Prenatal auditory stimulation induces physiological stress responses in developing embryos and newly hatched chicks

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ABSTRACT Prenatal stress may evoke considerable physiological consequences on the developing poultry embryos and neonates. The present study aimed to determine prenatal auditory stimulation effects on serum levels of ceruloplasmin (**CPN**), alpha-1-acid glycoprotein (**AGP**), corticosterone (**CORT**), and heat shock protein 70 (**Hsp70**) regulations in developing chicken embryos and newly hatched chicks. Hatching eggs were subjected to the following auditory treatments; 1) control (no additional sound treatment other than the background sound of the incubator's compressors at 40 dB), 2) noise exposure (eggs were exposed to pre-recorded traffic noise at 90 dB) (NOISE), and 3) music exposure (eggs were exposed to Mozart's Sonata for Two Pianos in D Major, K 488 at 90 dB) (MUSIC). The NOISE and MUSIC treatments were for 20 min/h for 24 h (a total of 8 h/d), starting from embryonic days (**ED**) 12 to hatching. The MUSIC (1.37 ± 0.1 ng/mL) and NOISE (1.49 ± 0.2 ng/mL) treatments significantly elevated CPN at ED 15 compared to the Control (0.82 ± 0.04 ng/mL) group and post-hatch day 1 (Control, 1.86 ± 0.2 ng/mL; MUSIC, 2.84 ± 0.4 ng/mL; NOISE, 3.04 ± 0.3 ng/mL), AGP at ED 15 (Control, 39.1 ± 7.1 mg/mL; MUSIC, 85.5 ± 12.9 mg/mL; NOISE, 85.4 ± 15.1 mg/mL) and post-hatch day 1 (Control, 20.4 ± 2.2 mg/mL; MUSIC, 30.5 ± 4.7 mg/mL; NOISE, 30.3 ± 1.4 mg/mL). CORT significantly increased at ED 15 in both MUSIC (9.024 ± 1.4 ng/mL) and NOISE (12.15 ± 1.6 ng/mL) compared to the Control (4.39 ± 0.7 ng/mL) group. On the other hand, MUSIC exposed embryos had significantly higher Hsp70 expression than their Control and NOISE counterparts at ED 18 (Control, 12.9 ± 1.2 ng/mL; MUSIC, 129.6 ± 26.4 ng/mL; NOISE, 13.3 ± 2.3 ng/mL) and post-hatch day 1 (Control, 15.2 ± 1.7 ng/mL; MUSIC, 195.5 ± 68.5 ng/mL; NOISE, 13.2 ± 2.7 ng/mL). In conclusion, developing chicken embryos respond to auditory stimulation by altering CPN, AGP, CORT, and Hsp70. The alterations of these analytes could be important in developing embryos and newly hatched chicks to cope with stress attributed to auditory stimulation.

Key words: prenatal auditory stress, acute phase proteins, corticosterone, heat shock protein 70, chickens embryos

INTRODUCTION

The prenatal period is crucial because any external stimuli imposed at a specific time during the stage may influence the development process (Dobbing and Sands, 1979; Rice and Barone, 2000). Stress during development could alter animals' physiological and behavioral responses throughout life (Cirulli et al., 2009; Henriksen et al., 2011; Dixon et al., 2016). Prenatal stress in avian species is a concern because embryos could be more vulnerable to unpredictable environmental fluctuations than post-hatching individuals due to their development outside the mother's body (Henriksen et al., 2011; Dixon et al., 2016; Hanafi et al., 2022). In the case of the avian species, a multitude of environmental or climatic factors, such as the hen's pre-lay habitat and the environment to which the embryo is exposed between ovulation and hatching, can

have long-term consequences on the offspring (Lay and Wilson, 2002; Henriksen et al., 2011). Prenatal exogenous corticosterone (**CORT**) treatment may also influence neonatal chicks. Sui et al. (1997) reported that pre-hatch CORT enhanced memory retention in hatchlings, but Rodricks et al. (2006) reported otherwise. The conflicting findings could be attributed to differences in the timing of CORT treatment (Henriksen et al., 2011).

Previously, most of the work on prenatal stress involved rodents and primates (Schneider et al., 2001; Jafari et al., 2017; Weinstock et al., 2017). The findings showed that prenatal stress was detrimental to offspring's stress-coping ability, learning ability, social behavior, and personality (Henriksen et al., 2011). Elevated maternal cortisol levels and programming of the hypothalamic-pituitary-adrenal (**HPA**) axis are possible underlying fundamental mechanisms (Seckl, 2004; Kapoor et al., 2006). Adversity in early life, especially in the critical developmental window of intrauterine life, may have programming effects on health outcomes in postnatal life.

Interestingly, earlier work in poultry suggests that a certain magnitude of prenatal stress may positively affect neonatal chicks. For example, thermal manipulation at sensitive embryonic phases during the last days of incubation (Tzschentke and Plageman, 2006; Fernandes et al., 2016), such as using certain degrees and durations of heat or cold exposure, induced long-lasting thermotolerance in poultry during postnatal life (Shinder et al., 2002; Piestun et al., 2008; Al-Zghoul, 2018). Loyau et al. (2014) suggested the importance of epigenetic in modulating the thermal tolerance mechanisms. Recently, there has been an increasing interest in the consequences of prenatal auditory stimulation in poultry (Kesar, 2013; Sanyal et al., 2013a,b; Tong et al., 2015). The auditory system development in domestic chickens began as early as day 10 of incubation (Alladi et al., 2002), and chicken embryos responded to external sound below 90 dB from day 16 of incubation (Jones et al., 2006). Previous studies showed that prenatal sound stimulation with species-specific calls and sitar music at 65 dB enhanced morphological and biochemical alterations in the chicks' hippocampus and improved spatial orientation and learning at 12 h post-hatch (Chaudhury et al., 2006). Sanyal et al. (2013b) subjected developing chicken embryos to either patterned and rhythmic sitar sound (music) or unpatterned and arrhythmic sound (noise) of a similar sound pressure level (110 dB). The authors found that music was beneficial to the neuronal morphology of the auditory areas and hippocampus in chicken embryos, and the contrary was reported for noise exposure. Donofre et al. (2020) reported that exposing developing embryos to loud noise (at 90 dB) improved hatchability rate from 2% to 8% and chick quality at hatching time. However, the consequences of auditory stimulation on physiological stress response in the developing embryos are unknown. Such information is necessary before prenatal auditory stimulation can be considered a practical strategy to improve the health and well-being of chicks in the industry.

