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Hematological Values and Body, Heart and Liver Weights of Male and Female Broiler Embryos of Young and Old Breeder Eggs

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ABSTRACT

This study analyzed broiler breeder age (29 or 60 wk-old) effects on physical characteristics of eggs (initial mass, mass loss, volume, diameter, surface area and density) and of eggshells(weight, volume, thickness, conductance, and porosity), as well as the influence of embryo sex on hematological parameters and body, liver and heart weights during incubation (at days 13, 15, 18 and 21). Physical parameter values were lower in 29-wk-old broiler breeder eggs than those of 60-wk-old breeders, except for relative eggshell weight, which was higher. In both male and female embryos, erythrocytic parameters and the body, liver, and heart weights increased during the incubation. The embryos and their organs were heavier when derived from 60-wk-old breeder eggs as compared to 29-wk-old breeder eggs. At hatching, hematocrit values were higher in males than in females. Thrombocytes were the most frequent leukocytes in the blood. Thrombocyte percentage decreased and lymphocyte percentage increased during the last days of incubation. The results showed maternal age influence only on body, heart and liver weights, focal sex-related influence the hematocrit at hatching, and temporal effect of incubation on body and organ weights, as well as on red blood cell count, hematocrit, hemoglobin, plasma glucose, and lymphocytes, which increased during the incubation period, while mean corpuscular volume and thrombocyte values decreased.

INTRODUCTION

The functional structure of fertile eggs must provide adequate embryo development outside the maternal body. This requires the egg to contain adequate and available nutrients, energy source, and water, as well as to allow gas exchange across the eggshell pores throughout incubation. The avian eggshell is a protective barrier against mechanical shock and microorganisms, and the main source of minerals. In addition, it allows O_2 diffusion into the egg and release of CO_2 and steam from the eggs into the environment. Gas exchanges in embryos are carried out by the chorioallantoic membrane (Wangensteen & Rahn, 1970/1971) mainly by diffusion (Metcalfe *et al*., 1981; Rahn *et al*., 1987). Gases are exchanged through the eggshell pores, which are unique communication channels between the internal and external egg environment, and have to cross the barriers of the chorioallantoic membrane, outer and inner shell membranes, palisade layer, and cuticle (Wangensteen & Weibel, 1982; Seymour & Piiper, 1988). The main limiting features of gas exchange are shell thickness and porosity (Ar *et al*., 1974).

Maternal physiological conditions during egg production, together with genetic factors, influence eggshell characteristics and, consequently, embryo development (Maiorka *et al*., 2003). In broilers, it is known that older breeders produce eggs with higher total pore number

than young breeders (Shanawany, 1984). Eggshell pore size and number are established during egg formation (La Scala, 2003). According to Christensen *et al*. (1995), changes in eggshell thickness, pore number and diameter decrease the conductance of O_2 available for embryo growth.

Nutritional and respiratory deficiencies can be hematologically diagnosed by analyzing the erythrocytic and leukocytic series. Literature data show that organisms are able to respond to stressor agents by eliciting an increase in plasma glucose and hematocrit, a reduction in lymphocyte number and, glycogen level and hepatic index, as well as changes in heterophil: lymphocyte ratio (Barton *et al*., 1987; Chamblee *et al*., 1989; Maxwell *et al*., 1990).

The objectives of the present study were to analyze broiler embryo blood cell counts and body, liver, and heart weights, to determine if there are differences in eggshell structural characteristics between 29- and 60-week-old breeders and if these differences, including embryo sex, influence embryo hematological parameters and body and organ weights during the incubation period.

MATERIALS AND METHODS

Fertile eggs (white eggshell) of Cobb500® broiler breeders were collected from the same hens at 29 and 60 weeks of age (56±3.0g and 74.6±2.7g, respectively) and obtained from a commercial hatchery. Eggs were analyzed and incubated at the Laboratory of Histology and Embryology, Department of Animal Morphology and Physiology, School of Agrarian and Veterinary Sciences, São Paulo State University (UNESP), Jaboticabal campus. The present study was conducted in 2006 and 2007.

