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# Heat Exposure Affects the mRNA Levels of Antioxidant Enzymes in Embryonic and Adult Broiler Chickens

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**Abstract**

Artificial selection pressures utilized by the poultry industry have resulted in broilers capable of fast growth but possessing poor thermoregulatory capacity. Modern broiler strains are, therefore, especially vulnerable to heat exposure during the incubation, rearing, and transport processes. The objective of the present study was to investigate the effects of heat exposure (HE) during embryonic and adult life on catalase, NADPH oxidase 4 (*NOX4*), and superoxide dismutase 2 (*SOD2*) expression. Briefly, the experimental design involved two phases: HE during embryonic development as well as HE during adult life. For the first phase, Ross eggs were divided into control (n=268) and HE (n=270) groups that were incubated under standard (37.8$^{o}$C and 56% relative humidity (RH)) and HE (39$^{o}$C and 65% RH for 18 h/day from embryonic day (ED) 10 to 18) conditions, respectively. At ED 18, embryos (n=6) from each group were randomly selected and euthanized to obtain heart, liver, and spleen samples. For the second phase, 28 day-old adult Cobb chickens were exposed to an elevated temperature of 40$^{o}$C for 7 hours, and, at 0, 1, 3, 5, and 7 hours of HE, 5 chickens were randomly selected and euthanized to obtain liver samples. Embryonic heat exposure resulted in dysregulated catalase, *NOX4*, and *SOD2* mRNA expression. Catalase mRNA expression was significantly higher in the hearts (p=0.014) and spleens (p=0.0299) but significantly lower in the livers (p=0.002) of heat-exposed embryos. Likewise, in heat-exposed embryos, *SOD2* mRNA expression was significantly higher in the hearts (p=0.0002) and spleens (p=0.041) but significantly lower in the livers (p=0.009). Although *NOX4* mRNA levels were significantly higher in the hearts (p=0.003) and significantly lower in the livers (p=0.03) of heat-exposed controls, this expression did not change in the spleens (p=0.79). In contrast, heat stress during adult life affected only the catalase mRNA levels during certain time points, namely after 7 hours of heat exposure (p=0.0001). Since the aforementioned genes play essential roles in the prevention of oxidative stress, the present study could help elucidate the mechanism behind heat-induced oxidative stress in the context of broiler chickens.

**Keywords**: heat stress; broiler; antioxidant; liver; catalase; superoxide dismutase.

## 1. Introduction

The broiler industry is the fastest-growing meat industry on a global scale (Chang and Hui-Shung, 2007). Over the past half-century, artificial selection pressures have increased the growth rates of broiler chickens by more than 400% (Zuidhof *et al.*, 2014). Despite these advances, broiler growth is still dependent upon environmental rearing conditions such as feed and water provision, flock density, and ambient temperature, the latter of which can seriously impact broiler welfare (Lara and Rostagno, 2013). In fact, it has been suggested that artificially selecting for increased growth rates has compromised the thermoregulatory capacity of broilers, making them especially susceptible to heat stress (Sandercock† *et al*., 2006). Heat stress is a major cause of broiler mortality during rearing and transportation, resulting in major economic losses and reduced meat quality (St-Pierre *et al.*, 2003; dos Santos *et al*., 2017).

In broilers, heat stress often manifests in the form of oxidative stress, which occurs as a result of the imbalance between an organism's antioxidant defence system and its reactive oxygen species (ROS) (Estévez, 2015). NADPH oxidase 4 (NOX4) is a constitutionally active membrane-bound complex that helps maintain oxidative homeostasis by acting as an oxygen sensor (Nisimoto *et al*., 2014). In the process, NOX4 generates superoxide ($O_2^-$) radicals and hydrogen peroxide ($H_2O_2$), and their unchecked production contributes to damage of cellular DNA, lipids, and proteins (Mujahid *et al*., 2005; Mishra and Jha, 2019). Although they are damaging in excess, both superoxide and hydrogen peroxide play an essential role in innate immune defence mechanisms, and *NOX4* under-expression increases an organism's susceptibility to microbial infection (Rada and Leto, 2008). A plethora of mechanisms are involved in the prevention of oxidative stress in the case of elevated *NOX4* expression, the most notable of which are superoxide dismutase 2 (*SOD2*) and catalase (Al-Zghoul *et al*., 2019).