Acute phase proteins (**APP**) are highly conserved plasma proteins that are increasingly secreted by the liver in response to trauma, infection, stress, neoplasia, and inflammation (Murata et al., 2004; O'Reilly et al., 2014). The synthesis of APPs is regulated by both endogenous glucocorticoids and the production of pro-inflammatory cytokines (Rosa et al., 2019). Stress caused by higher stocking densities (Shakeri et al., 2014; Najafi et al., 2015), heat stress (Najafi et al., 2015; Olubudun et al., 2015), and feed deprivation (Najafi et al., 2016) increased circulating ceruloplasmin (**CPN**) and alpha-1-acid glycoprotein (**AGP**) concentrations in broiler chickens. Hence, APPs could gauge physiological stress in avian species and are thus another biomarker for well-being. Recent work in our laboratory showed that APPs are present in "unstressed" chicken embryos (Hanafi et al., 2021). However, auditory stress and blood APPs in the avian species during the prenatal or neonatal stage have not been documented.

Heat shock proteins are molecular chaperones with multiple physiological roles. The expression of heat shock proteins in response to stress protects against the initial insult, augment recovery, and produce a state of resistance to subsequent stresses (Kregel, 2002). Hanafi et al. (2021) reported the detection of heat shock protein 70 (**Hsp70**) in chicken embryos on day 14 of incubation. The effects of heat and cold stresses on Hsp70 expressions in chicken embryos have been demonstrated (Givisiez et al., 2000; Leandro et al., 2004; Al-Zghoul et al., 2013). However, the effects of prenatal auditory stimulation on Hsp expression in avian embryos remain unclear. Hoekstra et al. (1998) reported that 60 min of exposure to loud rock music (76 dB) for 5 d increased myocardial Hsp70 expression in Japanese quail. Therefore, the present study aimed to elucidate the effects of music and noise exposure during late embryonic development on serum levels of CPN, AGP, CORT, and Hsp70 in developing broiler embryos and neonatal chicks. The findings of this study are essential to shed light on the potential implication of prenatal sound stimulation, which may influence the ability of chicks to cope with subsequent stressors later in life. We postulate the hypothesis that music and noise may have disparate effects on the physiological response of embryos and neonatal chicks.

MATERIALS AND METHODS

Ethical Statement

The experiment was conducted following guidelines of the Research Policy of Universiti Putra Malaysia, as outlined in the Guide for the Care and Use of Experimental Animals (UPM/IACUC/AUP-R058/2019).

Experimental Incubators

A total of 3 incubators (Brinsea Ovo-Easy Incubator) with a carrying capacity of 144 eggs per incubator were used in this study (Brinsea Products Inc., Titusville, FL).

These incubators were equipped with electronic temperature control and humidity control with 3 trays on tilted platforms, ensuring that the eggs could be turned as preset. The preset incubation temperature was 37.8°C for each incubator (precision of 0.5°C), and the relative humidity ranged between 55% on embryonic day (**ED**) 1 and 17% to 65% on ED 18-hatching. Eggs were turned automatically every 2 h from ED 1 to ED 18. On ED 18, eggs were placed into hatching trays and were not turned for the remainder of the incubation process. All incubators were located in the same ventilated room with a controlled environmental temperature of 24°C to 25°C (accuracy of 2°C). The incubators were installed with double-walled, sound-proof medium density fibreboard (**MDF**) insulator to prevent interference with sound treatments (Figure 1). Sound pressure levels (**SPL**) were measured inside and outside the operating incubators using the sound meter level. The SPL outside the operating incubators dropped below 50 dB, whereas inside the operating incubator was maintained at 90 dB.

Auditory Stimulation Protocols

The background noise was measured at the center of the incubation chambers using a digital sound level meter (TECPEL DSL-330, New Taipei City, Taiwan). Sound treatments were applied inside the incubator chambers through a 10 W wireless speaker with 24 h of playtime (Tronsmart Element Groove, Shenzen, China) placed in the incubation chamber emitting sounds recorded in MP3 format at 44.1 kHz sampling frequency stored in SD card memory. The intensity level of the sounds emitted by the speaker was calibrated using the sound meter level at the center of the incubation

chamber at the beginning of the sound exposure. The recorded MP3 music and noise signals were analyzed using MATLAB software (version 2020B) installed in a computer with an intel core i7 CPU 2.6 GHz processing speed and 16 GB RAM.

The signal spectrums of the right channel of the signals were analyzed using the fast Fourier transform (**FFT**) and short-time Fourier transform (**SHFT**) methods utilizing FFT and spectrogram functions in the signal processing toolbox, respectively.

Hamming window of 1,000 length, 50% overlap, and 256 FFT points was applied to generate the spectrogram. Figures 2 and 3 depicted the spectrogram of the music and noise signals, respectively. It can be observed that the noise had a larger range of frequency components up to 16 kHz compared to the music of 13 kHz. Furthermore, the noise had a higher energy density, especially in the lower frequency band. This is also evident from the signal spectrum determined using FFT for the entire signal duration, as shown in Figures 4 and 5. The noise was broadband with uniformly distributed frequency components compared to the music with peaks at selected frequencies.