The physical characteristics of the egg and eggshell from 29- and 60-wk-old breeders were compared (N = 15 eggs per breeder age). The length of egg longitudinal axis corresponded to the distance between the end of the egg containing the air cell and the pointed end, while its transversal axis was measured transversally to the former and corresponded to maximum width. Both axes were measured in centimeters using a vernier caliper. Initial egg mass (g) was the egg mass measured immediately after puncturing the air cell with a hypodermic needle and injecting distilled water to displace the air volume (Christensen *et al*., 1996). Egg volume (cm^3) was determined by the Archimedes principle as the difference in mass between dry egg weight and egg weight submersed in a graduated test tube of distilled water divided by water density at water temperature (Rahn *et al*., 1981). Egg surface area (cm^2) was estimated by the allometric formula: Area = $4.835W^{0.662}$, where W is initial egg mass (g) (Paganelli *et al*., 1974). Egg density was obtained by dividing egg mass by egg volume.

Eggshell weight (g) included eggshell membranes, and was obtained after the eggs were emptied, internally washed with water to completely remove the albumen, and dried to a quarter of the temperature for two weeks. Eggshell thickness and pore number per cm^2 were obtained from pieces of the eggshell taken from the pointed, equatorial, and enlarged areas of the egg. Eggshell pieces were boiled for 10 minutes in a 5% NaOH solution (to remove eggshell cuticle and membranes), rinsed with water, dried, stained for 2 minutes with a water solution of 1% methylene blue, rinsed again with water, and dried (Rahn *et al*., 1981). Eggshell thickness did not include membranes, and was measured using a digital micrometer (Mitutoyo - 0.001 mm accuracy). Mean eggshell thickness was obtained for each individual egg from its shell pieces. The number of pores per cm^2 was determined under a stereomicroscope using a reticular eye piece. Mean pore number was also established for each egg based on the values of its three areas. The mean value was multiplied by total egg surface area to estimate the total number of pores per egg. Eggshell volume was calculated by the formula: Volume = A x L, where A is the egg surface area (cm^2), and L is the thickness of the shell (cm) (Rahn *et al*., 1981).

Daily egg mass loss and eggshell conductance were analyzed under normal incubation conditions (at 37.5ºC and 60% RH). Thirty fertile eggs per breeder age were distributed in two incubators (IP70, Premium Ecológica) with automatic temperature control and egg turning every 2 hours. The relative humidity was measured by an automatic thermo-hygrometer. Daily egg mass loss and eggshell conductance were calculated for every 10 eggs randomly chosen per breeder age during the first week of incubation. Eggshell conductance (mg H_2O per day per mm Hg) was obtained by dividing daily egg mass loss by a saturation vapor conductance of 23.86mm/Hg at 25ºC (Rahn *et al*., 1981), correcting the values to a barometric pressure of 1 atmosphere, as described by Ar *et al*. (1974).

Body, heart, and liver weights, and blood parameters were analyzed according to a completely randomized experimental design with a 2 (sex: male and female) x 2 (breeder age: 30 and 60 wk of age) x

4 (embryo age: 13, 15, 18, and 21 days of incubation) factorial arrangement. Three hundred eggs (150 eggs per breeder age) were placed in four incubators (two per breeder age) (IP70, Premium Ecológica), maintained at 37.5ºC and 60% RH controlled by a hygrometer until hatching, and turned every two hours.

