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Breeder age and bone development in broiler chicken embryos

[*Idade da matriz e desenvolvimento ósseo em embriões de frango de corte*]

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ABSTRACT

The effect of breeder age on long bone development was studied in chicken embryos from 12 days of incubation until hatching. Fertile eggs were incubated and randomly assigned in a 2 x 6 factorial arrangement (two breeder ages - 38 and 60 weeks and six incubation days - 12, 14, 16, 18, 20, and 21). Enzymatic activity of acid and alkaline phosphatases in tibial epiphyses and weights as well as length and width in tibias and femurs of the embryos were determined. Tartrate-resistant acid and alkaline phosphatases activity in epiphyses was not affected by breeder age. Absolute weight and width of femur and tibia were larger in 60-week-old embryos compared to 38-week-old. Enzymatic activity and morphometric measurements increased with incubation day, independently of breeder age. The results showed that the process of endochondral ossification during the last two thirds of embryo development was not influenced by the age of the breeders. Although, in terms of absolute weight, the long bones of embryos from older breeders were heavier, which was associated with the larger width of the bones, but and not with their length.

Keywords: chicken embryo, alkaline phosphatase, epiphyses, growth plate, long bones

RESUMO

O efeito da idade da matriz sobre o desenvolvimento dos ossos longos foi estudado em embriões de frango de 12 dias de incubação até o nascimento. Ovos férteis foram incubados e distribuídos em delineamento inteiramente casualizado em arranjo fatorial 2 x 6 (duas idades de matriz – 38 e 60 semanas e seis dias de incubação – 12, 14, 16, 18, 20 e 21 dias). Determinou-se a atividade enzimática das fosfatase alcalina e ácida-resistente ao tartrato no peso e nas epífises da tíbia, no comprimento e na largura da tíbia e do fêmur. A atividade das fosfatases não foi afetada pela idade da matriz. O peso absoluto e a largura de fêmur e tíbia foram maiores nos embriões das matrizes com 60 semanas de idade. Atividade enzimática e medidas morfométricas aumentaram com o dia de incubação independentemente da idade da matriz. Concluiu-se que o processo de ossificação endocondral durante os dois últimos terços de desenvolvimento embrionário não foi influenciado pela idade das matrizes. No entanto, em termos de peso absoluto, os ossos longos de embriões provenientes de matrizes velhas foram mais pesados o que foi associado à maior largura e não ao maior comprimento dos ossos.

Palavras-chave: embrião de frango, fosfatase alcalina, disco de crescimento, ossos longos

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INTRODUCTION

Leg problems in broilers have very high prevalence in conventional production systems (Sanotra et al., 2001) and cause economic losses to the poultry industry, which have resulted in increased mortality and a higher number of culled birds and carcass condemnations (Cook, 2000). Abnormalities in the skeleton such as kyphosis, scoliosis, and torticollis (twisted neck) are occasionally observed in newly hatched broiler chicks and poults. Such defects might have genetic, congenital, or metabolic origin. Congenital and metabolic skeletal defects may be associated with pre-incubation conditions, such as storage time, or to incubation conditions, like temperature and relative humidity (Julian, 2005). Thus, the bone development study might contribute to identify the causes of skeletal abnormalities (Cook, 2000).

Many studies have reported the effects of pre-incubation conditions, such as breeder age and nutrition, storage time, and egg weight on overall embryo development (Christensen and Donaldson, 1994; Vieira and Moran, 1998a, b; Tona et al., 2004). It is known that storage time and breeder age affect egg quality and consequently hatchability and chick quality. Those characteristics are negatively affected as breeder age increases and the impairment is exacerbated when eggs are stored for one week compared to no storage time (Tona et al., 2004). Therefore, heavier eggs and chicks from old breeders should not be considered an advantage for broiler performance (Tona et al., 2004). On the other hand, it has been proven that the performance of broilers from heavy eggs is better compared to lighter eggs if both have originated from breeders with similar age (Vieira and Moran, 1998a).

According to Vieira and Moran (1998b) the increase of breeder age enhances the yolk percentage and albumen and decreases eggshell percentages; thus, these factors may contribute to impaired bone development in embryos. Although egg size increases with breeder age, the amount of calcium deposited on the eggshell is constant during the laying cycle and therefore eggshell thickness decreases as well as its percentage in relation to egg weight. Considering that eggshell is the primary source of calcium for the formation of the skeleton in the embryo (Lavelin et al., 2000), different percentages of calcium in the eggshell might affect bone development in embryos; however, this statement should be confirmed.

