JWPR
Journal of World's Poultry Research

2017, Scienceline Publication
J. World Poult. Res. 7(3): 104-113, Sept 25, 2017
Research Paper, PII: S2322455X1700013-7

Effect of Aging on Mitochondrial Gene Expression in Chicken Breast Muscle

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Received: 01 Aug 2017
Accepted: 04 Sept 2017

ABSTRACT

Efficient conversion of food into body mass has been associated with altered gene expression of some proteins of the electron transport chain. We evaluated the effect of age on the mRNA expression of Cytochrome oxidase III(COX III), avian adenine nucleotide translocator (avANT), avian PPAR-γ coactivator-1α (avPGC-1α), Peroxisome proliferator-activated receptor-γ (PPARγ) and avian uncoupling protein (avUCP) in chicken. A total of 90 male birds each from Nandanam B2, Rhode Island Red, Aseel and White Leghorn, were divided into three replicates containing 30 birds each and used for the study. Production parameters consist of body weight, body weight gain, cumulative feed consumption and cumulative feed efficiency at fourth and eighth weeks of age were recorded. Total RNA was extracted from the breast muscle tissue of male birds and reverse transcribed into cDNA. Real-time PCR analysis was performed using specific primers for the genes. The greatest reduction was observed when comparing fourth and eighth week old birds in COX III, avANT mRNA expression levels were then followed by avPGC-1α and increased mRNA expression levels were observed in PPARγ followed by avUCP at eighth week of age. The study revealed phenotypic differences in production traits as well as the difference in expression of mitochondrial gene like COX III, avANT, avPGC-1α, PPARγ and avUCP expression level change with age in chickens.

Key words: Ageing, Mitochondia, PPARγ, COX III, avANT, avPGC-1α, avUCP

INTRODUCTION

Genetic improvement has greatly enhanced the production performance of broiler in recent years, by drastically reducing the slaughter age. In the poultry industry, feed efficiency is a major criterion for defining the optimum performance to broiler chicken. It is considered as one of the most important traits in poultry farming activities since feed represents about 50 to 70% of the total cost of production. Moreover, because feed cost has increased dramatically in recent years, decreasing the amount of feed per unit of weight gain will improve efficiency of production and increase profits. Efficient conversion of food into body mass was reported to be associated with altered gene expression of some proteins of the electron transport chain (Gasparino et al., 2012). All cells need energy to perform their activities. Mitochondria are responsible for producing 90% of the energy needed for cells. Series of studies are conducted to understand relationships of mitochondrial function and biochemistry with the phenotypic expression of feed efficiency in broilers (Ojano-Dirain et al., 2004, 2005a, 2005b; Iqbal et al., 2004, 2005; Lassiter, 2006). These organelles are responsible for transforming chemical energy from metabolites into easily accessed energy to be used by the cell (Schauss et al., 2010).

Increased production of mitochondrial ROS, which occurs with advancing age, is related to greater oxidative damage in the macromolecules, as well as depletion in the energy production machinery. Birds with lower ATP

production due to lower mitochondrial efficiency in producing ATP from substrates show less efficiency or feed conversion. Therefore, mechanisms that favor a reduced ROS production may be useful in the prevention of age-related issues (Gasparino et al., 2012).

The aim of this study was to evaluate the effect of ageing on mitochondrial genes related to energy production, ATP synthesis and mitochondrial biogenesis of the genes Cytochrome oxidase III (COX III), avian adenine nucleotide translocator (avANT), avian PPAR-γ coactivator-1α (avPGC-1α), Peroxisome proliferator-activated receptor-*γ* (PPARγ) and avian uncoupling protein (avUCP) were analyzed at the fourth and eighth weeks of age in breast muscle of different chicken breeds that are known to have differential phenotypic expression.

MATERIALS AND METHODS

Ethical approval

The experimental protocol was approved by the Institutional Animal Ethics Committee, Tamil Nadu, India.