On days 13, 15 and 18 of incubation, blood samples were collected from the yolk pedicle, whereas at 21 days of incubation (corresponding to newly-hatched chicks, which were used as soon as the down dried) blood samples were collected from jugular vein (N= 6 embryos/day of incubation/breeder age). Blood samples were placed in plastic vials containing EDTA (GLISTAB, 15µl/ml blood), stored on ice, and submitted to the lab in order to determine hematocrit (HCT, %), red blood cell count (RBC, x mm^3/mL), hemoglobin (HGB, g/dL), mean corpuscular volume (MCV, μ^3), and plasma glucose (mg/dL). Erythrocytic values were obtained using a blood-cell counter (Celm, Mod. 550), with two readings per bird (20µL of blood per reading). For glucose determination, blood samples were centrifuged at 1.500 rpm at 4ºC for 15 min. Plasma samples were placed in plastic vials and stored at -20ºC until analysis. Plasma glucose was determined using a glucose PAP liquiform kit (Labtest, Cat. N.84). Two readings per embryo were carried out at 505nm. Specific leukocytes were counted in blood smears stained with Rosenfeld solution. Monocyte, lymphocyte, heterophil, eosinophil, and basophil counts were determined by counting the number of each leukocyte type in 100 analyzed cells, and expressed as estimated percentages of the 100 analyzed cells.

Immediately after blood collection, embryos were weighed (without the yolk sac), as well as their livers and hearts (Marte, 0.0001g). Relative heart and liver weights were calculated as a percentage of embryo body weight.

Egg physical characteristics were analyzed by thet-student test ($p<0.05$). A 2x2x4 factorial arrangement was employed to analyze weights and erythrocytic parameters. Data were analyzed for outliers and tested for normality and variance. All analyses were performed using the GLM procedure of SAS package (2002), with a different number of replications. The fitted means were compared by the F-test and Tukey's test ($p<0.05$).

RESULTS AND DISCUSSION

Table 1 shows egg physical parameter data. All analyzed physical parameters were lower ($p<0.05$) in eggs from 29-wk-old as compared to those of 60-wk-old breeders (29WB and 60WB, respectively), except for relative eggshell weight and eggshell thickness, which were lower ($p<0.05$) in 60WB eggs than in 29WB eggs, and egg density, which was not significantly different($p>0.05$) between breeder ages.

The similar density observed between 29WB eggs and 60WB eggs resulted from a proportional increase

Table 1 - Physical characteristics of eggs of 29- (29WB) and 60- (60WB)-week-old broiler breeders.

	IM (g)	MxD (cm)	MinD (cm)	SA (cm^2)	Vol (cm^3)	D (g/cm^3)	ASW (g)
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.8433	<0.0001
T	-14.5665	-8.1335	-5.4012	-14.3951	-14.8790	0.2005	-7.9111
29WB	56.2 b	5.3 b	4.0 b	69.6 b	52.5 b	1.0 a	5.2 b
60WB	74.7 a	6.1 a	4.3 a	84.1 a	70.0 a	1.0 a	6.7 a
	RSW (%)	**SVol (ml)**	**MST (mm)**	**STP (mm)**	**STE (mm)**	**STL (mm)**	**TPN**
p	<0.0001	<0.0001	0.0094	0.0007	0.0238	0.0029	0.0114
T	-14.3951	-6.0511	-1.734	-4.0521	-2.4673	-3.4324	2.1100
29WB	9.3 a	3.2 b	0.6 a	0.6 a	0.6 a	0.6 a	1741 b
60WB	9.0 b	4.9 a	0.5 b	0.4 b	0.5 b	0.5 b	2364 a
	PNP ($pore/cm^2$)	**PNE ($pore/cm^2$)**	**PNL ($pore/cm^2$)**	**MPN ($pore/cm^2$)**	**CON (g)**	**TWL (g)**	**DWL (g)**
p	0.1293	0.4765	0.7533	0.2279	<0.0001	0.2371	0.0001
T	-1.5893	-0.7270	-0.3190	-1.2481	1.985	-1.9762	1.5521
29WB	24.8 a	23.6 a	26.8 a	25.1 a	11.6 b	0.28 a	1.39 b
60WB	31.4 a	26.7 a	27.6 a	28.6 a	14.9 a	0.31 a	1.78 a