The total signals energies are computed by summing up signal energy at individual frequencies using a method by McLoughlin (2016).

$$E = \frac{1}{N} \sum_{k=1}^{N} |X(f_k)|^2$$

where $X(f_k)$, f_k, and N are the spectral amplitude density, discrete frequency and the number of discrete frequency elements, respectively. The results indicated that the total energy of the noise of 0.2898 Joules is higher than the music of 0.1044 Joule.

Figure 1. The external view: experimental incubators with acoustic padding isolation; internal view: incubator platform with automatic turning and the speaker placed in the incubator chamber.
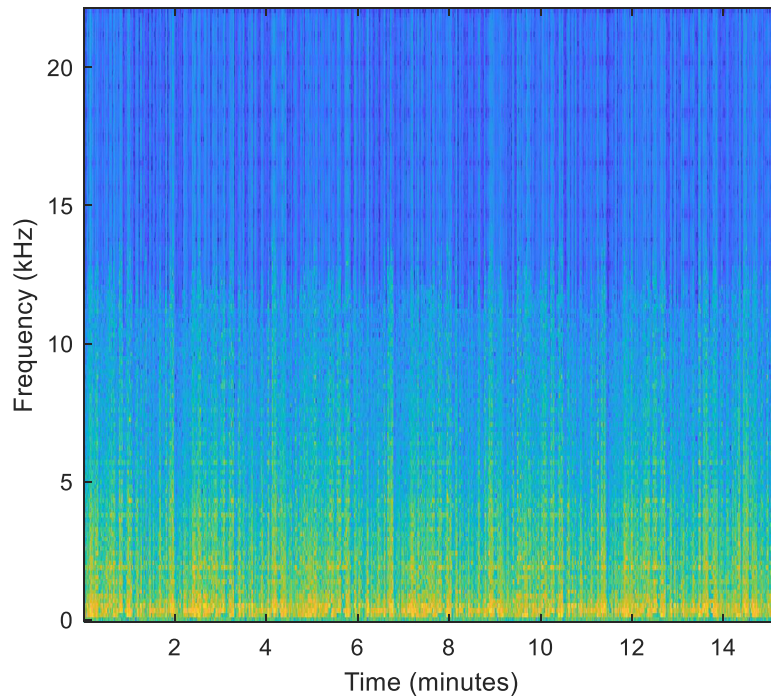
Figure 2. Spectrogram of music signal.

Experimental Groups

The study involved 3 prenatal auditory stimulation treatments. The prenatal auditory stimulation groups are as follow:

1) Control: The eggs were incubated and received no additional sound stimuli except the compressor's (40 dB) sound, which is unavoidable.
2) Noise stimulation: The eggs were incubated under similar conditions to the controls. From ED 12 to ED 21, the eggs were exposed to pre-recorded traffic noise at 90 dB ($\pm$ 2 dB) for 20 min/h over 24 h (a total of 8 h/d). This
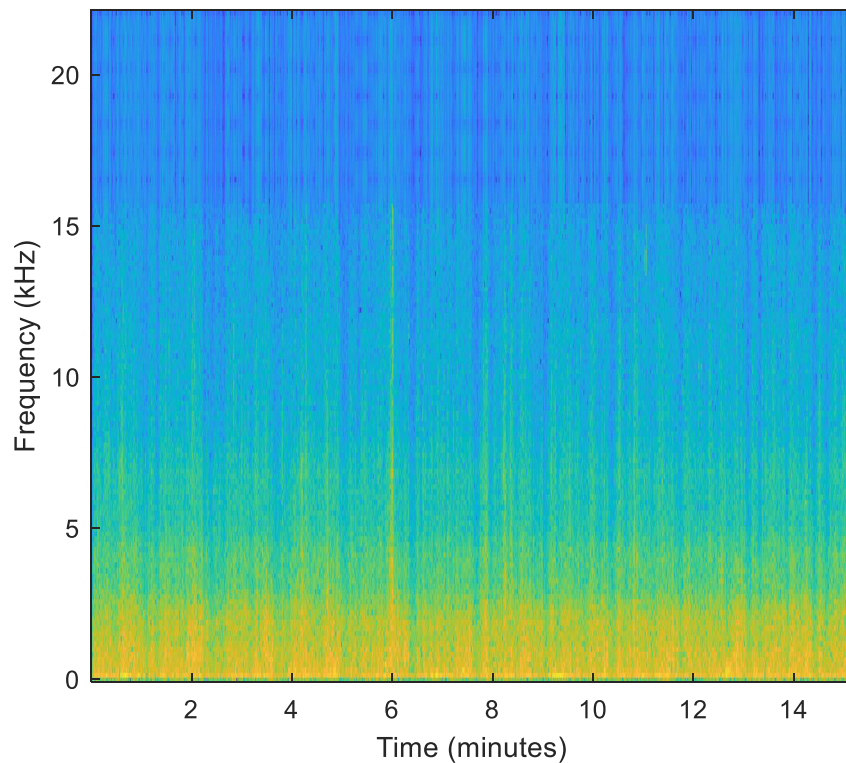