The growth of long bones in the embryo occurs through endochondral ossification, which consists of the proliferation and differentiation of chondrocytes in the epiphyseal growth plate (Van der Eerden et al., 2003). The activity of the enzyme alkaline phosphatase is recognized as the marker of chondrocyte differentiation/maturation (Farquharson and Jefferies, 2000), and is involved in cartilage mineralization (Anderson, 1995), while the activity of tartrate-resistant acid phosphatase is used as the marker of osteoclastic activity and cartilage resorption (Roach, 1997).

Thus, the aim of the present study was to evaluate the effect of the breeder age (38 and 60 weeks) on bone development in embryos of broiler chickens by determining the activities of the enzymes alkaline and tartrate-resistant acid phosphatases and by performing morphometric measurements on long bones.

MATERIALS AND METHODS

Hatching eggs from 38 and 60-week-old broiler breeders[1] were used. Mean egg weights were 63.1±4.8g and 70.8±4.1g, respectively. The eggs were incubated at 37.5±0.2°C, under a relative humidity of 55-60%, in a commercial incubator[2] with turning at each hour from one to 19 days of incubation. Temperature was decreased by 0.5ºC and the relative humidity was increased by 5% from the 19th day of incubation until hatching. At day 9 of incubation, candling was performed to discard infertile eggs or early dead embryos.

Twenty-two embryos from each breeder age were chosen randomly on the 12, 14, 16, 18, 20, and 21 days of incubation. Half of these groups (n=11) were used to prepare the enzymatic extract from the tibias, which

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contained alkaline and tartrate-resistant acid phosphatases; and the other group (n=11) for morphometric measurements. Each embryo was sacrified by cervical dislocation and tibias were quickly and carefully removed, all adjacent tissue was also removed. Epiphyses with cartilage were collected in a microtube and snap-frozen using liquid nitrogen. Afterwards, the samples were stored at -70ºC until processing.

Alkaline phosphatase activity was determined as an indicator of biological calcification, whereas acid phosphatase activity was used to indicate the activity of reabsorption cells (osteoclasts) on the tibial epiphyses of embryos. To this end, epiphyses were homogenized in ice-cold 10mM TRIS[3] HCl buffer, pH 7.5, containing 2mM $MgCl_2$ and 1μM $ZnCl_2$ (25mL of buffer/g of epiphysis) in a high speed shearing homogenizer, at 20,000rpm for three times (30 seconds each). The homogenate was centrifuged at 5,000xg for 3min, at 4ºC. The supernatant was stored at 4°C and the pellet was resuspended and homogenized in original (wt vol^{-1}) homogenization buffer, and then centrifuged at 5,000xg for 3min, at 4ºC. This process was repeated and all the supernatants were pooled. Aliquots (1.0mL) were frozen in liquid nitrogen and stored at -70ºC.

Alkaline phosphatase activity was discontinuously assayed at 37ºC in a spectrophotometer[4] by the production of pNPP[3] at 410nm. Standard conditions were 50mM AMPOL[3] buffer pH 10.0 containing 2mM $MgCl_2$ and 1mM pNPP in a final volume of 1.0mL. The reaction was initiated by the addition of the enzyme and stopped with 1.0mL of 1.0M NaOH at appropriate times.

For tartrate-resistant acid phosphatase activity, it was also discontinuously assayed by the liberation of pNPP at 410nm. Standard assay conditions were 100mM acetate buffer pH 5.5 containing 10mM sodium tartrate and 2mM pNPP in a final volume of 1.0mL. The reaction was initiated by the addition of the enzyme and stopped with 1.0mL of 1.0M NaOH at appropriate times.

Controls without added enzyme were included to evaluate non-enzymatic hydrolysis of substrate. The enzymatic activity was expressed in nmols by mg of total protein. The Hartree procedure (Hartree, 1972) was used to determine total protein, using bovine serum albumin as standard.

The embryos used to perform morphometric measurements of long bones were sacrificed, and the tibias and femurs were carefully dissected. Epiphyseal cartilages were kept and adjacent tissues were removed. The bones were immediately weighed using an analytical scale to the nearest 0.01mg and kept at -20ºC for further analysis. Bone weights were expressed as absolute weight (mg) and relative weight (% of embryo weight). Bone images were obtained using a stereoscope[5] and analyzed with image software[6] to obtain the morphometric measurements. Tibia length was measured from the proximal end (*Eminentia intercondylaris)* to the distal end and the width at the medial diaphysis. It was also measured the width of the proximal and distal tibia epiphyses. Femur length was measured from the *Trochanter major* to *Condylus lateralis* and the width at the medial diaphysis.