Serving as the first line of defence against heat-induced oxidative stress, the antioxidant enzymes SOD2 and catalase dismutate superoxide and disproportionate hydrogen peroxide, respectively (Matsumoto *et al*., 2009;

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Ighodaro and Akinloye, 2018). As can be seen from Figure 1, SOD2 and catalase are essential to the prevention of oxidative damage in the aftermath of *NOX4* expression. Furthermore, heat-stressed broiler chickens were reported to have modulated levels of *SOD2* and catalase expression that differed between different types of tissues (Surai, 2016; Del Vesco *et al.*, 2017; Kikusato and Toyomizu, 2019). Compared to other organs, the liver is especially vulnerable to the effects of oxidative stress because of its key role in maintaining homeostasis (Bonkovsky, 2015; Li *et al.*, 2015). It has been previously shown that the liver of broiler chickens is more susceptible than the heart during periods of acute heat stress (Lin *et al*., 2006).

The optimum incubation temperature for a chicken embryo is 37.8°C ± 0.3°C; higher temperatures accelerate growth while lower temperatures inhibit it (Yalcin and Siegel, 2003). To combat the effects of heat stress, thermal manipulation (TM), which is the increase of incubation temperature for a certain period of time during embryonic development, enhances a broiler's ability to tolerate heat stress as an adult (Moraes *et al*., 2003; Morita *et al*., 2016; Narinç *et al*., 2016; Al-Zghoul *et al*., 2019; Saleh and Al-Zghoul, 2019; Al-Zghoul *et al*., 2019). Such thermal manipulation should be intermittent and not continuous, as the latter negatively affected the hatchability and overall performance parameters of broilers (Piestun *et al.*, 2008). Although there are previous reports of the post-hatch benefits of embryonic thermal manipulation, the exact effect on embryo physiology and metabolic function is not well-understood.

Despite warming global temperatures and a rapidly growing broiler industry, there is only a small number of studies on the effect of heat-induced oxidative stress on broiler liver function. Similarly, there is a dearth of information on the effects of oxidative stress on the embryonic cardiac, hepatic, and splenic antioxidant function of broilers. Therefore, the main objectives of the present study are to investigate the effects of embryonic heat exposure and the impact of post-hatch heat stress on the cardiac, hepatic and splenic expression of the catalase, *NOX4*, and *SOD2* genes in broiler chickens.

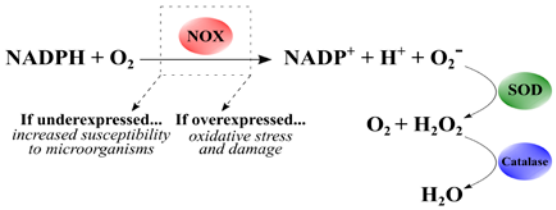


**Figure 1**. NOX4 acts as an oxygen sensor to protect against oxidative damage. However, its activity generates superoxide and hydrogen peroxide, which must then be dismutated and disproportionated by superoxide dismutase 2 (SOD2) and catalase, respectively.

## 2. Material and methods

All experimental procedures employed in the current study were approved by the Animal Care and Use Committee at Jordan University of Science and Technology. **Figure 2** illustrates the details of the experimental design described in this section.

### *2.1. Heat exposure during embryonic development*

Fertile Ross eggs (n=600) were acquired from Al-watannia poultry certified breeder in Amman, Jordan, and thoroughly examined for any abnormalities. After excluding damaged eggs (n=17), the remaining eggs were incubated in two Type I HS-SF commercial incubators (Masalles, Spain) under standard conditions (37.8°C and 56% relative humidity (RH)) until embryonic day (ED) 10. The viability of the incubated eggs was checked on embryonic day (ED) 7 by candling, in which infertile eggs and eggs with dead embryos were excluded from the present study (n=35). On ED 10, the eggs in the first incubator were considered as the control group (n=268), and the eggs in the other incubator were considered as the heat-exposed (HE) group (n=280). The eggs of the control group were maintained at 37.8°C and 56% RH for the entirety of the incubation period, while those in the HE group were incubated at 39°C and 65% RH for 18 h/day during ED 10 to 18. On ED 18, six embryos were randomly selected from each group and their hearts, livers, and spleens were collected.