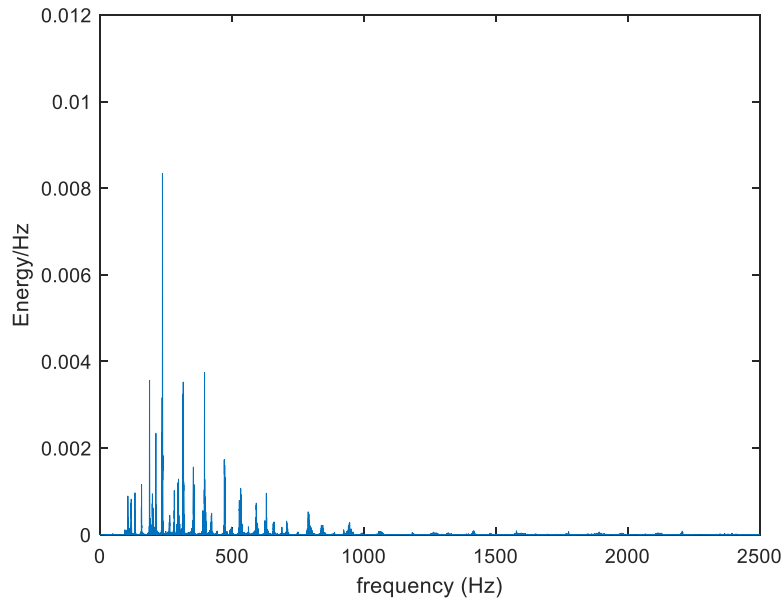**Figure 3.** Spectrogram of noise signal.

Figure 4. Single-sided spectrum of music signal displayed up to 2,500 Hz.

treatment was termed the "NOISE" group. The last cycle of noise stimulation was approximately at 09:00.

3) Music stimulation: The eggs were incubated under similar conditions to the Control and NOISE groups. From ED 12 to ED 21, the eggs were exposed to low-level classical music (Mozart's Sonata for Two Pianos in D Major, K 488) at 90 dB ($\pm$ 2 dB) for 20 min/h over 24 h (a total of 8 h/d). This treatment was termed the "MUSIC" group. The last cycle of music stimulation was approximately at 09:00.

Pre-hatching Responses

A total of 420 Cobb 500 broiler chicken eggs (65.90 $\pm$ 0.47 g) were obtained from a commercial hatchery in Negeri Sembilan, Malaysia. The age of the breeding flocks was 44 wk. The eggs were fumigated and incubated in 3 double-insulated incubators (Brinsea Ovo-Easy 190 Advance Series) (140 eggs per incubator). Ventilation was provided with a forced draft of air, and the levels of temperature and humidity were assumed uniform throughout the chamber. At ED 10, all eggs were candled, and 30 infertile eggs or eggs with dead embryos were removed from the incubators. The remaining 390 eggs were equally allotted to 3 similar incubators. Commencing from ED 12, eggs from the NOISE and MUSIC group were exposed to background noise and music at 90 dB emitted by the portable speaker placed in the assigned incubators. Maturation of the embryo's auditory system occurred at ED 12 (Jones et al., 2006; Sanyal et al., 2013a). The sound pressure level recorded emitted auditory stimulation at 90 dB for 20 min intervals every hour from ED 12 until ED 21. Blood sampling was carried out on 90 individual
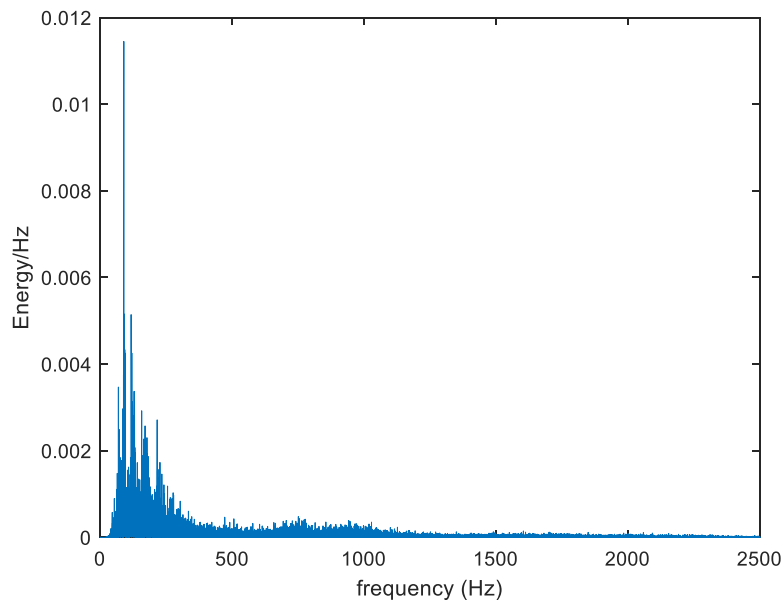

Figure 5. Single-sided spectrum of noise signal displayed up to 2,500 Hz.

embryos from each treatment group on ED 15 and ED 18 at approximately 09:00 h. The embryos were removed from the eggs, and blood was collected directly into 10 × 75 mm centrifuge tubes from the vitelline artery and vein. The samples were then centrifuged at 3,000 × g for 10 min at room temperature to separate the serum. Serum samples were kept at −80°C until analyzed. The embryos were then individually weighed after the bleeding procedure.

Hatching Responses

The remaining number of eggs in the incubator was allowed to hatch. During hatching, the number of chicks hatched was recorded every hour. The hatching windows were between day 20th and day 21st of incubation. The percentage of hatchability was calculated as the number of chicks hatched from the fertile eggs. After feather drying (approximately 2 h after hatching, considered as day-old chick), the chicks were individually weighed. Fifteen chicks per group (a total of 45 chicks) were randomly selected and their blood samples were collected by decapitation upon hatching. The blood samples were then centrifuged at 3,000 × g for 10 min at room temperature to separate the serum. Serum samples were kept at −80°C until analyzed. Serum samples were used to determine CPN, AGP, CORT, and Hsp70.