Data were analyzed according to a completely randomized design in a 2x6 factorial arrangement, including two breeder ages (38 and 60 weeks) and six incubation days (12, 14, 16, 18, 20, and 21 days). There were 11 embryos for each treatment. Before analysis, the presence of outliers, the normal distribution of studentized errors (Cramer-Von Mises test) and homogeneity of variances (Brown-Forsythe test) were evaluated (Littell et al., 2002). The assumptions were violated for tibia and femur absolute weight, and these data were transformed ($x^{0.23}$)**.** The other variables did not violate the assumptions. The effects of breeder age were tested by the F value of analysis of variance. The effects of incubation day were analyzed using polynomial regressions. All analysis was performed using the General Linear Model procedure of SAS software (Littell et al., 2002).

[3]Sigma-Aldrich - St. Louis, USA.
[4]Hitachi U-2000 - Japan.
[5]MZ 12, Leica - Wetzlar, Germany.
[6]Motic - Xiamen, China.

RESULTS AND DISCUSSION

In the present study, the alkaline and acid phosphatase enzymatic activities of embryos were determined and are shown in Table 1. There was neither significant interaction between breeder age and incubation day nor main effect of breeder age on such variables. These findings suggest that breeder age is not a pre-incubation factor that regulates the longitudinal bone growth by endochondral ossification during the embryonic life of broiler chickens.

Table 1. Means ± standard errors of enzymatic activity of alkaline and acid phosphatases (nmols/mg) in tibial epiphyses of broiler embryos according to the breeder age

Incubation day	Alkaline phosphatase		Acid phosphatase[1]	
	38 weeks	60 weeks	38 weeks	60 weeks
12	180.6±14.0	217.1±12.0	11.6±1.4	12.2±0.8
14	208.8±10.9	219.6±20.7	15.0±1.1	12.0±0.9
16	329.0±14.8	363.5±22.4	20.9±1.3	22.1±2.2
18	435.1±16.6	436.7±23.6	31.1±1.3	33.1±1.2
20	549.0±21.0	504.2±18.8	29.3±1.4	29.4±1.8
21	586.1±16.0	594.6±30.3	33.0±1.7	35.2±1.9
Analysis of variance	Probability[2]			
Incubation day (ID)	<0.0001		<0.0001	
Breeder age (BA)	0.50		0.53	
Interaction ID*BA	0.31		0.54	
CV (%)	15.1		18.5	
Equation[3]	Y = -329.211+42.195DI		Y= -17.061+2.404DI	
R^2	0.84		0.70	

[1]Tartrate-resistant acid phosphatase.
[2]Test F (P<0.05).
[3]P<0.05 for the parameters estimated.

On the other hand, it was found that there was a linear increase in the activity of both enzymes after 12 days of incubation. In agreement, Firling et al. (1999) also observed a similar activity of alkaline phosphatase in embryos from 10 to 16 days of incubation. This increase in the activity of alkaline phosphatase in the growth plate represents the last stages of the bone formation cascade (phase of cell hypertrophy), immediately after the cell division phase had finished, and the subsequent enzymatic activity increased further until bone mineralization (Lovitch and Christianson, 2000). Concomitantly, the width of the hypertrophic zone of the epiphyseal growth plate progressively increased during the embryonic development, with no simultaneous resorption of the cartilage, as opposed to what happens with mammals (Roach, 1997). In the present study, it was observed a linear growth of the width of tibial epiphyses (Table 2). As previously explained, this increase might be attributed to a wider hypertrophic zone of the growth plate. According to Yalçin et al. (2001), such linear increase in the width of tibial epiphyses in embryos continues after hatching and lasts until market-age in broilers.

The enzyme acid phosphatase was found to be active during all periods, from 12 days of incubation until hatching. These findings do not corroborate with those reported by Firling et al. (1999), who stated that osteoclastic activity does not begin before 17 days of incubation. Roach (1997) also reported that the process of bone resorption in the growth plate of long bones of broiler embryos occurs only after bone has been formed. Nevertheless, the presence of acid phosphatase simultaneously with alkaline phosphatase after 12 days of incubation might have occurred in the mineralized matrix of the endochondral bone, when it could have a role in preventing early bone remodeling, due to its ability to cause dephosphorylation of osteopontin and bone sialoprotein, which are present in the endochondral matrix as opposed to mammals (Roach, 1997).