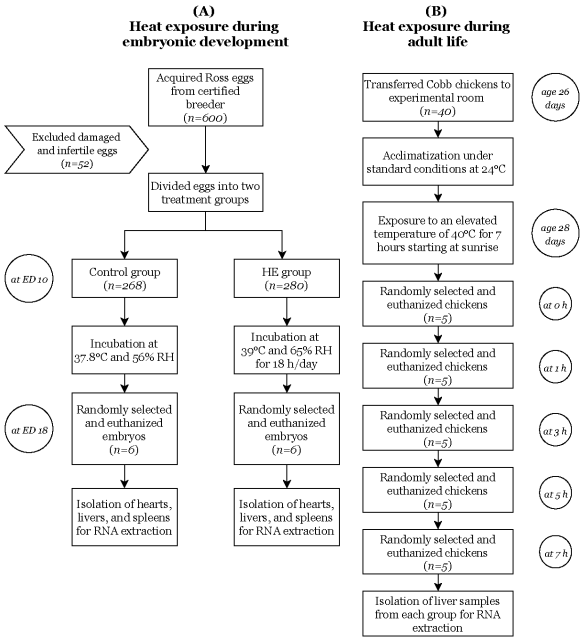


**Figure 2**. Summary of the experimental procedures employed in the present study. ED: embryonic day; HE: heat-exposed; RH; relative humidity.

### *2.2. Heat exposure during adult life*

At the age of 26 days, healthy Cobb chickens (n=40) were transferred to the experimental room in order to acclimate under standard conditions and at a room temperature of 24°C. At the age of 28 days, the chickens were exposed to an elevated temperature of 40°C for 7 hours starting at sunrise. After 0, 1, 3, 5, and 7 hours of heat exposure, 5 chickens were randomly selected and euthanized in order to extract liver samples. The chickens euthanized at 0 hours of heat exposure were considered as the control group to be compared with all other time intervals.

### *2.3. Total RNA extraction and reverse transcription*

Total RNA was extracted using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, USA) alongside a TRI

Reagent Kit (Zymo Research, USA). RNA concentrations were determined using the PowerWave XS2 microplate spectrophotometer (BioTek, USA). 2 µg of total RNA from each sample was used to carry out reverse transcription by means of the Power cDNA Synthesis Kit (iNtRON Biotechnology, South Korea).

### *2.4. Relative quantification via real-time RT-PCR*

The QuantiFast SYBR Green PCR Kit (Qiagen, USA) was used on the Rotor-Gene Q MDx 5plex HRM instrument (Qiagen, USA). Briefly, the 20 µl reaction mix was prepared from 10 µl of master mix, 1.2 µl of forward primer, 1.2 µl of reverse primer, 1 µl of sample cDNA, and 6.6 µl of nuclease-free water. The PCR process involved a single cycle of 95°C for 5 mins, 40 cycles of 95°C for 10s followed by 30s at 55°C, and 72°C for 10s with final melting at 95°C for 20s. The fluorescence emission detection was carried out during the extension step. 28S rRNA was used as an internal control to which the fold changes in mRNA levels were normalized. The single target amplification specificity was assessed using the generated melting curve. The relative quantitation was calculated automatically. Table 1 shows the primer sequences that were used in the real-time RT-PCR analysis (Al-Zghoul *et al*., 2019).

### *2.5. Statistical analysis*

IBM SPSS Statistics v. 23 was utilized for all statistical analyses. Catalase, *NOX4*, and *SOD2* mRNA levels were expressed as means ± SD. One-way analysis of variance (ANOVA) was used to compare between the control and HE groups during embryonic development. However, ANOVA followed by the all-pairs Bonferroni test was used to compare the difference between time intervals of heat exposure (0, 1, 3, 5, and 7 h). Parametric differences were considered to be statistically significant at $P < 0.05$.