Determination of Circulating APPs, CORT, and Hsp70

Acute Phase Proteins Assay The AGP level was determined using a commercial ELISA kit specific to chicken (Cat. No. AGP-5, Life Diagnostics Inc., West Chester, PA). The level of CPN was determined, as previously explained (Zulkifli et al., 2014). About 20.375 g of sodium acetate trihydrate was dissolved in 250 mL of distilled water and adjusted to pH 6.2 using glacial acetic acid. Subsequently, 0.615 g of 1,4-phenylenediamine dihydrochloride (Sigma P1519; Sigma Aldrich, St. Louis, MO) was added to the prepared buffer and kept in the dark for at least 45 min. Then, 100 μL of the described buffer was mixed with 10 μL of samples or standards and were added to each microplate wells, gently shaken and kept in the dark for 20 min. After 20 min, the plate was read using a microplate reader at 550 nm (Multiskan FC Microplate Photometer; Waltham, MA). Serial dilution of standards was prepared using a known CPN concentration against purified CPN (Sigma Chemical Co.). In order to reach concentrations of 12.75 mg/ml of CPN, 20 μL of pig serum was added with 60 μL of saline buffer. Serial dilutions were prepared accordingly to reach various concentrations of 6.375, 3.1875, 1.59375, 0.79608, 0.39804, 0.199, and 0.099 mg/mL of CPN.

CORT Assay According to manufacturer recommendations, the CORT level was determined using commercially available high sensitivity EIA kits (AC-15F1, IDS, Boldon, UK). Cross-reactivity of the CORT antiserum

was less than 6.7% and 7.8%, respectively, and the detection limit was 27 ng/mL.

Hsp70 Assay Hsp70 determination was performed using a commercial ELISA kit specific to chicken (Cat. No. 201-16-0033, Shanghai Sunred Biological Technology, Shanghai, China) according to manufacturer recommendations. All samples were run in the same assay to prevent inter-assay variability.

Statistical Analysis

All statistical analyses were performed with the aid of Statistical Analysis System (SAS Institute, Inc., Cary, NC) software (SAS, 2005), using 1-way ANOVA. When the analysis of variance indicated the presence of significant differences between the treatments, means were separated using the least significant difference (**LSD**) test. The hatchability data was subjected to a chi-square test. Significance was declared at $P < 0.05$.

RESULTS

The prenatal auditory stimulation had a negligible effect on the hatchability (Control, 89%; MUSIC, 90.7%; NOISE, 96%) (Table 1). The body weights of embryos at ED 15 (Control, 17.1 ± 0.6 g; MUSIC, 18.38 ± 0.3 g; NOISE, 18.06 ± 0.49 g), ED 18 (Control, 29.75 ± 0.6 g; MUSIC, 31.1 ± 0.7 g; NOISE, 30.8 ± 0.7 g) and post-hatch day 1 (Control, 58.1 ± 0.4 g; MUSIC, 58.2 ± 0.3 g; NOISE, 59.9 ± 0.5 g) were not significantly influenced by the prenatal auditory stimulation (Figure 6). The MUSIC (9.024 ± 1.4 ng/mL) and NOISE (12.15 ± 1.6 ng/mL) groups had significantly higher CORT than controls (4.39 ± 0.7 ng/mL) at ED 15 (Figure 7). The prenatal auditory stimulation did not significantly affect the CORT at ED 18 (Control, 5.77 ± 0.4 ng/mL; MUSIC, 6.70 ± 0.5 ng/mL; NOISE, 5.73 ± 0.3 ng/mL) and post-hatch day 1 (Control, 13.68 ± 1.4 ng/mL; MUSIC, 13.05 ± 1.2 ng/mL; NOISE, 11.45 ± 1.1 ng/mL). The MUSIC and NOISE treatments significantly elevated CPN (Figure 8) and AGP (Figure 9) at ED 15 [(CPN: Control, 0.82 ± 0.04 ng/mL; MUSIC, 1.37 ± 0.1 ng/mL; NOISE, 1.49 ± 0.2 ng/mL) (AGP: Control, 39.1 ± 7.1 mg/mL; MUSIC, 85.5 ± 12.9 mg/mL; NOISE, 85.4 ± 15.1 mg/mL)] and post-hatch day 1 [(CPN: Control, 1.86 ± 0.2 ng/mL; MUSIC, 2.84 ± 0.4 ng/mL; NOISE, 3.04 ± 0.3 ng/mL) (AGP: Control (20.4 ± 2.2 mg/mL); MUSIC, 30.5 ± 4.7 mg/mL; NOISE, 30.3 ± 1.4 mg/mL)] compared to the control group. All embryos showed similar CPN (Control, 2.66 ± 0.6 ng/

Table 1. The effect of auditory stimulation on hatchability in day-old chicks.

Groups	Control	Music	Noise
Non-hatched eggs	9	9	3
Total (hatch)	71	88	69
Hatchability	89%[a]	90.7%[a]	96%[a]

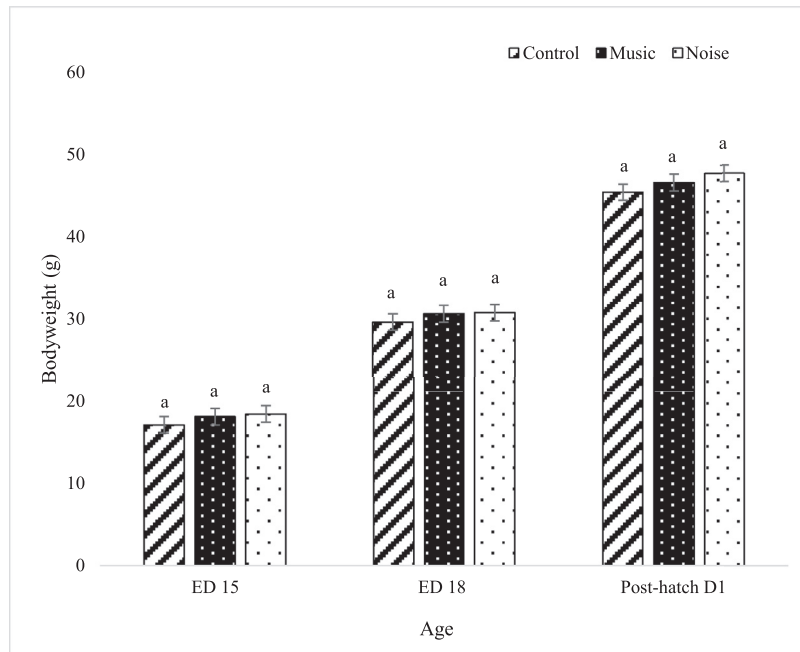[a]Means between a column subgroup with no common superscripts are significantly different at $P < 0.05$.