Experimental Birds

A total of 90 male birds each selected from Nandanam B2, Rhode Island Red, Aseel and White Leghorn divided into three replicates containing 30 birds each were used for the study. Nandanam B2 is a commercial hybrid dual purpose strain developed by Tamil Nadu Veterinary and Animal Sciences University, India. The concerned breed/strain was divided into four treatment groups with three replicates in each group, containing 30 birds each. The breeds were selected based on observed high and low feed efficiency over generations in Poultry Research Station, Madhavaram Milk Colony, Chennai-51, with the aim to evaluate mRNA expression of genes that are involved in mitochondrial energy metabolism and mitochondrial biogenesis that are known to have differential phenotypic expression.

All the experimental birds were wing banded and reared up to eight weeks of age following standard management practices in cages. All the chicks were immunized against Ranikhet disease by using F_1 and Lasota strain at 7th day and 28th day respectively. Known quantity of feed was provided *ad libitum* with feed containing 3100Kcal ME/kg and 22 percent dietary crude protein. Clean potable water was provided *ad libitum.* The study was carried out during October- December months 2015 where the average daily high temperature in the study area (13.1623° N, 80.2433° E) was below 31^{o}C. Data on phenotypic performance and gene expression studies were recorded.

Phenotypic assessment

The day old experimental chicks were weighed with 0.1 g accuracy. Body weight was again recorded at fourth and eighth weeks of age. Based on day old body weight, body weight gain was calculated. All the birds were provided with *ad libitum* quantity of experimental feed during the experimental period. At the end of every two weeks period, left over feed was weighed back and net feed consumption was estimated for each group. Feed efficiency was calculated at 4th and 8th weeks of age.

Genotypic assessment

Mitochondrial mRNA expression of COX III, avANT, avPGC-1α, PPARγ and avUCP genes were studied in breast muscle tissue at fourth and eighth weeks by following the protocol below. Two male birds from each replicate were randomly selected, birds were killed by cervical dislocation, and tissue from the breast muscle (pectoralis superficialis) was collected and submerged in RNA later and kept at -80^{o} C.

The reagents were used for RNA extraction were TRIzol® LS Reagent (Invitrogen, USA) Catalog number: 15596026, Chloroform (Sigma, USA), Isopropanol (Sigma, USA), 70 per cent ethanol prepared from 99.9 per cent absolute ethanol (Jiamgsu Huasi International, China) and Nuclease free water (QIAGEN, USA)

The muscle tissues were initially triturated with, 1ml of Trizol^{TM} reagent in a mortar and pestle. The mixture was then incubated for 5 min at room temperature and 200µl of chloroform was added and vortexed for 1 min. The vortexed mixture was then centrifuged at 12000 rpm for 15 min at 4^{0}C to separate the aqueous phase. The aqueous phase was then transferred to a fresh tube and equal volume of isopropanol was added and mixed by slightly inverting the tube. The tube was then incubated at room temperature for 10 min. The mixture was then centrifuged at 12000 rpm for 10 min at 4^{0}C and the supernatant was discarded. To the RNA pellet obtained, 1ml of 70% Ethanol was added and then stored at -80^{0}C until further use. For immediate purposes, the tubes were centrifuged; the RNA pellet air dried and re-suspended in nuclease free water, quantified and equal volume of RNA was used across the different samples.

The quantity of RNA was measured by using eppendorf BioPhotometer Plus. The spectrophotometer was blanked with 1µl of nuclease free water and 1 µL of extracted RNA was used for quantification. The final concentration of the RNA (in stock) was determined by multiplying with the dilution factor. The quantity of RNA

was measured by taking ODs at 260 and 280 and then by the ratio of 260/280.

The cDNA was synthesized from the extracted total RNA using High capacity cDNA Reverse Transcriptase kit, United States (Thermo Scientific Revert Aid H Minus First Strand cDNA Synthesis Kit #K1632) according to the manufacturer's instructions. The following biological reagents were added as presented in table 1.

