ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 14 (7): 387-393, 2015
ISSN 1682-8356

Ascorbic Acid in Egg Injection Minimizes the Effects of Fasting Between Hatching and Housing of Broiler Chicks

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Abstract: The duration of pre-housing fasting may determine the chick's weight loss, impairing their performance during the rearing. Management's practices to minimize the effect of this fast are extremely important to the success of broilers rearing. The aim of this study was to check if the ascorbic acid injection (AA) in egg avoids or minimizes the effects of pre-housing fasting in broilers chicks. Fertile Cobb® broiler eggs from hens at 29 weeks of age were used. The experimental design was completely randomized in a factorial arrangement 2 x 4 x 2, and two sex (males and females), with 4 treatments (non-injected eggs and chicks submitted to fasting; not injected eggs and chicks fed with water and feed *ad libitum*; eggs injected with 1 µg de AA/150 µL of water and chicks submitted to fasting; eggs injected with 3 µg de AA/150 µL of water and chicks submitted to fasting) with 2 different ages (48 and 72 h after hatching). Eggs injected with 1 mg of AA preincubation has increased the hatching by 14.2% avoiding the negative effect of fasting on the weights of the chicks till 48 h after hatching, however, feed intake after this period of fasting is critical, independent of the AA injection, the association of AA injection and feed intake, are important managements to avoid weight loss of pre-housing chicks.

Key words: Post-hatching, relative weight, vitamin C, yolk sac

INTRODUCTION

The weight at hatching or weight at the time of housing is highly correlated to weight gain during growth of broiler chickens. Different periods of post-hatching fasting leads to different body weights in chicks housing and can hinder their performance during rearing (Junqueira *et al*., 2001; Maiorka *et al*., 2003). Several factors may determine the length of the fasting and pre-housing, as the variability of the number of hours until the rupture of the shell, incubation temperature, egg size and year season. These factors within the same incubator lead time differences in hatching of 36 to 48 hours (Sklan *et al*., 2000; Vieira *et al*., 2005). There are some operational factors of the hatchery, as the time required for selection, sexing, vaccination of chicks and transportation. All these factors can determine Chick weight loss by up to 10% (Cancado and Baiao, 2002a,b) and, in this cases, the presence of yolk sac is vital to ensure the survival of the bird during the first hours of life (Vieira and Pophal, 2000).

The developing embryo is nutritionally dependent of the egg composition which will influence the hatching rate, the quality of the chicks in the hatching and body weight (Finkler *et al*., 1998). In egg administration of nutrients could be an alternative method to manipulate the quality of the chicks and their post-hatch performance. If ascorbic acid is an anti-stress agent and improves the performance of the bird (Pardue and Thaxton, 1986; Mahmoud *et al*., 2004), it is possible that the ascorbic acid injection in egg is beneficial to egg embryos under stress conditions of fasting pre-housing. Although there are registers in the literature for the effects of ascorbic acid on bird's performance (Zakaria and Al-Anezi, 1996; Ghonim *et al*., 2009; Mohammed *et al*., 2011; Nowac-zewski *et al*., 2012), they also refer to the ascorbic acid injection in later stages of embryonic development. There are a lacking of literature on the effects of ascorbic acid injection in pre-hatching egg on the histological characteristics, weight of hatched chicks and pre-housing fasting. Given the above context, the aim of this study was to analyze the ascorbic acid injection in egg before hatching, to minimize or avoid the effects of stress by pre-housing fasting, on hematological parameters, body weight and organ in post hatching chicks.

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MATERIALS AND METHODS

Eggs and trial design: This study was conducted in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and with approval of the local Committee for Ethical Animal Use (CEUA-protocol no. 7377/10, College of Agricultural and Veterinary Sciences-Sao Paulo State University-UNESP, Jaboticabal, SP).

Three hundred and twenty (320) fertile Cobb® broiler eggs from hens at 29 weeks of age from a commercial company (Globoaves Itirapina, SP) were weighted and distributed according the average egg weight (61.3±6.3g). The completely randomized design used a 2 x 4 x 2 factorial, using 2 sex (male and female), 4 treatments (non-injected eggs and chicks submitted to fasting; not injected eggs and chicks fed with water and feed *ad libitum*; eggs injected with 1 µg de AA/150 µL of water and chicks submitted to fasting; eggs injected with 3 µg de AA/150 µL of water and chicks submitted to fasting) with 2 different ages (48 and 72 h after hatching) in four incubators (Premium Ecologica, IP120), equipped with automatic temperature, humidity and egg turning control (the eggs were turned every 01 h) in total of 20 eggs/ treatment/incubator). The eggs were incubated at 37.8°C and maintained at 60% relative humidity during all incubation period.