SA: surface area, MxD and MinD: maximum and minimum diameters, D: density, IM: initial mass, DWL and TWL: daily and total water loss, Vol: volume of eggs. CON: eggshell conductance; STP, STE and STL: shell thickness in the pointed end, equatorial and widest regions of the egg; MST: mean eggshell thickness; TPN: total pore number; PNP, PNE and PNL: pore number in the pointed end, equatorial and widest regions of the egg; ASW and RSW: absolute and relative eggshell weights; SVol: eggshell volume. a-b: means followed by similar letters in the same column are not significantly different ($p<0.05$) by t-student test.

in egg mass and volume in both ages. According to Woods (1999), when there is an isometric increase in size, volume increases more rapidly than surface area. The results of the present study show a mean difference of approximately 17.5 cm^3 in volume and of 14.5 cm^2 in surface area between 29WB and 60WB eggs, corresponding to an increment of 33.3% in volume and of 21% in surface area of eggs in a 31-wk interval (between 29 and 60 weeks of age). These data suggest an isometric growth in egg size, and show a lower surface:volume ratio in 29WB eggs as compared to 60WB eggs (1.32 and 1.20, respectively). Eggshell volume increased 50.8% between 29 and 60 weeks of age, while egg volume increased only 33%, resulting from the reduction in relative eggshell weight during the same period.

The data relative to eggshell thickness obtained in the present study agrees with those of Maiorka *et al*. (2003), who also found that eggshell thickness decreases with breeder age. According to Baião & Aguilar (2001), calcium content remains constant in the eggshell during the laying cycle, which may explain the lower mean eggshell thickness observed in eggs from older breeders as compared to younger breeders. In the present study, we verified a high positive correlation between eggshell thickness and weight, as well as between eggshell thickness and egg longitudinal axis, volume, and surface area, indicating that eggshell thickness reduction is related to the physical dimensions of the eggs.

An equal number of pores per cm^2 was observed in the three analyzed areas of the eggshell, independently of breeder age, in the present experiment. Eggshells from 29-wk-old breeders, however, presented a lower total pore number than those from 60-wk-old breeders. This higher egg porosity in older breeders, however, results from the higher volume and surface area of their eggshells. 60WB eggs had lower eggshell thickness, and higher porosity and conductance. Considering that conductance corresponds to capacity of gas exchange between the internal and external egg environment, and that gas exchanges are related to water mass loss (Campos *et al.*, 2003), the higher conductance reported in 60WB eggs resulted simultaneously from their higher pore number and lower shell thickness. Increases in conductance and water loss during incubation were also observed by Ar & Rahn (1980).

There was significant interaction ($p<0.05$) between breeder age and sex, and breeder and embryo age for absolute (ABW) and relative (RBW) body weights (Tables 2 and 3). Independently of the breeder age, males and females were not different as to ABW and RBW, and both presented higher ABW and lower RBW when derived from 60WB eggs as compared to 29WB eggs. Embryo ABW and RBW increased continuously and significantly ($p<0.05$) after day 13 of incubation in both breeder ages. At day 18 of incubation, embryos from 29WB eggs had higher RBW ($p<0.05$) than those from 60WB eggs, and at day 21, their ABW was lower ($p<0.05$). The absence of any significant difference in RBW between males and females, and between the two breeder ages at hatching shows that the body

Table 2- Effects of embryo age (13, 15, 18 and 21 days of incubation), sex and breeder age (29 and 60 weeks of age: 29WB and 60WB, respectively) on absolute (ABW, g) and relative (RBW, %) body weights; absolute (ALW, g) and relative (RLW, %) liver weights; absolute (AHW, g) and relative (RHW, %) heart weights of broiler embryos.