Table 1. Composition of cDNA synthesis reaction

RNA	8µL
Primer (oligo dT)	1 µL
5X Reaction buffer	4 µL
dNTPs	2 µL
Reverse Transcriptase enzyme	1 µL
Ribolock RNAse inhibitor	1 µL
RNAse free water	3 µL
Total volume	20 µL

The RNA pellet was air dried and re-suspended in 10µl DEPC water and denatured at 65^0C for 5 min and snap cooled in ice for 1 – 2 min. The cDNA master mix (10µl) was added to the denatured RNA on ice. The total reaction mixture was incubated at 25^0C for 5 min followed by 42^0C for 1 hour for the reverse transcription and finally at 70^0C for 5 min to inactivate the enzyme. The cDNA synthesized was then used for the amplification of the house keeping gene β actin or stored at -20°C until further use. Quantitative PCR (qPCR) was carried out using SYBR® Green following the manufacturer's instruction. The real time plates were obtained from Roche, India. The mRNA expression levels of the mitochondrial genes COX III, avANT, avPGC-1α, PPARγ and avUCP. The primer sequences were adopted from Ojano-Dirain et al. (2007). The Real-time PCR mix was prepared as presented in table 2.

Table 2. Composition of Real-time PCR mix

2X SYBR Green Mix	10 µL
Forward Primer	0.5µL (10pmol/ µL)
Reverse Primer	0.5µL (10pmol/ µL)
Template cDNA	2 µL
Water	up to 20 µL

The components were mixed gently by vortexing and were briefly centrifuged to collect all the components at the bottom of the tube. PCR reaction was performed in duplicates for each sample. The cycling protocol was 40 cycles of de-naturation at 94°C for 2 min followed by 94°C for 10 seconds, annealing temperature at 58°C for 10 s with a melting program and finally held at 37°C.

The relative mRNA expression levels of the target genes such as COX III, avANT, avPGC-1α, PPARγ and avUCP gene were shown as Ct values in the muscle tissue. The β-actin Ct value for each sample was subtracted from the Ct value of the target gene to normalize for the host basal levels. Following normalization the mRNA expression levels of the target genes COX III, avANT, avPGC-1α, PPARγ and avUCP of each breed are expressed as fold change ($2^{-\Delta\Delta Ct}$) over the respective levels in Nandanam B2 and logarithmic transformation was applied to all the genes evaluated.

Statistical analysis

The results were expressed as mean ± Standard Error (SE). The differences between groups were assessed by using the Statistical Package for Social Sciences (SPSS version 17.0) software package for windows as per Snedecor and Cochran (1994). The difference within the means were estimated using Duncan's multiple range test (Duncan, 1955) by considering the differences at significant level ($P < 0.05$).

RESULTS AND DISCUSSION

Body weight and body weight gain

In the present study, highly significant difference was observed in biweekly body weight at the fourth and the eighth week which was due to different types of chicken. Broiler type Nandanam B2 had attained the highest body weight at eighth week of age. Nandanam B2 was followed by Rhode Island Red, Aseel and lowest in White leghorn as shown in table 3.

This finding was in agreement with the earlier results obtained by Sangilimadan et al. (2014) who had studied the performance of Nandanam B2. Few other researchers compared the performance of different breeds like Aseel and Kadaknath in their locality like Huanshi et al. (2011) and got similar results. Khawaja et al. (2012) who compared the growth performance of Rhode Island Red had revealed comparable results.

Feed consumption and feed efficiency

Effect of different types of chicken in cumulative feed consumption and feed efficiency at different periods were significantly different and better feed efficiency was seen in Nandanam B2 followed by RIR, White leghorn and Aseel. This may be due to their difference in the genetic makeup of different types of chicken as shown in table 4.