For the AA injection, the eggs were held horizontally and after cleaning with 100% ethanol, the shell was perforated with a sterile needle [Injex, 13 x 0.38 (27.5 G1/2")]. The solution with Ascorbic Acid (Synty, 99% purity) was injected in the albumen, about 6 mm below the membrane, the eggs were held horizontally and the solutions were applied near the thin end, the end opposite side of the air cell. After injection, the hole was sealed with a label identifying the treatment and repetition. The AA solution was prepared with Milli-Q water and autoclaved in the dark due to its photosensitivity.

The birds feed after hatching received a started feed following the nutritional requirements established by Rostagno *et al*. (2011).

Were evaluated the incubation parameters (hatching), quality of the chicks, (body and organs weight) and hematological parameters (hematocrit value (HCT), hemoglobin levels (HGB), average corpuscular volume of red blood cells (MCV), total number of red blood cells (RBC) and concentration of plasma protein and glucose).

Hatching and chick's quality: Were evaluated the hatching (number of birds born/number of eggs incubated).

The absolute fresh weights of the liver, yolk sac, spleen and bursa of Fabricius were obtained at the end of the ages of 48 and 72 h, where six chicks/treatment/sex were weighed (body weight with Marte balance; 0.0001) and sacrificed by cervical dislocation, followed by bleeding, for collecting and weighing the organs. The relative weights of the organs were calculated in relation to the body weight of the chicks.

Hematological parameters: For blood tests (HCT, HGB, RBC and MCV) were used six chicks per treatment.

Blood was collected from the jugular vein and maintained in plastic tubes such as "Eppendorf" anticoagulant containing 15 µL/1 ml of blood (Glistab, Cat. 29, Labtest Diagnostica) on ice for use in the analysis on a blood cell counter (Celm, Mod. 550), using two readings per bird.

Additionally was evaluated the plasma protein and glucose concentration. To obtain these values, the blood samples were centrifuged for 15 min (5000 rpm at 4°C) using a microfuge (Costar). The plasma (supernatant) was removed with the micro pipette and stored in tubes type "eppendof", properly identified and frozen at -20°C. For plasma glucose levels (CGP) (mg/dL) was used Glucose PAP kit Liquiform, 500 ml (Labtest, cat. 84). Samples were prepared and calculations performed according to the manufacturer's specifications.

The protein concentration in plasma (PP) (µg/µL) was determined by conventional biochemical assay of Bradford (Bradford, 1976) using different concentrations of serum albumin to obtain the standard curve. From the standard curve obtained from the analysis, plasma samples were prepared using 15 µl plasma added to 20 µL of water and 1 ml solution Bradford (Coomassie Bright Blue G 50 mg +25 ml +50 ml Ethanol 95% orthophosphoric acid 85% +400 ml double distilled water). For both parameters duplicates were performed and the readings taken in to a spectrophotometer using 595 nm.

Statistical analysis: The data were submitted to variance analysis by procedure General Linear Model (GLM) of software SAS® (SAS Institute, 2002). In case of significant effect, comparison of means was performed by Tukey test at 5% probability.

RESULTS

Hatching and chick quality: There was a significant effect ($p<0.05$) from eggs injected with ascorbic acid (AA) and the hatching rate (Fig. 1). For chicks quality parameters there were interaction ($p<0.05$) between treatments and age for body weight of spleen, liver and yolk sac (Table 1).

According to the interaction unfolding (Table 2) for all variables, except for the yolk sac, the chicks from not injected eggs and fed with water and feed *ad libitum*, showed higher weights than the chicks of other

Table 1: Effect of treatments, age and gender on the quality of chicks

	Body weight	Spleen	Liver	Yolk sac	Fabricius Bursa
	---------- (g) ----------				
Treatments (T)					
0 µg AA/feed	53.95	0.0283	2.13	2.66	0.0705[a]
0 µg AA/fastening	38.50	0.0152	1.03	2.76	0.0453[b]
1 µg AA/fastening	38.55	0.0143	1.08	2.67	0.0443[b]
3 µg AA/fastening	38.28	0.0142	1.07	3.37	0.0404[b]
Ages (A) (hour)					
48	41.31	0.016	1.15	3.38	0.0486
72	43.05	0.019	1.37	2.41	0.0484
Sex (S)					
Female	41.89	0.018	1.28	2.86	0.0466
Male	41.47	0.017	1.25	2.92	0.0504
Probability					
Treatment	<0.0001	<0.0001	<0.0001	0.0063	<0.0001
Age	0.0137	0.0399	<0.0001	<0.0001	0.8659
Sex	0.6930	0.327	0.7757	0.7401	0.0664
Interaction T x A	<0.0001	0.0396	<0.0001	0.0128	0.1311
Interaction T x S	0.2293	0.8162	0.2042	0.8247	0.9911
Interaction A x S	0.1387	0.5516	0.1992	0.9931	0.3770