Figure 6. The effect of auditory stimulation on bodyweight in broiler embryos and day-old chicks. Sampling group means; Control (n = 71), Music (n = 88), and Noise (n = 69), respectively, with no common letters differ at $P < 0.05$.

mL; MUSIC, 1.93 ± 0.2 ng/mL; NOISE, 2.43 ± 0.3 ng/mL) and AGP (Control, 44.1 ± 5.2 mg/mL; MUSIC, 31.9 ± 3.6 mg/mL; NOISE, 37.7 ± 4.5 mg/mL) at ED 18. Prenatal auditory stimulation had a negligible effect on serum levels of Hsp70 at ED 15 (Control, 34.8 ± 3.1 ng/mL; MUSIC, 33 ± 1.9 ng/mL; NOISE, 28.2 ± 3.1 ng/mL) (Figure 10). However, the MUSIC embryos had significantly higher Hsp70 expression than their control and NOISE counterparts at ED 18 (Control, 12.9 ± 1.2 ng/mL; MUSIC, 129.6 ± 26.4 ng/mL; NOISE, 13.3 ± 2.3 ng/mL) and post-hatch day 1 (Control, 15.2 ± 1.7 ng/mL; MUSIC, 195.5 ± 68.5 ng/mL; NOISE, 13.2 ± 2.7 ng/mL).

DISCUSSION

The present findings confirmed earlier work that neither prenatal music (Sanyal et al., 2013b) nor noise (Donofre et al., 2020) exposure influence the embryo's weight and chick's weight. On the contrary, Kesar et al. (2014) reported that the bodyweight of chicks at post-hatch day 1 was adversely affected by prenatal noise exposure. However, the authors exposed developing embryos to intermittent noise at a pressure level of 110 dB for 15 min/h from ED 10 until hatching. Thus, the treatment imposed by Kesar et al. (2014) was more severe than ours.
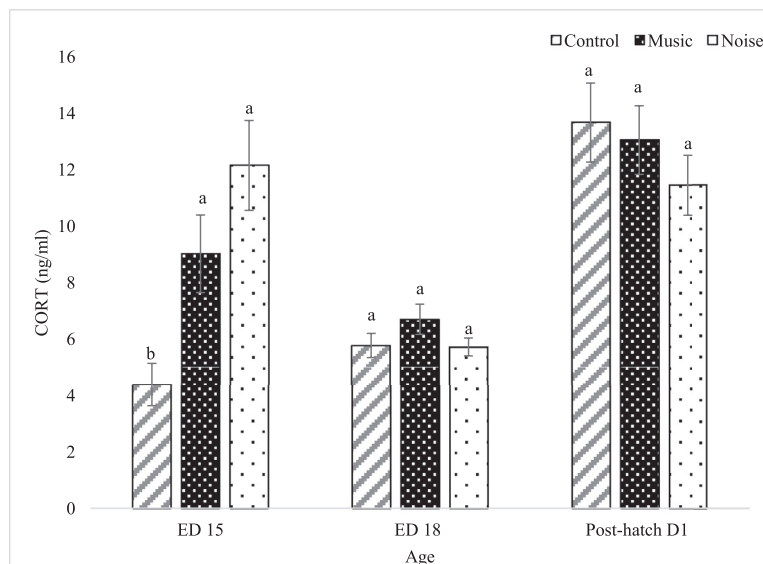

Figure 7. The effect of auditory stimulation on serum levels of corticosterone (CORT) in broiler embryos and day-old chicks. Sampling group means (n = 15) with no common letters (a-b) differ at $P < 0.05$.
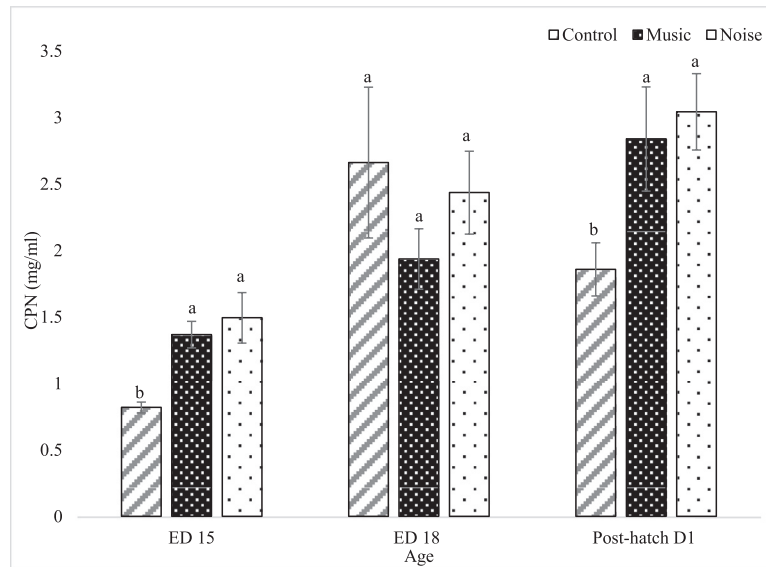
Figure 8. The effect of auditory stimulation on serum levels of ceruloplasmin (CPN) in broiler embryos and day-old chicks. Sampling group means (n = 15) with no common letters (a-b) differ at $P < 0.05$.