**Table 1**. Primer sequences used in the PCR analysis.

| Gene | Forward (5′ to 3′) | Reverse (5′ to 3′) |
|---|---|---|
| *NOX4* | CCAGACCAACTTAGAGGAACAC | TCTGGGAAAGGCTCAGTAGTA |
| *SOD2* | CTGACCTGCCTTACGACTATG | CGCCTCTTTGTATTTCTCCTCT |
| Catalase | GAAGCAGAGAGGTTCCCATTTA | CATACGCCATCTGTTCTACCTC |
| 28S rRNA | CCTGAATCCCGAGGTTAACTATT | GAGGTGCGGCTTATCATCTATC |

## 3. Results

### *3.1. Effect of embryonic heat exposure*

The effects of embryonic heat exposure on the mRNA levels of *NOX4*, *SOD2*, and catalase in broiler hearts, livers, and spleens are shown in **Figure 3**. Catalase and *SOD2* mRNA levels were significantly higher in hearts (p=0.014; p=0.0002) and spleens (p=0.0299; p=0.041) but significantly lower in the livers of HE embryos compared to controls (p=0.002; p=0.009). In addition, *NOX4* mRNA levels were significantly higher in the hearts (p=0.003) and significantly lower in the livers of HE embryos compared to controls (p=0.03). However, the splenic mRNA levels of *NOX4* were not significantly different between the two groups (p=0.79).

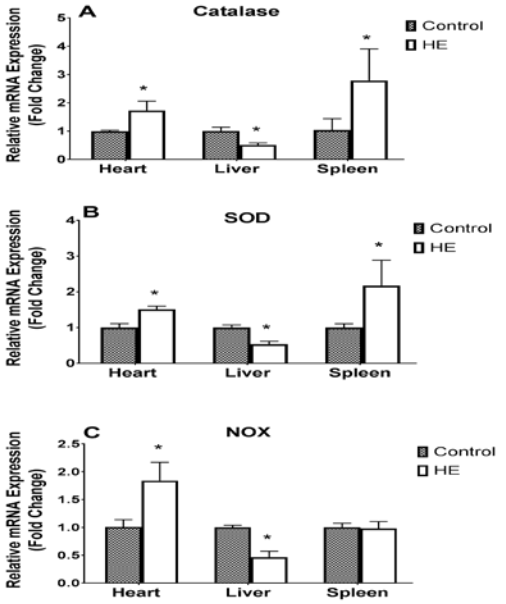


**Figure 3**. mRNA expression levels of the catalase, *NOX4*, and *SOD2* genes in broilers exposed to heat during embryonic development. In panels A and B, cardiac and splenic catalase and *SOD2* expression is significantly higher in the HE group, while hepatic expression is significantly lower. In panel C, there was no difference in splenic *NOX4* expression between the control and HE groups, but cardiac and hepatic expressions were respectively higher and lower in the HE group. * mean ± SD of HE group is significantly different with control group.

### *3.2. Effect of adult heat exposure*

The effects of heat exposure on the mRNA levels of *NOX4*, *SOD2*, and catalase in the livers of 28 day old chickens are shown in **Figure 4**. Heat exposure did not result in significant changes in the mRNA levels of *NOX4* and *SOD2* in broilers (p>0.05). Moreover, heat exposure did not significantly change catalase mRNA levels after 1, 3, and 5 h (p>0.05). In contrast, the mRNA levels of catalase were significantly higher after 7 h of heat exposure compared to 0 h (p=0.0001).

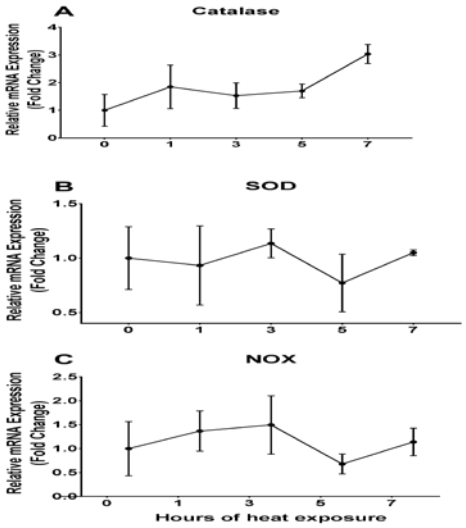


**Figure 4**. Hepatic mRNA expression levels of the catalase, *NOX4*, and *SOD2* genes in broilers exposed to heat during adult life. In panel A, catalase expression was significantly higher only after 7 h of HE compared to 0 h. In panels B and C, HE did not significantly affect *SOD2* and *NOX4* expression.