[a-b]Means followed by different letters differ significantly

Table 2: Unfolding of the interactions between treatments and ages on the quality of chicks

	Age (h)	0 µg AA/ feed	0 µg AA/ fastening	1 µg AA/ fastening	3 µg AA/ fastening
Body weight (g)	48	48.25^{bA}	38.98^{aB}	39.62^{aB}	39.95^{aB}
	72	59.90^{aA}	38.07^{aB}	37.64^{aB}	36.61^{bB}
Spleen (g)	48	0.023^{bA}	0.014^{aB}	0.015^{aB}	0.014^{aB}
	72	0.033^{aA}	0.016^{aB}	0.014^{aB}	0.014^{aB}
Liver (g)	48	1.51^{bA}	1.03^{aB}	1.09^{aB}	1.10^{aB}
	72	2.60^{aA}	1.02^{aB}	1.07^{aB}	1.04^{bB}
Yolk sac (g)	48	3.39^{aB}	2.94^{aB}	2.98^{aB}	4.223^{aA}
	72	2.08^{bA}	2.57^{aA}	2.391^{aA}	2.522^{bA}

[a-b], [A-B]Means followed by different letters (line and columns) differ significantly

treatments at both ages. Additionally, the chicks from not injected eggs and fed with water and feed *ad libitum*, showed body weight, liver and spleen, larger and smaller yolk sac with 72 h after hatching. The chicks from eggs injected with 3 mg of AA and fasting post-hatching showed body weight, liver and yolk sac lower with 72 h when compared with the 48 h of age.

The weight of the Fabricius bursa was affected ($p<0.05$) only for the treatments and lower for chicks submitted to the fasting management (Table 1).

Blood parameters: For blood parameters, there was no effect ($p<0.05$) between the ages for the RBC and HCT, which were smaller values for the 72 h chicks (Table 3). Additionally there was an interaction ($p = 0.05$) between treatments and sex for these parameters, whose lowest values were observed for post-hatch fed females without AA injection (Table 4).

There was effect ($p>0.05$) between treatments, age and gender on the values of MCV and HGB (Table 3).

In addition, there was an interaction ($p\leq0.05$) between treatments and age for PP and CGP. According to the split of interaction (Table 4), PP of chicks analyzed with 48 h of age, the lowest values were observed for fasting birds, with an injection of 1 and 3 mg of AA in egg while

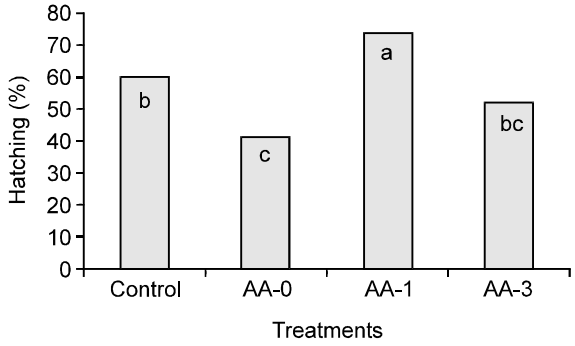

Fig. 1: Hatching rate according to the treatments (%). a-c: comparison between treatments. Different letters indicate significant differences ($p<0.05$) between treatments. Control: no injection; AA-0, AA-1 and AA-3: Injection of 0, 1 and 3 mg of ascorbic acid in egg

the larger values were observed for the birds that were fed after hatching and with 72 h old. For the CGP variable, the highest values were observed for the birds that were fed after hatching and did not have AA injection in both ages, 48 and 72 hold. By analyzing the fed chicks, not injected, the highest value was observed for birds with 72 h old. In addition, there was an interaction ($p<0.05$) for CGP between sex and age (Table 3). According to the split of interaction (Table 5) with 48 h old the females have a higher concentration of CGP. However, both females and males aged 72 h have higher values for CGP when compared to chicks analyzed with 48 h old.