Noise represents a potential stressor to farm animals, and their growth and reproductive performance may be adversely affected by the noxious stimulus (Broucek, 2014). Chloupek et al. (2009) reported a significant increase in the blood CORT levels of broilers after 10 min of noise exposure at 80 dB and 100 dB. Bedanova et al. (2010) indicated that 100 dB noise evoked plasma CORT reaction in broilers at market age, but they habituated to the noise exposure after the first 14 min. In the present study, at ED 15, the MUSIC and NOISE embryos had elevated CORT compared to the control group. According to Wise and Frye (1973), the adrenal glands had autonomous functional capability before day 14 of incubation, and the pituitary was critical in maintaining circulating CORT levels during resting and stressful events between ED 14 and ED 16. It is not clear why prenatal auditory stimulation had a negligible effect on CORT at ED 18 in this study.

Moraes et al. (2004) subjected chicken embryos to 39°C from ED 13 to ED 17 for 2 h/d and noted a significant increase in CORT on ED 14 only, although daily sampling was done until hatching. The authors suggested that the elevated circulating CORT at day 14 was an initial reaction to heat stress which could be a part of the epigenetic heat adaptation mechanism. Critical epigenetic reprogramming activities may occur during early embryogenesis and germ cell development (Mifsud et al., 2011). The noted lack of prenatal auditory stimulation on CORT in the newly hatched chicks concurs (Sanyal et al., 2013a). However, Sanyal et al. (2013a) reported that music and noise stimulations elevated plasma noradrenaline levels in neonatal chicks moderately and dramatically, respectively.

The noted increases in CPN and AGP at ED 15 are the first evidence of APPs response to sound stimulation in developing avian embryos. Interestingly, the pattern
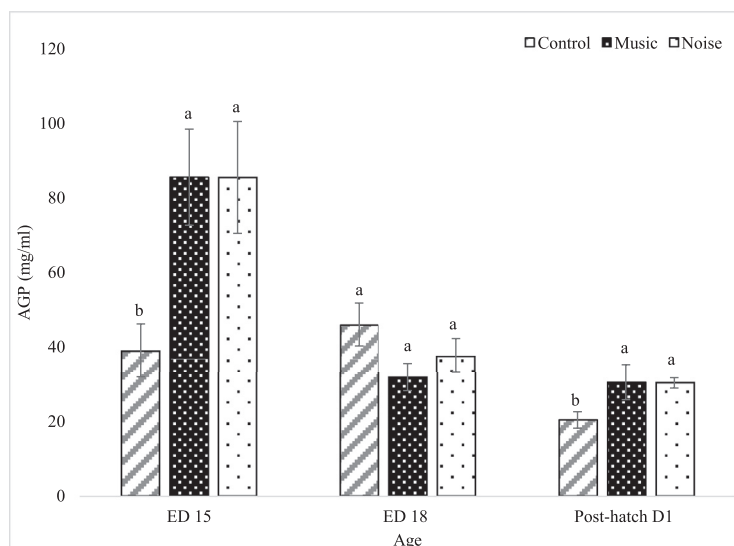

Figure 9. The effect of auditory stimulation on serum levels of alpha-1-acid glycoprotein (AGP) in broiler embryos and day-old chicks. Sampling group means (n = 15) with no common letters (a-b) differ at $P < 0.05$.
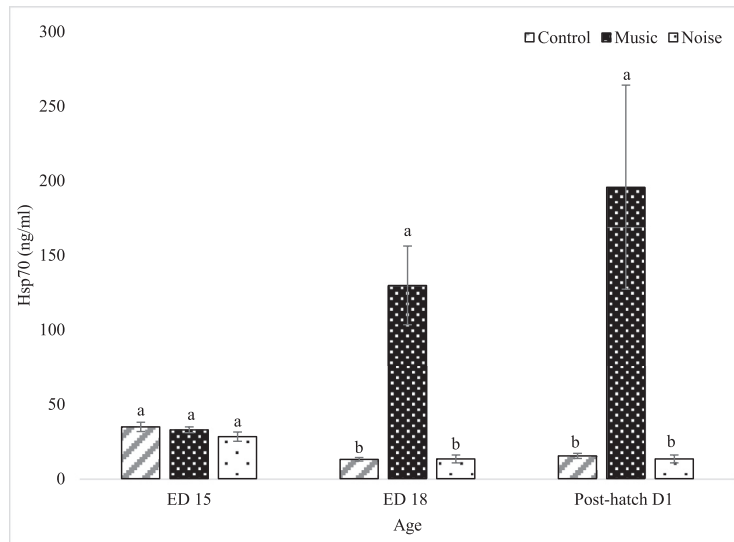
Figure 10. The effect of auditory stimulation on serum levels of heat shock protein 70 (Hsp70) in broiler embryos and day-old chicks. Sampling group means (n = 15) with no common letters (a-b) differ at $P < 0.05$.

of CPN and AGP responses to MUSIC and NOISE at ED 15 and ED 18 were similar to CORT. Murata et al. (2004) reported that pro-inflammatory cytokines such as interleukin-6 (**IL-6**) stimulated APP synthesis in the liver. They suggested that stressors' activation of the HPA axis may trigger IL-6 production and the release of APPs into the bloodstream. In chickens treated with exogenous CORT, Zulkifli et al. (2014) showed significant elevations in AGP and CP, and plasma IL-6. Najafi et al. (2018) reported that stress attributed to feed deprivation without a concurrent increase in CORT had a negligible effect on serum levels of APPs and IL-6. Hence, it appears that the mechanism behind the stress-induced APPs response in developing embryos is related to activation of the HPA axis resulting in increased production of glucocorticoids. Our study suggests that measuring blood APPs levels can provide unique insights about stress physiology in developing avian embryos.