The results of this study coincided with work carried out by Jha and Prasad (2013) that had studied the performance of Aseel under deep litter and reported FCR of 5.46 upto 20 week of age. Whereas the findings in the present study were contrary to the work carried out early by Sangilimadan et al. (2014) who studied the performance of Nandanam B2 and reported that feed efficiency at 8th week of age was 2.49. Halima et al. (2006) also compared the feed consumption of native chicken and Rhode Island Red but found no significant difference in feed intake.

The primers for the COX III, avANT, avPGC-1α, PPARγ and avUCP genes proved in this study to be adequate for real-time PCR analysis and expression of fold change in 4th and 8th weeks of age (Figure 1,2,3,4 and 5). The analysis of the dissociation curves did not reveal the presence of any unspecific products or the formation of primer dimers, demonstrating the reliability of the data in determining mRNA expression of the genes evaluated. Mean and standard deviation of the CT values obtained in the samples of muscle tissue for analysis of the expressions of genes are shown in tables 5 and 6.

Table 3. Body weight and body weight gain of different chicken breeds during rearing periods

Genetic groups	Body weight(g)		Body weight gain(g)	
	4th week	8th week	0-4 weeks	0-8weeks
Nandanam B2	315.84^{c} ±5.40*** (84)	971.23^{c} ±20.87 (72)	265.75^{c} ±5.32 (84)	925.16^{c} ±24.43 (72)
RIR	219.31^{b} ±4.52 (79)	515.13^{b} ±9.19 (72)	183.99^{b} ±4.49 (79)	479.77^{b} ±9.13 (72)
Aseel	208.89^{b} ±4.16 (67)	502.81^{b} ±9.55 (55)	177.32^{b} ±4.12 (67)	471.54^{b} ±9.50 (55)
WLH	181.68^{a} ±2.71 (80)	442.95^{a} ±6.33 (69)	150.88^{a} ±2.71 (80)	412.19^{a} ±6.33 (69)
F value	139.61**	355.13**	139.16**	263.74**

a,b,c – means within coloumn bearing different superscripts differ significantly (P<0.05); ******– Highly Significant (P<0.01), *** Mean Weight ± Standard Error

Table 4. Feed consumption and feed efficiency of different chicken breeds during rearing periods

Genetic groups	Feed consumption (g)		Feed efficiency	
	0-4 weeks	0-8weeks	0-4 weeks	0-8weeks
Nandanam B2	633.06^{b}±2.15***	1920.57±96.02	1.69^{a}±0.01	1.95^{a}±0.24
RIR	559.00^{a}±25.85	1863.72±43.95	1.73^{a}±0.19	2.78^{c}±0.12
Aseel	521.60^{a} ±20.54	1682.20±47.63	1.89^{a}±0.08	3.34^{b}±0.04
WLH	534.85^{a}±37.18	1789.61±42.64	2.94^{b}±0.34	4.50^{b}±0.08
F value	13.875*	4.376^{NS}	8.533**	193.10**

a,b,c – means within column bearing different superscripts differ significantly (P<0.05); *****–Significant (P<0.05)**, ****– Highly Significant(P<0.01), NS-Non-Significant (P>0.05); *** Cumulative mean ± Standard Error

COX III

Expression levels of mRNA as reflected by fold change positively link with metabolic regulating factor for energy production of AMP and ATP at mitochondria, subsequently for the body weight gain and feed efficiency traits. Percent fold change was lowest in white leghorn and highest in Aseel at 8th week compared to 4th week of age. White leghorn being a layer type had a negative trend in expression level. This may be due to greater ROS production and greater protein oxidation that are consistently found in birds with lower feed efficiency thereby decreasing the cellular efficiency.

This study coincides with the work done by Iqbal et al. (2004) who discussed that COX III mRNA levels in breast muscle were lower in poor feed efficiency compared with the birds that had better feed efficiency. Similarly, Ojano-Dirain et al. (2007) suggested that a greater ROS production and greater protein oxidation are

consistently found in birds with lower feed efficiency, indicating that this factor may alter the expression of mitochondrial genes.