DISCUSSION

The injection of 1 µg AA increased by 14.2% hatching rate compared to the control treatment. AA injection in pre-incubation egg can be used, therefore, in order to increase the hatching rate. This result is interesting since it indicates the occurrence of dose-dependent effect of hatching rate compared to the AA injection in eggs also recorded by Zakaria and Al-Anezi (1996) and Nowaczewski *et al*. (2012), although these authors has registered response that are concentration-specific to hatching performing and AA injection in egg, in advanced periods of development

According to Jochemsen and Jeurissen (2002), the age that is made the inoculation procedure may affect the location where the product is applied. Ohta *et al*. (2001) observed reduction in hatching when the eggs were inoculated with amino acids before incubation.

Elibol *et al*. (2001) and Ipek *et al*. (2004), analyzed the AA injection in early embryonic development stages and found similar results to present study, in which the effect of this vitamin in the egg development does not vary with the stage of embryonic development at which the injection is performed. However, there are few data

Table 3: Effects of treatments, ages and sexes on the blood parameters of chicks

	RBC ($x10^6/mm^3$)	MCV (μm^3)	HGB (g/dl)	HCT (%)	Protein (µg/µl)	Glucose (mg/dl)
Treatments (T)						
0 µg AA/feed	2.81	109.44	13.98	30.81	489.57	391.98
0 µg AA/fastening	3.52	109.21	18.28	38.34	572.15	159.68
1 µg AA/fastening	3.43	110.51	16.48	37.70	450.57	158.61
3 µg AA/fastening	3.30	109.71	16.15	36.37	437.45	154.75
Age (A) (hours)						
48	3.45[a]	108.89	16.82	37.61[a]	503.22	172.53
72	3.12[b]	110.60	15.76	34.47[b]	466.29	230.55
Sex (S)						
Female	3.28	108.77	15.86	35.56	485.29	208.81
Male	3.30	110.77	16.71	36.55	484.20	195.32
Probability						
Treatment	0.1353	0.9684	0.2366	0.1207	<0.0001	<0.0001
Age	0.0287	0.1805	0.2247	0.0429	<0.0001	<0.0001
Sex	0.0982	0.0779	0.5980	0.6869	0.8662	0.3365
Interaction T x A	0.1773	0.5744	0.2009	0.1207	<0.0001	<0.0001
Interaction T x S	0.0010	0.2737	0.2842	0.0012	0.7138	0.3759
Interaction A x S	0.0952	0.8848	0.1755	0.0711	0.067	0.0212

[a-b a-b]Means followed by different letters in the columns differ significantly

Table 4: Unfolding of the interaction between treatments and sexes of the interaction between treatments and ages on the blood parameters

	Sex	0 µg AA/feed	0 µg AA/fastening	1 µg AA/fastening	3 µg AA/fastening
RBC ($x10^6/mm^3$)	Female	2.36[bC]	3.98[aA]	3.75[aAB]	2.99[aBC]
	Male	3.34[aA]	3.04[bA]	3.14[aA]	3.62[aA]
HCT (%)	Female	25.54[bC]	43.38[aA]	40.02[aAB]	32.82[aBC]
	Male	37.40[aA]	32.36[Ba]	35.48[aA]	39.93[aA]
	Age (hours)				
Protein	48	564.16[aA]	579.26[aA]	447.43[aB]	431.96[aB]
(µg/µl)	72	415.07[bB]	566.36[aA]	452.95[aB]	442.94[aB]
Glucose	48	247.63[bA]	165.74[aB]	157.17[aB]	148.80[aB]
(mg/dl)	72	504.11[aA]	154.68[aB]	160.77[aB]	160.71[aB]

[a-b, A-B]Means followed by different letters (line and columns) differ significantly

Table 5: Unfolding of the interaction between sexes and ages on the blood parameters

		---------- Age (hours) ----------	
	Sex	48	72
Glucose	Female	184.15[aB]	230.24[aA]
(mg/dl)	Male	163.43[bB]	228.81[aA]

[a-b, A-B]Means followed by different letters (line and columns) differ significantly

in the literature with the injection of AA pre-incubation. According to the results of the present study, the exogenous intake of feed is essential for animal growth after 72 h of hatching, due to the greater nutritional intake provided. However, supplemented chicks in egg with AA had a nutritional intake for up to 48 h post-hatch, while remaining in fastening feed and water, showed higher body weight, liver and yolk sac. Chicks with different body weights during the housing have different growth curves, which can result in differences at performance, normally favorable to the birds with higher initial body weight, knowing that the post outbreak weight is highly correlated with body weight during the growth of broilers it is of great interest to the poultry industry (Junqueira *et al*., 2001; Noys and Sklan, 2002; Maiorka *et al*., 2003; Gomes *et al*., 2008; Teixeira *et al*., 2009).