Table 2. Means ± standard errors of the epiphyseal width (mm) of the tibia of broiler embryos according to the breeder age

Incubation day[1]	Proximal epiphysis width		Distal epiphysis width	
	38 weeks	60 weeks	38 weeks	60 weeks
14	2.49±0.20	2.34±0.11	2.40±0.16	2.48±0.15
16	3.89±0.16	3.71±0.18	3.32±0.10	3.50±0.18
18	4.57±0.13	4.82±0.17	4.50±0.11	4.60±0.10
20	5.22±0.11	5.37±0.10	5.30±0.10	5.37±0.10
21	6.10±0.13	6.11±0.10	5.74±0.12	5.94±0.05
Analysis of variance	Probability[2]			
Incubation day (ID)	<0.0001		<0.0001	
Breeder age (BA)	0.84		0.18	
Interaction ID*BA	0.57		0.95	
CV (%)	8.9		6.0	
Equation[3]	y= -4.035 + 0.478DI		y= -4.313 + 0.486DI	
R^2	0.86		0.94	

[1]The distal and proximal epiphyses were not recorded at day 12 of incubation.
[2]Test F (P<0.05).
[3]P<0.05 for the parameters estimated.

In regard to morphometric measurements, there was no significant interaction between incubation day and breeder age on tibia (Table 3) and femur (Table 4) absolute and relative weights; on tibia (Table 5) and femur (Table 6) width and length; and on the width of proximal and distal tibias (Table 2). It was observed a breeder age effect on the absolute weight and the width of long bones. These measurements were greater in embryos from 60-week-old breeders compared to 38-week-old breeders. The greater bone weight in embryos from older breeders might result from a greater mineral density, such as that observed by Yalçin et al. (2001) in newly hatched chicks. However, as previously discussed, the mineralization activity in long bones was not different due to breeder age, and when weight was expressed as a percentage of embryo weight (relative weight), the breeder age effect disappeared. Moreover, there was no significant breeder age effect on tibia and femur lengths, and on the width of proximal and distal tibial epiphyses. The width of proximal and distal tibial epiphyses linearly increased with embryo age. The other variables had quadratic effects as a function of incubation day.

Table 3. Means ± standard errors of absolute weight (mg) and relative weight (% of embryo weight) of the tibia of broiler embryos according to the breeder age

Incubation day	Absolute[1]		Relative	
	38 weeks	60 weeks	38 weeks	60 weeks
12	17.99±1.23	20.54±0.75	0.32±0.02	0.35±0.01
14	49.26±1.90	49.77±2.43	0.40±0.01	0.40±0.01
16	104.90±2.21	107.09±5.12	0.51±0.02	0.51±0.02
18	195.26±7.07	212.48±6.20	0.71±0.02	0.74±0.01
20	270.10±6.52	289.38±7.26	0.60±0.02	0.59±0.02
21	332.89±6.46	372.75±5.58	0.76±0.01	0.76±0.01
Analysis of variance	Probability[2]			
Incubation day (ID)	<0.0001		<0.0001	
Breeder age (BA)	0.0004		0.38	
Interaction ID*BA	0.38		0.74	
CV (%)	2.2		8.9	
Equation[3]	y_{38}= -0.57+0.10DI-0.002DI^2 y_{60}= -0.46+0.09DI-0.001DI^2		y= -0.81+0.12DI-0.002DI^2	
R^2	0.99	0.99	0.78	

[1]Data were transformed to $y^{0.23}$ before variance and regression analysis; the means ± standard errors showed is not transformed.
[2]Test F (P<0.05).
[3]P<0.05 for the parameters estimated; the adjusted models are separated for 38 and 60 weeks when the effect of breeder age was significant by analysis of variance.

Table 4. Mean ± standard error of absolute weight (mg) and relative weight (% of embryo weight) femur of broiler embryos according to the breeder age

Incubation day	Absolute[1]		Relative	
	38 weeks	60 weeks	38 weeks	60 weeks
12	15.63±0.71	15.85±0.80	0.27±0.01	0.28±0.01
14	41.19±1.98	38.31±1.64	0.33±0.01	0.31±0.02
16	72.41±2.53	75.52±3.57	0.39±0.01	0.39±0.01
18	122.12±6.04	140.94±3.32	0.47±0.02	0.50±0.01
20	175.83±5.44	182.81±5.29	0.40±0.01	0.37±0.01
21	220.93±3.86	243.13±3.65	0.49±0.01	0.49±0.01
Analysis of variance	Probability[2]			
Incubation day (ID)	<0.0001		<0.0001	
Breeder age (BA)	0.005		0.64	
Interaction ID*BA	0.08		0.06	
CV (%)	2.1		8.2	
Equation[3]	y_{38}= -1.41+0.33DI-0.005DI^2 y_{60}= -1.85+0.38DI-0.006DI^2		y= -0.56+0.09DI-0.002DI^2	
R^2	0.99	0.99	0.68	