The findings concurred with Barazzoni et al. (2000) who verified a reduction in COX III mRNA expression related to altered oxidative capacity of mitochondria in older animals. This would indicate that maintaining transcription levels may be essential to mitochondrial oxidative capacity and the maintenance of efficient use of nutrients. Also similar to Bottje et al., (2002) who stated that increased oxidative stress and protein oxidation in the low-FE phenotype is likely due to increased mitochondrial reactive oxygen species.

The finding was in agreement with Kemp et al., (2003) who opined that COX III plays an important role in mitochondrial energy efficiency. Also similar to Scheffler (1999) who had reported that COX III may play a key role in energy production.

Zhang et al. (2010) reported that ROS production and expression of proteins of the respiratory chain complexes involved in metabolism, with the feed efficiency of animals. Bottje and Carstens (2009) reported that the low-FE phenotype generated more mitochondrial ROS than the high-FE phenotype. The low-FE broiler phenotype exhibited site-specific defects in electron transport, resulting in increased mitochondrial ROS production and increased protein oxidation in several tissues.

av (ANT)

Fold change was increased in Rhode Island Red and decreased in Aseel and WLH birds at eighth week of age. This protein is responsible for moving ADP from the cytosol to the mitochondria and for moving ATP through the inner mitochondrial membrane (Ojano-Dirain et al., 2007). Therefore, ANT has the function of increasing the quantity of ADP to be transformed into ATP by means of ATP synthase. The mitochondrial function may be impaired by the incapacity of ADP/ATP exchange between the cytosol and the membrane, thus there may be some connection between the ANT expression with the phenotypic expression of feed efficiency (Bottje et al., 2006). In the present study, we found that older birds displayed a lower ANT mRNA expression in muscle tissue, and poorer feed conversion, just as Ojano-Dirain et al. (2007) also reported that birds with a lower ANT expression had a poor feed efficiency due to the lower ATP production efficiency. Nicoletti et al. (2005) also found a reduced ANT expression correlated to aging. According to these authors, alterations in the expression of respiratory chain subunits may represent an adaptive cellular response to the accumulated damage to proteins and/or mitochondrial DNA that occurs due to the increased quantity of ROS in older birds.

av (PGC 1α)

Fold change was up-regulated in Aseel and down-regulated in White leghorn and Rhode Island Red at eighth week in comparison to fourth week of age. The finding was in agreement with Nisoli et al. (2003) who stated thatPGC-1α stimulates nuclear respiratory factor-1 and mitochondrial transcription factor A expression, that in turn up-regulate expression of nuclear and mitochondrial genes that encode mitochondrial proteins. It agreed with the work done by Wu et al. (1999) who reported thatPGC-1α is the most dominant regulatory protein in mitochondrial biogenesis. In general it is coinciding with the work done by Richards (2003) who discussed about the genes associated with controlling feed intake and energy balance. Also similar to Lassiter et al. (2006) who provided evidence of increased oxidation associated with low FE and further evidence of differential protein expression associated with the phenotypic expression.

PPARγ

Percent fold change was increased in Rhode Island Red, Aseel and WLH birds at eighth week in comparison to fourth week of age. This may be due to the advancement of age there is more fatty acid uptake and metabolism.

The result coincides with the Sato (2004) who studied chicken PPARγ mRNA expression in abdominal adipose tissue tended to increase with age, as shown by higher expression levels at 6 week than at 1 and 2 week of age. It also coincides with the work of Rosen et al. (1999) who stated that PPAR-γ is activated by fatty acids that control adipocyte differentiation as well as fatty acid uptake and metabolism. The result contradicts with Ojano-Dirain et al. (2007) who reported that there were no differences in breast muscle PPAR mRNA expression.

av(UCP)

Fold change was down-regulated in Aseel and up-regulated in Rhode Island Red and White leghorn at eighth week in comparison to fourth week of age. This result coincides with the work done by Raimboult et al. (2001) who reported that chickens divergently selected for low feed efficiency has higher avUCP mRNA expression than in birds from a high feed efficient line. Also coincides with Bottje et al. (2006) who also stated that avUCP mRNA expression in breast muscle from low feed efficient birds may be a mechanism to reduce the higher hydrogen peroxide production. The findings were in

agreement with Abe et al. (2006) who reported that increase in avUCP content could be associated with altered ROS production by mitochondria. Also similar to Ojano-Diran et al. (2007) who reported that greater UCP mRNA expression can impair feed conversion, as it can reduce ATP production.