	ABW	RBW	ALW	RLW	AHW	RHW
Embryo age (EA)						
13	9.11 a	14.19 a	0.15 a	1.65 b	0.06 a	0.68 b
15	15.47 a	24.29 a	0.29 a	1.90 a	0.13 a	0.84 a
18	27.89 a	37.00 a	0.57 a	2.04 a	0.20 a	0.73 b
21*	49.48 a	75.17 a	0.80 a	1.63 b	0.26 a	0.53 c
Sex (S)						
Male	26.03 a	37.15 a	0.49 a	1.79 a	0.17 a	0.68 a
Female	24.95 a	38.16 a	0.47 a	1.80 a	0.17 a	0.69 a
Breeder age (BA)						
29WB	23.88 a	40.15 a	0.42 a	1.80 a	0.14 a	0.65 a
60WB	27.1 a	34.79 a	0.48 a	1.79 a	0.17 a	0.72 a
Probability						
EA	< 0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
S	< 0.0001	<0.0001	0.4043	0.6135	0.6729	0.6135
BA	< 0.0001	<0.0001	<0.0001	0.6904	<0.0001	0.6904
BA x S	0.0076	0.0487	0.0500	0.4729	0.3445	0.4729
BA x EA	<0.0001	0.0023	<0.0001	0.2362	0.0051	0.2362
S x EA	0.3307	0.7956	0.9318	0.4454	0.0553	0.4454

a-d: means followed by similar letters in the same column are not significantly different ($p<0.05$) by Tukey's test and analysis of variance. *Newly-hatched chicks.

weight of newly-hatched chicks was equivalent to approximately 75% of the initial egg mass. This value is consistent with the interval (75-80%) considered normal for chicks by Henry & Burke (1997).

Table 3 - Interaction between breeder age (29: 29WB and 60: 60WB weeks of age) and sex (male and female) for absolute (ABW) and relative (RBW) body weights, and interaction between breeder and embryo age (13, 15, 18, and 21 days of incubation) for ABW, RBW, absolute liver weight (ALW), and absolute (AHW) heart weights.

	Breeder age X Sex			
	ABW		RBW	
	29WB	60WB	29WB	60WB
Male	23.92 Ba	28.14 Aa	40.61 Aa	33.70 Ba
Female	23.82 Ba	26.06 Aa	40.45 Aa	35.88 Ba
	Breeder age X Embryo age			
	ABW		RBW	
	29WB	60WB	29WB	60WB
13	8.90 Ad	9.30 Ad	15.44 Ad	12.94 Ad
15	15.27 Ac	15.68 Ac	26.56 Ac	22.01 Ac
18	26.69 Ab	29.10 Ab	44.47 Ab	29.54 Ba
21*	44.65 Ba	54.32 Aa	75.66 Aa	74.83 Aa
	ALW		AHW	
	29WB	60WB	29WB	60WB
13	0.05 Ad	0.06 Ad	0.15 Ad	0.14 Ad
15	0.12 Ab	0.13 Ab	0.28 Ac	0.31 Ac
18	0.18 Bc	0.21 Ac	0.54 Ab	0.59 Ab
21*	0.23 Ba	0.28 Aa	0.72 Ba	0.88 Aa

a-b (columns), A-B (lines): means followed by similar letters are not significantly different (p<0.05) by Tukey's test and analysis of variance..*Newly-hatched chicks.

The observed significant interaction (p<0.05) between embryo and breeder ages shows that the absolute liver weight (ALW) increased continuously and significantly (p<0.05) from day 13 of incubation in embryos from 29WB and 60WB eggs. At days 18 and 21, ALW was higher (p<0.05) in embryos from 60WB eggs than from 29WB. Relative liver weight was significantly influenced (p<0.05) only by embryo age (Table 2), increasing between day 13 and 15 of incubation and from day 18 to hatching. For absolute heart weight (AHW), there was a significant (p<0.05) interaction between embryo and breeder ages (Table 2 and 3), showing that AHW continuously increased during the incubation period, reaching higher values in newly-hatched chicks from 60WB eggs than those from 29WB eggs. Relative heart weight was significantly influenced (p<0.05) only by embryo age, increasing between day 13 to 15 of incubation, and decreasing from day 15 to hatching, and reaching the lowest values at hatching as compared to the other three analyzed ages.