## 4. Discussion

Due to artificial selection pressures imposed by the commercial poultry industry, modern broiler breeds possess significantly enhanced weight gain and feed efficiency rates compared to their predecessors (Zuidhof *et al.*, 2014). However, these selection pressures did not result in similar improvements to the cardiovascular, immune, and respiratory systems, all of which contain organs that play essential roles in broiler thermoregulation (Havenstein *et al.*, 2003). Heat exposure during adult life poses a serious and imminent threat to the global poultry industry, resulting in substantial commercial losses (St-Pierre *et al.*, 2003). Therefore, the aim of the present study was to investigate the effects of heat exposure during the embryonic and adult lives of broiler chickens on the mRNA levels of certain antioxidant enzymes, mainly with regard to the catalase, *NOX4*, and *SOD2* genes.

Catalase mRNA levels were elevated in the heart and spleen but reduced in the liver of embryos exposed to heat. Likewise, catalase mRNA levels significantly increased in the livers of adult broilers after 7 hours of heat exposure. Previously, catalase expression has been reported to increase in heat-stressed broilers in response to heat-induced oxidative stress (Ismail *et al*., 2013; Del Vesco *et al.*, 2017). Moreover, catalase activity in heat-stressed broilers can be enhanced by means of dietary supplementation (Kumbhar *et al*., 2018). However, it was found that heat stress during embryonic and adult life resulted in reduced catalase mRNA expression levels in the livers of Cobb and Hubbard breeds (Al-Zghoul *et al.*, 2019).

After heat exposure, *NOX4* mRNA levels were found to significantly increase and decrease in embryo hearts and livers, respectively, although splenic *NOX4* expression remained the same. Contrastingly, *NOX4* mRNA expression levels decreased in the livers of heat-stressed broilers exposed to embryonic thermal manipulation (Al-Zghoul *et al*., 2019). In adult broilers, heat exposure did not result in significant changes to hepatic *NOX4* expression (Habashy *et al.*, 2018). Nevertheless, *NOX4* mRNA expression levels increased in avian skeletal muscle cells upon exposure to heat stress (Kikusato and Toyomizu, 2019).

Our findings show that heat exposure caused *SOD2* mRNA levels to increase in the hearts and spleens and decrease in the livers of broiler embryos. In contrast, heat exposure during adulthood did not significantly alter *SOD2* expression in broilers. Increased *SOD2* expression in certain types of skeletal muscle was reported in 21-day-old broilers exposed to heat stress (Kikusato and Toyomizu, 2019). In contrast, thermally manipulated Cobb and Hubbard broilers exposed to heat stress exhibited reduced hepatic *SOD2* mRNA expression levels compared to controls (Al-Zghoul *et al*., 2019).

Differences between antioxidant gene expression in embryos and adults could be due to the fact that embryos are poikilothermic and, therefore, highly sensitive to temperature changes (Yalcin and Siegel, 2003; Noiva *et al*., 2014). In fact, TM during embryonic development results in lower plasma triiodothyronine concentrations and reduced oxygen consumption, both of which control metabolism and the production of heat in fast-growing chickens (Loyau *et al*., 2014). Moreover, the liver has a chief role in the metabolic function and homeostatic maintenance of broilers, particularly in response to cyclic heat stress (Jastrebski *et al*., 2017).

## 5. Conclusions

The present findings indicate that heat exposure during embryonic and adult broiler life can have significant effects on a broiler's response to oxidative stress. Of special importance is the catalase gene, as its expression was dysregulated during heat challenge in both the embryonic and adult phases of a broiler's life. Limitations of the present study include the fact that it only utilized one broiler breed (Cobb or Ross) for each experimental phase. In the future, different broiler strains should also be investigated in the context of antioxidant mRNA expression after embryonic and post-hatch heat exposure.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

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