This study contradicts with Gasparino (2012) who observed a gradual reduction in UCP mRNA as the quails aged. Also Ferrandiz et al. (1994) suggested that with increase in age, more failures in ATP production occur due to the impaired activity of the respiratory chain complexes.

Table 5. CT values obtained in the samples of muscle tissue of different chicken breeds for analysis of COX III, avANT, PGC1α, PPARγ and avUCP expressions at 4 weeks of age

Genetic groups	Endogenous control β-actin.				
	COX III	**avANT**	**PGC1α**	**PPARγ**	**avUCP**
Nandanam B2	16.37±1.15*	29.65±2.60	30.03±2.58	21.83±2.02	29.81±2.26
RIR	24.91±0.75	29.34±0.79	30.45±0.80	32.70±0.75	29.42±0.74
Aseel	27.73±2.80	27.74±2.82	32.46±0.82	34.70±0.71	29.92±1.66
WLH	20.91±1.95	25.59±1.94	25.96±1.98	30.46±2.01	26.65±1.94

***** Mean ± Standard Error

Table 6. CT values obtained in the samples of muscle tissue of different chicken breeds for analysis of COX III, avANT, PGC1α, PPARγ and avUCP expressions at 8 weeks of age

Genetic groups	Endogenous control β-actin.				
	COX III	**avANT**	**PGC1α**	**PPARγ**	**avUCP**
Nandanam B2	29.93±2.61*	27.51±0.96	27.81±1.30	33.47±0.61	29.81±1.67
RIR	38.72±1.02	28.29±0.88	29.18±0.91	33.38±1.00	29.42±1.44
Aseel	32.52±2.62	26.17±2.45	25.73±2.50	29.99±1.18	29.92±1.09
WLH	38.69±0.38	27.69±0.48	27.40±0.36	34.56±1.17	26.65±0.36

***** Mean ± Standard Error

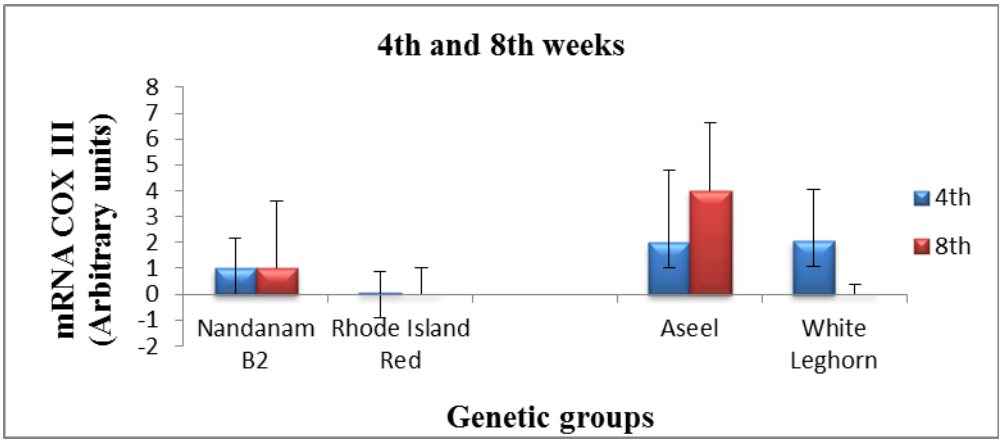

Figure 1. mRNA expression of Cytochrome oxidase III (COX III) in the breast muscle of different chicken breeds at 4 and 8 weeks of age