Data relative to ALW and AHW show that both organs gradually grow during the second half of the incubation period, independently of sex or breeder age. In addition, it was shown that RLW is not related to breeder age, as previously observed by Maiorka *et al*. (2000), or to embryo sex. In broilers (Nichelmann *et al*., 1998; Decuypere *et al*., 1979) and in emus (Prinzinger *et al*., 1997), energy production, and consequently, metabolism, markedly increases from day 10 of incubation on. Therefore, the higher liver weight values determined at days 15 and 18 of incubation seem to be related with the higher metabolic activity presented by the embryos during the last quarter of incubation.

Increases in environmental oxygen during the last quarter of incubation increases oxygen consumption, as well as embryo growth and survival (Tazawa *et al*., 1992; Christensen *et al*., 1997). It is known that a lower number of pores per egg limits the oxygen available for the embryo (Tullett & Deeming, 1982) and reduces its growth rate (Burton & Tullett, 1983). In avian embryos, higher oxygen consumption and, consequently, higher metabolic rates and gas exchange, depends on two structural eggshell features: thickness and pore number. Therefore, the higher body, liver, and heart weights recorded in embryos from 60WB eggs seems to be related to the lower thickness and the higher porosity and conductance of the eggshell of 60WB eggs.

Tables 4 and 5 show the erythrogram results. No significant (p<0.05) effect of sex or breeder age was observed on RBC, MCV and HGB, but these parameters were significantly (p<0.05) influenced by embryo age. RBC and HGB values increased both between days 13 and 15 of incubation and days 18 and 21, reaching the highest values at hatching. MCV values were lower at 21 than at 13 days of incubation. There was no effect of breeder age on HCT, but a significant (p<0.05) interaction between sex and embryo age was observed, showing that both female and male HCT values increased from days 13 to 15 and days 18 to 21 of incubation, reaching the highest values at hatching; however, males had higher HCT values than the females at 15 and 21 days.

In broilers (Nichelmann *et al*., 1998; Decuypere *et al*., 1979) and in emus (Prinzinger *et al*., 1997), heat production, as estimated by oxygen consumption, and therefore metabolism, markedly increases in the second half of the incubation period. This indicates that the higher RBC, HCT and HGB values recorded in embryos after day 13 in the present study are related to a higher embryonic metabolic rate during this period. The positive correlations between these erythrocytic parameters and heart weight during the incubation

period suggest an adaptation of heart mass to changes in heart potential (output) related to increases in metabolic and respiratory rates during this period, according to Decuypere (1979). As already mentioned here,, with isometrical increases in size, volume increases more rapidly than surface area (Woods, 1999), indicating that lower MCV values imply in higher erythrocyte surface areas. Therefore, it is possible that the reduction in the MCV values presented by the embryos at the end of incubation (our data) is related to an increase in oxygen supply, in an attempt to supply the increasing embryonic oxygen demand resulting from the higher embryonic metabolic rate after day 13 of incubation. Therefore, the results of the present study indicate that older embryos respond to higher oxygen demand resulting from their higher metabolic rate elevating oxygen transportation rate by increasing their cardiac output and also reducing their blood cell volume.

Table 4- Effects of embryo age (13, 15, 18, and 21 days of incubation), sex and breeder age (29: 29WB and 60: 60WB weeks of age) on red blood cell counts (RBC, $10^6/mm^3$), mean corpuscle volume (MCV, μm^3), hematocrit (HCT, %), hemoglobin (HGB, g/dl), and plasma glucose concentration (PG, mg/dl).

	RBC	VCM	HCT	HGB	PG
Embryo age (EA)					
13	0.45 c	134.08 b	5.61	2.8 c	0.18 c
15	1.23 bc	127.29 ab	15.29	6.5 b	0.17 c
18	1.54 b	126.85 ab	15.92	7.33 b	0.31 b
21*	2.57 a	123.8 a	25.88	16.01 a	0.49 a
Sex (S)					
Male	1.59 a	128.96 a	17.47 a	9.2 a	0.28 a
Female	1.29 a	127.44 a	13.88 a	7.11 a	0.28 a
Breeder age (BA)					
29WB	1.33 a	127.74 a	15.77 a	6.82 a	0.29 a
60WB	1.55 a	128.66 a	15.59 a	9.49 a	0.28 a
Probabilility					
EA	<0.0001	0.0308	<0.0001	<0.0001	< 0.0001
S	0.1701	0.7369	0.0956	0.6622	0.9420
BA	0.3131	0.6635	0.1055	0.1637	0.5883
BA x S	0.7846	0.7893	0.8332	0.3791	0.7843
BA x EA	0.7017	0.5087	0.5369	0.3554	0.6696
S x EA	0.8829	0.6921	0.0067	0.1276	0.1399

