The sperm amount measured by pipetting. No significant difference was determined between the HTHS and HS group. (p=0.5075). B: The concentration of sperm (10⁶/ml) in the collected ejaculations (the spectrophotometer was calibrated before the experiment). No significant difference was determined between the HTHS and HS group (p=0.1077). C: The sperm motility was measured by subjective estimation. The estimation was in 40x magnification. The sperm motility classifies in 0-5 scale. Every estimation was made by the same researcher. No significant difference was determined in the motility data between the HTHS and HS group (p=0.6972). D: Finally, the live-dead ratio of sperm was measured in the collected ejaculates. Eosin-aniline vitality staining was used. The results of live-dead ratio represented in %. No significant difference was determined in the live-dead ratio of HTHS and HS group (p=0.8816).
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In this figure the results of heat-treated and heat stressed (HTHS) and only heat stressed but not treated (HS) mature cockerel’s sperm parameters are summarized. Significant difference between the HT and HTHS group was not found. A: The quantity of sperm (ml) collected with dorso-abdominalis massage from cockerels. The sperm amount measured by pipetting. No significant difference was determined between the HTHS and HS group. (p=0.5075). B: The concentration of sperm (10⁶/ml) in the collected ejaculations (the spectrophotometer was calibrated before the experiment). No significant difference was determined between the HTHS and HS group (p= 0.1077). C: The sperm motility was measured by subjective estimation. The estimation was in 40x magnification. The sperm motility classifies in 0-5 scale. Every estimation was made by the same researcher. No significant difference was determined in the motility data between the HTHS and HS group (p=0.6972). D: Finally, the live-dead ratio of sperm was measured in the collected ejaculates. Eosin-aniline vitality staining was used. The results of live-dead ratio represented in %. No significant difference was determined in the live-dead ratio of HTHS and HS group (p= 0.8816).
Demonstration of the daily egg production and the ratio of fertilized eggs in heat-treated and heat stressed (HTHS) and only heat stressed (HS) group. The eggs were collected every day, and were incubated to determine the fertilized egg ratio. A: Illustration of the daily egg production of HTHS and HS hens. In case of HTHS hens, significantly higher egg production was observed than in the HS group (p=0.0000235). B: We collected 1654 eggs all together. 54% derived from HTHS group, while 46% from HS group. C: 280 eggs were incubated all together. We found 257 fertilised eggs. 52% derived from HTHS group and 48% from the HS group.
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Figure 3

Delta Ct values of two Heat Shock Protein (HSP70 and HSP90) and four Heat Shock Factor HSF1, HSF2, HSF3, HSF4) genes were determined in male and female left gonads of (heat-treated) HT and (control) CTRL chickens and adults. In every case the GAPDH was the reference gene. The expression levels of 1: HSP70 (HSPA2), 2: HSP90 (HSP90AA1), 3: HSF1, 4: HSF2, 5: HSF3, 6: HSF4 genes in chicks and adults, females-males, left and right gonads in case of HT and CTRL. A: Male left gonads, B: Male right gonads, C: Female gonads. 1A: In case of the male left gonads, significant differences were determined in the expression of HSP70 between the CTRL chicks and CTRL adults (p=0.014), furthermore in case of adults between the CTRL and HT samples the differences were significant (p=0.0289). 1B: In male right gonads between the CTRL chicks and CTRL adult samples
(p=0.0228), and in case of the adults, between CTRL and HT was significant difference (p=0.0136). 1C: Comparing the female gonads no significant differences were determined. 2A: In case of the male left gonads, significant difference was determined in the expression of HSP90 between the CTRL chicks and CTRL adults (p=0.0072). 2B: No significant differences were determined in male right gonads. 2C: Between the CTRL and HT chicks’ significant difference (p=0.0355) was determined in the female gonads. 3A: The HSF1 expression of male left gonads were significantly different (p=0.0002) compared to the CTRL chicks and CTRL adult group. 3B: Significant HSF1 differences (p=0.0007) were determined in the male right gonads between the CTRL chicks and CTRL adults. 3C: In the female gonads, no significant differences were found. 4A: In case of male left gonads, we found significant differences between the CTRL chicks and the CTRL adult samples in HSF2 expression (p=0.0417). 4B: In the male right gonads there was no difference between the two groups. 4C: Comparing the female gonads significant difference was not found. 5A: Significant difference was found in the HSF3 expression in male left gonads between the CTRL chicks and CTRL adults (p=0.0002), furthermore between the adult CTRL and adult HT samples (p=0.0482), too. 5B: In the male right gonads, between CTRL chicks and adult group was significant difference (p=0.0013), furthermore between CTRL and HT group in case of adult samples was significant differences (p=0.0349) too. 5C: In case of female gonads, we did not find a significant diversion. 6A: In the left male gonads between the CTRL chicks and CTRL mature groups were significant differences (p=0.0113) in the HSF4 expression. 6B: In case of male right gonads there was no difference. 6C: Comparing the female gonads between CTRL chicks and CTRL adult a significant difference was found (p=0.0016), furthermore between CTRL and HT was significant difference (p=0.0342) in chicks. (* p<0.05, ** p<0.01 and *** p<0.001)
Delta Ct values of two Heat Shock Protein (HSP70 and HSP90) and four Heat Shock Factor HSF1, HSF2, HSF3, HSF4) genes were determined in male and female left gonads of (heat-treated) HT and (control) CTRL chickens and adults. In every case the GAPDH was the reference gene. The expression levels of 1: HSP70 (HSPA2), 2: HSP90 (HSP90AA1), 3: HSF1, 4: HSF2, 5: HSF3, 6: HSF4 genes in chicks and adults, females-males, left and right gonads in case of HT and CTRL. A: Male left gonads, B: Male right gonads, C: Female gonads. 1A: In case of the male left gonads, significant differences were determined in the expression of HSP70 between the CTRL chicks and CTRL adults (p=0.014), furthermore in case of adults between the CTRL and HT samples the differences were significant (p=0.0289). 1B: In male right gonads between the CTRL chicks and CTRL adult samples (p=0.0228), and in case of the adults, between CTRL and HT was significant difference (p=0.0136). 1C: Comparing the female gonads no significant differences were determined. 2A: In case of the male left gonads, significant difference was determined in the expression of HSP90 between the CTRL chicks and CTRL adults (p=0.0072). 2B: No significant differences were determined in male right gonads. 2C: Between the CTRL and HT chicks' significant difference (p=0.0355) was determined in the female gonads. 3A: The HSF1 expression of male left gonads were significantly different (p=0.0002) compared to the CTRL chicks and CTRL adult group. 3B: Significant HSF1 differences (p=0.0007) were determined in the male right gonads between the CTRL...
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**Figure 4**