Various environmental perturbations may influence avian embryonic development (Henricksen et al., 2011; Costa et al., 2020). Hence, the ability of the embryos to respond to noxious stimuli is crucial for survival (Scanes, 2016). It is well established that CORT plays a fundamental role in maintaining stress-related homeostasis and influences the organism's physiologic adaptive reaction against environmental stressors (Romero and Beattie, 2022). APPs may also be of physiological relevance for the developing embryos due to their unspecific antimicrobial effects (Murata et al., 2004) and association with restoring homeostasis (Cray et al., 2009). Deak et al. (1997) suggested that increases in blood APPs during stress could be considered an anticipatory defensive immune response to reduce infection, inflammation, and injury produced by the possible noxious stimuli.

In the present study, at post-hatch day 1, the CPN and AGP of MUSIC and NOISE chicks were similar and significantly higher than controls. It appears that the long-lasting effects of prenatal auditory stimulation on post-hatch stress levels can be detected by CPN and AGP but not CORT. There is no clear explanation for the lack of CORT response on post-hatch day 1. Nevertheless, the lack of CORT response to auditory stimulation at post-hatch day 1 does not preclude the possibility of a prior elevation of CORT. Elevation in CORT is known to precede the increase in CPN and AGP in chickens (Zulkifli et al., 2014; Najafi et al., 2015). It has been reported that CORT response was transient (Birrenkott and Wiggins, 1984; McFarlane and Curtis, 1989; Joëls et al., 2012). Birrenkott and Wiggins (1984) showed that plasma CORT levels attributable to the injection of dexamethasone to evoke stress has a short lifetime in chickens approximately 22 min. Work in ewes demonstrated a significant increase in acute phase proteins during the periparturient period but did not influence circulating cortisol levels (Gregula-Kania et al., 2020). Along with being seen as potential stress monitoring tools, APPs have also found potential applications in diagnosing infectious and noninfectious diseases in human beings and animals (Jain et al., 2011; Schmidt and Eckersall, 2015).

The present data confirmed (Hanafi et al., 2021) that Hsp70 is detectable in chicken embryos from ED 14. Evans et al. (2005) suggested that the chaperoning activity of Hsp was required in several different embryonic pathways. It is well documented that Hsps have various protective roles in the cell, including refolding partially unfolded proteins and removing denatured proteins due to stress-triggered events (Hartl, 1996). To date, the only documented work on noise and Hsp in poultry was by Hoekstra et al. (1998). They exposed Japanese quail to loud rock music (74 dB) for 60 min and noted a significant increase in the myocardial Hsp70 expression. Work in rats showed that exposure to loud noise (80−110 dB) significantly increased Hsp70 gene expression in the cochlea, which could be a

compensatory mechanism to counteract some of the detrimental effects of mechanical stress in the auditory sensory organ (Herwanto et al., 2016). Our present results suggest that the MUSIC exposed embryos but not their NOISE counterparts had a greater ability to express serum Hsp70 at ED 18 than the controls, and a similar trend was noted on post-hatch day 1. To the best of the authors' knowledge, this is the first report to collectively examine and study Hsp response to auditory stimulation in developing avian embryos and neonatal chicks. The synthesis of Hsp is considered an essential biochemical response to aid living organisms in coping with various environmental and biological stressors (Edwards et al., 2001; Kregel, 2002). The Hsp response is one of the critical pathways via which an animal attempts to survive and recover during and after stressful conditions (Yu et al., 2015; Hassan et al., 2019; Hu et al., 2020). Al-Zghoul (2018) reported that thermal manipulation of embryos altered the ability of neonatal chicks to express Hsp70 and enhanced their acquisition of thermotolerance later in life. Given the improved ability to express Hsp70, there is a possibility that the MUSIC chicks could cope better with subsequent stressors than the NOISE or Control group during postnatal life. Stresses early in life may have evoked Hsps mRNA transcription but the RNA may have been "sequestered" and not translated until exposure to heat challenge later in life (Zulkifli et al., 2002). On a cautionary note, however, overexpression of Hsp may be biologically costly as it could be detrimental to growth and fertility (Sørensen et al., 2003). Further work is required to clarify the consequences of up-regulated Hsps in the MUSIC exposed chicks during postnatal life.

The present findings showed that MUSIC and NOISE elicited similar CORT, CPN, and AGP reactions at ED 15. Thus, it appears that MUSIC and NOISE of equal sound pressure are equally stressful to developing chicken embryos. Interestingly, only the former showed changes in the blood expression of Hsp70 at ED 18 and post-hatch day 1. There is no clear explanation for the phenomenon. However, it appears that physical properties of a sound like rhythmicity, pattern, and frequency may have a disparate impact on Hsp reaction in developing embryos. Thus, the effects of different physical properties of sounds on Hsp expression in avian embryos merit further investigation. The varying effects of prenatal music and noise exposure on neurogenesis and brain development in chicken embryos (Sanyal et al., 2013b) and rat fetuses (Kim et al., 2006) have been reported.

CONCLUSIONS

The growth of embryos and the hatchability of broiler chicks are not affected by prenatal auditory stimulation. Both MUSIC and NOISE at 90 dB, as measured by CPN, AGP, and CORT, are stressful to the embryos at certain developing stages. The MUSIC but not NOISE treatment enhances the ability of embryos at ED 18 and neonatal chicks to express Hsp70. Importantly, there is

a need to clarify further our understanding of whether the physiological modifications we see in the developing embryos are adaptive or not and whether they vary with species life histories and environmental variability.

DISCLOSURES

The authors declare that they have no conflict of interest.

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