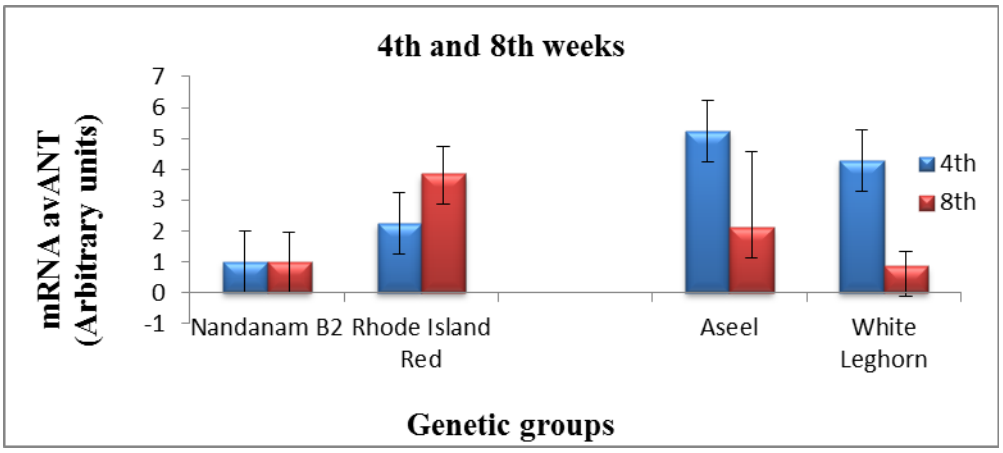

Figure 2. mRNA expression of avian adenine nucleotide translocator (avANT) in the breast muscle of different chicken breeds at 4 and 8 weeks of age

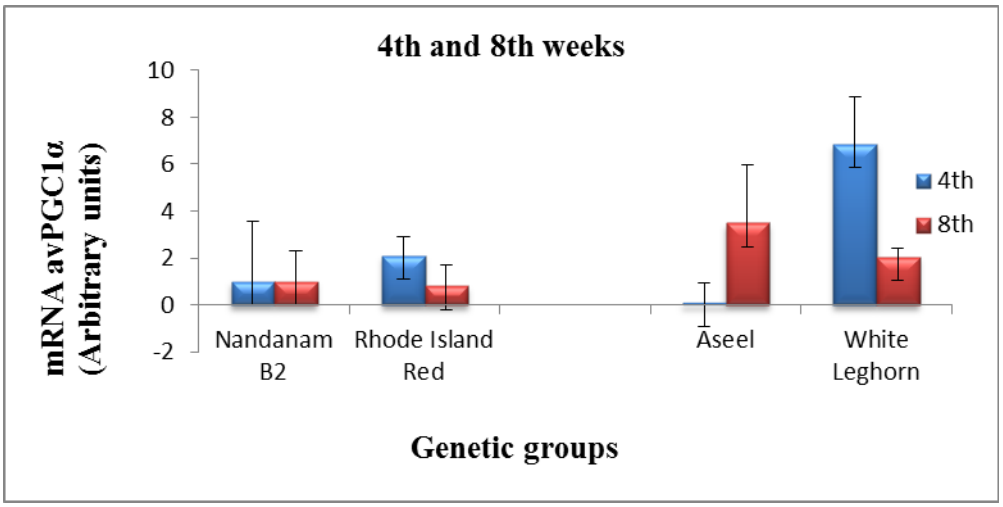

Figure 3. mRNA expression of avian PPAR-γ coactivator-1α (avPGC-1α) in the breast muscle of different chicken breeds at 4 and 8 weeks of age