The bar charts show the relative expression values in female and male chick and adult samples in control and heat-treated gonads relative to chicken embryonic fibroblast sample. The relative expression values of Heat Shock Protein (HSP70 and HSP90) and Heat Shock Factor (HSF1, HSF2, HSF3, HSF4) genes were determined in female and male gonads. GAPDH was chosen as reference gene. A: Relative expression values in right male chick gonads. No significant differences were found compare the heat-treated and control groups. B: Relative expression values in right male adult gonads. In adult HT male right gonads, all of the heat-shock related markers increased. Significant increase was observed in HSP70 ($p=0.0052$); HSF1 ($p=0.0333$); HSF3 ($p=0.0332$) and HSF4 ($p=0.0498$). C: In the left gonads of male chicks, we couldn't recognise any significant
difference between HT and CTRL groups. D: Relative expression values in left male adult gonads. HSP70 (p=0.0002); HSF1 (p=0.0013); HSF2 (p=0.0217) and HSF3 (p=0.0014) expressions showed significant difference. E: Relative expression values in female chicks. In case of HT female chick gonads, the HSP90 (p=0.0094) and HSF4 (p=0.0387) values were significantly different from CTRL. F: Relative expression values in female adult gonads. Comparing the relative expression values in the HT and CTRL adult female gonads only the HSF2 (p=0.0181) and HSF3 (p=0.0011) showed significant difference.

**Figure 4**

The bar charts show the relative expression values in female and male chick and adult samples in control and heat-treated gonads relative to chicken embryonic fibroblast sample. The relative expression values of Heat Shock Protein (HSP70 and HSP90) and Heat Shock Factor (HSF1, HSF2, HSF3, HSF4) genes were determined in female and male gonads. GAPDH was chosen as reference gene. A: Relative expression values in right male chick gonads. No significant differences were found compare the heat-treated and control groups. B: Relative expression values in right male adult gonads. In adult HT male right gonads, all of the heat-shock related markers increased. Significant increase was observed in HSP70 (p=0.0052); HSF1 (p=0.0333); HSF3 (p=0.0332) and HSF4 (p=0.0498). C: In the left gonads of male chicks, we couldn’t recognise any significant difference between HT and CTRL groups. D: Relative expression values in left male adult gonads. HSP70 (p=0.0002); HSF1 (p=0.0013); HSF2 (p=0.0217) and HSF3 (p=0.0014) expressions showed significant difference. E: Relative expression values in female chicks. In case of HT female chick gonads, the HSP90 (p=0.0094) and HSF4 (p=0.0387) values were significantly different from CTRL. F: Relative expression values in female adult gonads. Comparing the relative expression values in the HT and CTRL adult female gonads only the HSF2 (p=0.0181) and HSF3 (p=0.0011) showed significant difference.
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