a-c: means followed by similar letters in the same column are not significantly different (p<0.05) by Tukey's test and analysis of variance. *Newly-hatched chicks.

Table 5- Interaction between embryo sex and age (13, 15, 18, and 21 days of incubation) on hematocrit (HCT, %).

Embryo age	Sex	
	Male	Female
13	6.28 Ac	4.95 Ac
15	17.78 Ab	12.81 Bb
18	16.83 Ab	15.03 Ab
21*	29.01 Aa	22.76 Ba

a-c (columns), A-B (lines): means followed by similar letters are not significantly different (p<0.05) by Tukey's test and analysis of variance. *Newly-hatched chicks.

Plasma glucose (PG) values were not influenced (p>0.05) by embryo sex or breeder age, suggesting that embryonic carbohydrate metabolism as a function of embryo sex or breeder age did not result in any PG changes during embryo development. However, there was a significant (p<0.05) effect of embryo age on PG (Table 4). PG values remained unchanged until day 15 of incubation, and consistently increased between day 15 and 21. This increase in PG during incubation was also observed by Christensen *et al*. (2003). According to Christensen *et al*. (2001), during late incubation, oxygen demand by the embryonic tissues exceed oxygen conductance through the eggshell. During this period, more carbohydrate is required because of the higher oxygen demand of lipid metabolism (Pearce and Brown, 1971), which becomes the essential substrate during shell pipping (Freeman, 1965). Energy derived of anaerobic metabolism is necessary to sustain body growth and maintenance. However, if this energy is limiting, the embryo will have to choose between growth and vital activity maintenance (Ricklefs, 1987).

Our results are different from those obtained in turkeys by Christensen *et al*. (1996), which showed that embryonic blood plasma glucose concentrations declined as hens aged, but are consistent with the findings of Christensen *et al*. (2000). Also working with turkeys, the authors verified that PG values are correlated with body weight at hatching day. A significant (p<0.05) positive correlation (r=0.8765) between body weight and PG was found during embryonic development, independently of breeder age. Newly-hatched chicks derived from 60WB eggs were heavier than those of 29WB eggs, but there was no difference in their PGs. Therefore, despite the observed correlation between chick weight and PG, the absence of breeder age-related differences in the PG values indicates that the higher PG values observed during the last days of incubation are not related to body mass gain, but to the energy demands of tissue metabolism during the period of change from chorioallantoic to pulmonary respiration.

Data relative to differential leukocyte counts are presented in Table 6. There were no significant (p<0.05) effects of breeder age or sex on the counts of the different leukocyte types. There was influence embryo age only on monocyte, limphocyte and thrombocyte values. Monocyte counts were higher at day 15 as compared to the other three analyzed ages, which were not different from each other. Also, there were no differences in the lymphocyte counts between days 13 and 15, or between days 18 and 21; however,

Table 6- Effects of embryo age (13, 15, 18 and, 21 days of incubation), and sex, and breeder age (29: 29WB and 60: 60WB weeks of age) on differential leukocyte counts and H/L ratios in broiler embryos.