[1]Data were transformed to $y^{0.23}$ before variance and regression analysis; the mean ± standard error showed is not transformed.
[2]Test F (P<0.05).
[3]P<0.05 for the parameters estimated; the adjusted models are separated for 38 and 60 weeks when the effect of breeder age was significant by analysis of variance.

Table 5. Means ± standard error of length (mm) and width (mm) of the tibia of broiler embryos according to the breeder age

Incubation day	Length		Width	
	38 weeks	60 weeks	38 weeks	60 weeks
12	12.13±0.42	11.62±0.24	0.87±0.02	0.93±0.02
14	17.03±0.21	17.16±0.30	1.18±0.03	1.32±0.03
16	22.19±0.35	22.16±0.28	1.55±0.03	1.55±0.04
18	27.09±0.38	27.64±0.23	1.69±0.02	1.78±0.02
20	30.61±0.24	30.98±0.28	1.79±0.03	1.84±0.02
21	32.53±0.18	33.46±0.24	1.89±0.03	2.00±0.02
Analysis of variance	Probability[1]			
Incubation day (ID)	<0.0001		<0.0001	
Breeder age (BA)	0.24		<0.0001	
Interaction ID*BA	0.20		0.23	
CV (%)	3.2		5.0	
Equation[2]	y= -33.74+4.60DI-0.07DI^2		y_{38}= -2.72+0.41DI-0.01DI^2 y_{60}= -2.50+0.39DI-0.01DI^2	
R^2	0.99		0.95	0.95

[1]Test F (P<0.05).
[2]P<0.05 for the parameters estimated; the adjusted models are separated for 38 and 60 weeks when the effect of breeder age was significant by analysis of variance.

Table 6. Means ± standard errors of length (mm) and width (mm) of the femur of broiler embryos according to the breeder age

Incubation day	Length		Width	
	38 weeks	60 weeks	38 weeks	60 weeks
12	9.14±0.40	9.27±0.18	0.80±0.02	0.80±0.02
14	12.98±0.26	12.82±0.19	1.13±0.03	1.16±0.06
16	16.35±0.20	16.80±0.20	1.46±0.02	1.49±0.04
18	20.15±0.25	20.49±0.27	1.61±0.02	1.74±0.03
20	22.74±0.16	22.99±0.27	1.81±0.03	1.78±0.02
21	24.42±0.20	25.18±0.20	1.95±0.04	2.04±0.03
Analysis of variance	Probability[1]			
Incubation day (ID)	<0.0001		<0.0001	
Breeder age (BA)	0.06		0.03	
Interaction ID*BA	0.66		0.07	
CV (%)	3.9		6.1	
Equation[2]	$y= -18.79+2.67DI-0.03DI^2$		$y_{38}= -2.23+0.33DI-0.01DI^2$ $y_{60}= -2.52+0.36DI-0.01DI^2$	
R^2	0.98		0.95	0.93

[1]Test F (P<0,05).
[2]P<0.05 for the parameters estimated; the adjusted models are separated for 38 and 60 weeks when the effect of breeder age was significant by analysis of variance.

Yalçin et al. (2001) suggested that bone weight followed the overall embryo development. It is known that older breeders lay heavier eggs, consequently, chick weight also increases (Pinchasov, 1991; Vieira and Moran, 1998 a, b; Tona et al., 2004). Therefore, the greater weight of bones in embryos originating from 60-week-old breeders may be attributed to greater egg size associated with breeder age, but not as a direct effect of breeder age. The greater weight of bones in embryos from older breeders was due to a greater width instead of length. This finding is of practical interest since the wider bone of broilers from older breeders can increase the resistance to break. Even though, according to Yalçin et al. (2001), the effect of breeder age on bone morphometry is not present in later phases during broiler rearing, thus the findings of the present study warrant further investigations.

CONCLUSIONS

The process of endochondral ossification during the last two thirds of embryo development was not influenced by the age of the breeders. Although, in terms of absolute weight, the long bones of embryos belonging to older breeders were heavier, which was associated with the greater width of the bones, but not with the length.

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