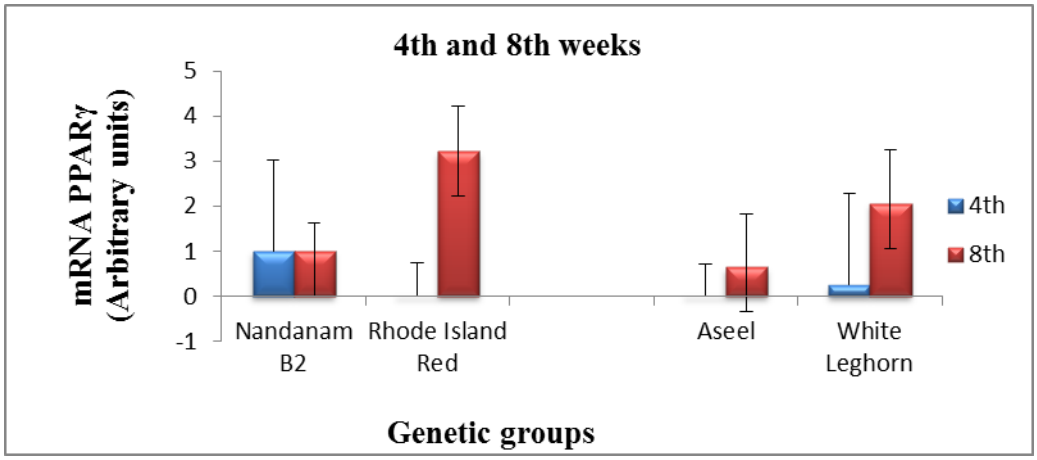

Figure 4. mRNA expression of Peroxisome proliferator-activated receptor-γ (PPARγ) in the breast muscle of different chicken breeds at 4 and 8 weeks of age

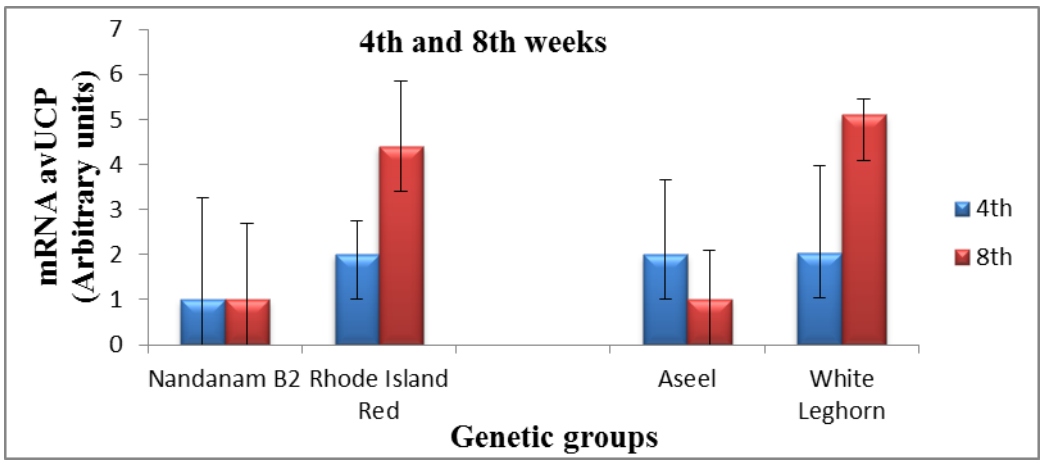

Figure 5. mRNA expression of avian uncoupling protein (avUCP) in the breast muscle of different chicken breeds at 4 and 8 weeks of age

CONCLUSION

In this study, aging influenced the expression of all genes analyzed; showing that the age of birds does influence the expression of electron transport chain genes, responsible for body energy production.

Consent to publish

All persons gave their informed consent prior to their inclusion in the study.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

This study is the part of M.V.Sc. Thesis of the first author Sarada Tarai, who carried out the research under the guidance of D.Thyagarajan who has helped in technical writing of the article and its final revision. G. Srinivasan has helped during the trial, processing of samples and analysis of data. All authors have read and approved the final version of the manuscript.

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