	Monocytes	Basophils	Eosinophils	Heterophils	Lymphocytes	Trombocytes	H/L
Embryo age							
13	1.49 B	2.09 A	0.61 A	13.05 A	13.97 B	64.93 A	0.79 A
15	3.56 A	7.23 A	0.80 A	2.89 A	4.46 B	73.90 A	0.56 A
18	1.65 B	2.44 A	1.51 A	9.01 A	38.54 A	48.69 B	0.24 A
21*	0.80 B	7.08 A	1.10 A	13.71 A	31.19 A	53.86 B	1.09 A
p	0.0500	0.5074	0.3386	0.5698	0.0005	0.0152	0.7886
Sex							
Male	1.68 A	3.14 A	0.90 A	7.41 A	29.79 A	59.89 A	1.03 A
Female	1.41 A	5.93 A	1.01 A	14.41 A	20.99 A	55.93 A	0.41 A
p	0.6591	0.3800	0.8234	0.1623	0.1463	0.4697	0.3415
Breeder age							
29WB	1.26 A	1.91 A	1.03 A	8.01 A	21.07 A	58.52 A	0.45 A
60WB	1.85 A	7.51A	0.87 A	14.27 A	30.02 A	57.16 A	1.02 A
p	0.3406	0.0753	0.7601	0.2125	0.1404	0.8046	0.3819

A-C: Means followed by similar letters in the same column are not significantly different ($p<0.05$) by Tukey's test . *: Newly-hatched chicks.

counts were higher in the latter than in the former. Thrombocyte counts between days 13 and 15, and days 18 and 21 were also similar, but in contrast with lymphocytes, a significant ($p<0.05$) decrease in thrombocyte counts was observed between day 15 and 18, resulting in lower values in the last days of incubation.

Heterophils, basophils and eosinophils do not present antigenic specificity, but they have an important role in the acute phase of infection (Charles Noruega, 2000). In the present study, the counts of these three cell types were very low during all periods, probably as a result of a contamination-free incubation.

Thrombocytes were markedly the most frequent leukocyte in the blood during incubation. This finding is very interesting, because it characterizes an immunological difference between broiler embryos and adult birds, where heterophils are the most frequent white blood cell (Brooks *et al*., 1996; Kogut *et al*., 1998). Avian thrombocytes have intense phagocytic activity (Morgulis, 2002), which together with their high percentage concurrent with lower heterophil and lymphocyte percentages in the blood, indicates that embryos have an unspecific immunity against pathogens that invade the blood.

Thrombocytes may also have a hemostatic role (Grecchi *et al*., 1980). A C3b-like receptor and analogs of mammalian platelet glycoproteins IIb and IIIa were identified in chicken thrombocytes (Kunicki & Newman, 1985). Avian thrombocytes have disperse fibrinogen receptors that become focally localized when thrombocytes are activated, allowing thrombocyte aggregation (O'Toole *et al*., 1994). During the first half of incubation, embryonic and extra-embryonic vessels and capillary nets increase substantially by angiogenesis, which appears to create the need of high thrombocyte numbers in the embryo circulation. Avian embryos obtain nutrients and oxygen through the vitelinic and the allantoic vessels, respectively. During eggshell pipping, embryos must change from corioallantoic respiration to pulmonary respiration, a process that involves the acquisition of a new functional structure in the circulatory system and involves the development or differentiation of new vessels and the degeneration of others. Therefore, it is possible that the maintenance of high thrombocyte percentage in the embryonic blood during the entire incubation period, as observed in the present study, also ensures adequate development of the embryonic circulatory and respiratory systems, as well as allows changes in both systems required during late incubation for the adaptation to pulmonary respiration.

Lymphocytes act in specific immunity and at the beginning of adaptive reactions (Cardoso, 2003). According to Kogut *et al*. (1998), during egg development and immediately after hatching, birds do not have acquired immunity. However, the increase in lymphocyte percentage from day 18 of incubation on and the concurrent reduction of thrombocyte percentage during the same period found in the present study suggest the occurrence of specific immune responses and simultaneous reduction of the unspecific immune response up to hatching, which is probably essential for the exposure to the external environment.

In conclusion, our data showed that body, heart, and liver weights are influenced by maternal age, that hematocrit is influenced by embryo sex at hatching,

and that body and organ weights, as well as red blood cell count, hematocrit, hemoglobin, plasma glucose, and lymphocytes values increase during the incubation period, whereas mean corpuscular volume and thrombocyte values decrease.

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