Furthermore, the residue yolk corresponds to 20% of the body weight of the chicks and it is a ready source of energy and protein (Figueiredo *et al*., 2003) for maintenance and growth (Sklan, 2003) and the presence of the yolk sac is vital to ensure the bird's survival in the first hours of life. As described by Edwards *et al*. (1962), birds that had the yolk sac surgically removed had a poorer performance compared to birds of a control group, also operated but with yolk bag kept, which proves the importance of this reserve for birds in the post-hatching period as the first source nutrients used by the chick after emergence, so the longer is the time between the hatch and the start of feed intake and water, the greater the reliance that the chick will have these reserves (Vieira and Pophal, 2000).

There was a decrease of the yolk sac at 72 h post-hatching for chicks that were fed. At the embryonic development and hatching period, yolk lipids are transported to the circulation by endocytosis (Lambson, 1970). After hatching, the yolk sac is transported through the pedicle into the small intestine (Esteban *et al*., 1991; Noys and Sklan, 1997). According to Noys and Sklan (2001), feed ingestion stimulates the yolk sac intestinal

absorption. According to the results obtained in this study, it appears that the exogenous supply enables or stimulates the yolk sac absorption, which can be an action or influence the intestinal nourishment flow on the intestinal absorption.

The proportional weight of the primary lymphoid organs is often adopted to evaluate cases of stress response (Revidatti *et al*., 2002). Donker and Beuving (1989) reported that stress causes regression of primary lymphoid organs such as the bursa. This effect was observed in this study for birds kept in post hatching fasting, indicating that the injection of pre-incubation AA did not minimize the stress caused to birds.

According to Moura and Pedroso (2003), AA is associated with increasing values of RBC, HCT and HGB, however, according to the results obtained in this study, there was no effect ($p>0.05$) of the AA injection pre-incubation on erythrocyte parameters, however, by the fasting, females showed higher values for RBC and HCT, with no changes in the values of HGB and MCV. In addition, RBC and HCT values were higher in isolation, for the chicks with 72 h old.

According to Campbell (1994), increased in HCT values may be associated with dehydration of the birds, however, no changes were observed in body weight of females that indicate this effect in the present study. Therefore the intensity of the red cell response in chicks due to fasting has strong correlation with sex. The results indicate higher sensitivity to fasting for females, compared to males, with erythrocytes released to the circulation, without changing the values of HGB and MCV. So the AA injection in egg did not stop the fall in the immune system of females, this effect was minimized by post-hatching feeding of the birds at 72 h of age.

Compared to a fasting state, for maintaining physiological status, there is a protein degradation as a consequence an increase in the concentration of PP, suggesting a greater protein degradation (Katanbaf *et al*., 1988). Therefore, according to the results obtained, 48 h post-hatching the injection of AA in egg reduced this degradation and after 72 h of hatching this effect was obtained with both managements, the AA injection in egg and the supply of feed to the birds.

Warriss *et al.* (1992) reported that chicks fasted for 48 h showed higher PP values compared to chicks that was fed. This result suggests that the degradation of the protein could be used for chicks fasted for obtaining energy and/or metabolic water.

Considering the CGP the chicks fed and not injected with AA in egg showed the same increase due to ingestion of nutrients such as protein and carbohydrates, especially after 72 h of hatching. The availability of glucose is particularly important to the time preceding and during the hatching, while occurs the passage of the chorion-allantoic breathing to the complete dependence of the pulmonary respiration (White, 1974). During this period, the glycogen levels are reduced to a minimum, due to the muscular demand (John *et al*., 1988) and the beginning of the activity in the central nervous system (Edwards and Roger, 1972), there is a positive correlation with gains in broiler performance (Vieira and Pophal, 2000).

Conclusion: The use of 1 µg injection of AA in egg at pre-incubation increase 14.2% the hatching rate and prevents the negative effect of fasting on the body weight of the chicks within 48 h after hatching. However, it is critical to have feed intake after this period of fasting, regardless of the AA injection and the association of AA application and feeding, these are important managements to avoid weight loss of pre-housing chicks.

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